In Search of a New Biomembrane Model

An International Symposium
The Royal Danish Academy of Sciences and Letters
August 13-16, 1997

Edited by OLE G. MOURITSEN *and* OLAF S. ANDERSEN

Biologiske Skrifter **49**

Det Kongelige Danske Videnskabernes Selskab
The Royal Danish Academy of Sciences and Letters

Kommissionær: Munksgaard · Copenhagen 1998

The Royal Danish Academy of Sciences and Letters

publishes four monograph series, an Annual Report and, occasionally, special publications. The format is governed by the requirements of the illustrations, which should comply with the following measures.

	Authorized Abbreviations
Historisk-filosofiske Meddelelser, 8°	Hist.Fil.Medd.Dan.Vid.Selsk. (printed area 175 x 104 mm, 2700 units)
Historisk-filosofiske Skrifter, 4° (History, Philosophy, Philology, Archaeology, Art History)	Hist.Filos.Skr.Dan.Vid.Selsk. (printed area 2 columns, each 199 x 77 mm, 2100 units)
Matematisk-fysiske Meddelelser, 8° (Mathematics, Physics, Chemistry, Astronomy, Geology)	Mat.Fys.Medd.Dan.Vid.Selsk. (printed area 180 x 126 mm, 3360 units)
Biologiske Skrifter, 4° (Botany, Zoology, Palaeontology, General Biology)	Biol.Skr.Dan.Vid.Selsk. (printed area 2 columns, each 199 x 77 mm, 2100 units)
Oversigt, Annual Report, 8°	Overs.Dan.Vid.Selsk.

The Academy invites original papers that contribute significantly to research carried on in Denmark. Foreign contributions are accepted from temporary residents in Denmark, participants in a joint project involving Danish researchers, or partakers in discussion with Danish contributors.

Instructions to Authors

Manuscripts from contributors who are not members of the Academy will be refereed by two members of the Academy. Authors of accepted papers receive galley proof and page proof which should be returned promptly to the Editor. Minidiscs etc. may be accepted; contact the Editor in advance, giving technical specifications.

Alterations causing more than 15% proof changes will be charged to the author(s). 50 free copies are supplied. Authors are urged to provide addresses for up to 20 journals which may receive review copies.

Manuscripts not returned during the production of the book are not returned after printing. Original photos and art work are returned when requested.

Manuscript

General. – Manuscripts and illustrations must comply with the details given above. The original ms. and illustrations plus one clear copy of both should be sent to the undersigned Editor.

NB: A ms. should not contain less than 32 *printed* pages. This applies also to the Mat.Fys.Medd., where contributions to the history of science are welcome.

Language. – English is the preferred language. Danish, German and French mss. are accepted and in special cases other languages. Where necessary, language revision must be carried out before final acceptance.

In Search of a New Biomembrane Model

An International Symposium
The Royal Danish Academy of Sciences and Letters
August 13-16, 1997

Edited by OLE G. MOURITSEN *and* OLAF S. ANDERSEN

Biologiske Skrifter **49**

Det Kongelige Danske Videnskabernes Selskab
The Royal Danish Academy of Sciences and Letters

Kommissionær: Munksgaard · Copenhagen 1998

Abstract

This volume is the written record of an international symposium *In Search of a New Biomembrane Model* held under the aegis of the The Royal Danish Academy of Sciences and Letters on August 13-16, 1997. It contains contributions from a diverse group of scientists, who approach the questions of membrane structure and function from a multitude of traditions and disciplines - ranging from cell biology and physiology, over traditional biochemistry and biophysics, to physical chemistry and theoretical physics. The purpose of the symposium was to synthesize current experimental and theoretical knowledge about the lipid bilayer component of cell membranes, and on this background explore whether the time has come to formulate a membrane model that is substantially refined compared to the Singer-Nicolson fluid-mosaic membrane model proposed 25 years ago. The various contributions cover basic concepts in membrane modeling, the use of simple lipid bilayers as membrane models, more complex bilayer models including peptides and membrane proteins, the cytoskeleton, complex biological functions associated with membranes, as well as membrane models used for drug-delivery systems.

OLE G. MOURITSEN
Department of Chemistry
Technical University of Denmark
DK-2800 Lyngby, Denmark
e-mail: ogm@kemi.dtu.dk

OLAF S. ANDERSEN
Department of Physiology and Biophysics
Cornell University Medical College
New York, NY 10021, USA
e-mail: sparre@mail.med.cornell.edu

Printed in Denmark by Special-Trykkeriet Viborg a-s
ISBN 87-7304-292-7

Contents

Preface 6

Do We Need a New Biomembrane Model?
Mouritsen & Andersen 7

Concepts in Membrane Modeling

Evolution of Membranes from a Physics Perspective
Bloom 13

Defining and Imaging Membrane Domains
Edidin 19

Membrane Organisation and Assembly - Perspectives From Spin Label ESR (and Other Biophysical Methods)
Marsh 23

The Bending Elasticity Concept of Biomembrane Function
Sackmann 31

Membranes are Fluid Mosaics With Transient Tiles (Dynamic Domains) Defined by Curvature, Traffic and Tension
Sheetz 35

Lipid Bilayers: Simple Membrane Models

Dynamic Heterogeneities of the Lipid Matrix of Biological Membranes and Associated Protein Function
Biltonen 41

Membranes Display Nano-scale Heterogeneity - Beating the Randomness of the Fluid Lipid Bilayer
Mouritsen 47

The Biological Membrane Structure Paradigm: One View of Its Current Condition
Thompson 55

Phospholipid-bilayer Vesicle Shapes and Shape Transformations: Theory vs. Experiment
Wortis 59

The Role of Minimal Models in the Context of a New Biomembrane Model
Zuckermann 65

Lipid Bilayers and Peptides: More Complex Models

Gramicidin Channels: Molecular Force Transducers in Lipid Bilayers
Andersen, Nielsen, Maer, Lundbæk, Goulian & Koeppe 75

Helix Stability and Interactions in Membrane Proteins
Engelman, Fleming & MacKenzie 83

Membrane Specificities of Antimicrobial Peptides
Huang 87

Tryptophan Anchors in Transmembrane Peptides
Koeppe, Killian, Greathouse & Andersen 93

Protein Folding in Membranes: Pondering the Nature of the Bilayer Milieu
White, Wimley, Ladokhin & Hristova 99

Membrane Proteins

The Courtship and Marriage of K^+ Channel Subunits
Deutsch 107

How Lipids Interact with an Intrinsic Membrane Protein: the Case of the Calcium Pump
Lee 115

Electrostatic Interaction of Myristoylated Proteins with Membranes
McLaughlin, Murray & Arbuzova 121

Lipid Sorting by Membrane Proteins and Membrane Organization
Tocanne 127

Not all Integral Proteins are Freely Diffusing in a Fluid Mosaic: How Lipids can Promote Protein Array Formation
Watts & Sabra 133

Membranes and the Cytoskeleton

Plasma Membrane Organization by the Membrane Skeleton
Kusumi 137

Molecular Basis for Red Cell Membrane Properties
Mohandas & Gimm 143

Membranes and Complex Biological Functions

IgE Receptor Signaling Utilizes Specialized Membrane Domains
Baird, Field & Holowka 147

Rapid Activation of the Na^+,K^+-pump - Mechanisms and Functional Significance
Clausen & Nielsen 153

Relationships Between Protein Domains and Lipid Monolayers in Membrane Fusion
Cohen, Melikyan, White & Lamb 159

Transmembrane Lipid Trafficking: Facts and Speculations
Devaux 167

Membrane Negative Spontaneous Curvature as an Ancient Signal for Cell Growth
Kinnunen 175

Luminal Ion Channels Involved in Isotonic Secretion by Na^+-Recirculation in Exocrine Gland-acini
Sørensen, Nielsen, Nielsen & Larsen 179

Active Membranes
Prost 193

Membrane Bilayer Properties in Acholeplasma laidlawii *are Sensed and Set by the Lipid-synthesizing Enzymes*
Berg, Karlsson, Li, Vikström & Wieslander 195

Membrane Models and Technology Transfer

Model Membrane Systems as Drug Delivery Vehicles
Cullis & Fenske 201

Index 209

Preface

These 31 contributions constitute the written record of the symposium *In Search of a New Biomembrane Model* held under the aegis of the The Royal Danish Academy of Sciences and Letters on August 13-16, 1997, at the premises of the Academy.

The purpose of the Symposium was to bring together a diverse group of scientists, who work on biological membranes - but from different points of view. The aim was to synthesize current experimental and theoretical knowledge about the lipid bilayer component of cell membranes, and on this background explore whether the time had come to formulate a membrane model that is substantially refined compared to the Singer-Nicolson fluid-mosaic membrane model proposed 25 years ago. The contours of a new membrane model have been emerging over the last 5-10 years based on progress in many laboratories that approach the questions of membrane structure and function from a multitude of traditions and disciplines - ranging from cell biology and physiology, over traditional biochemistry and biophysics, to physical chemistry and theoretical physics.

The symposium was conceived during the annual meeting of the Biophysical Society in Baltimore in February 1996. The undersigned organizers found, during an inspired conversation over lunch, that the time was ripe for summarizing the status of the field of membranes and to organize a symposium, which would bring together key scientists in the field. It was anticipated that a symposium with this scope could have a significant impact on membrane science, in particular with respect to shaping both the physicists' and the biologists' intuition about the relationship(s) between the physical properties of lipid bilayers on the one hand and biological membranes and membrane function on the other hand.

The response from the invited speakers and participants was extraordinarily positive. This positive and open-minded attitude permeated the symposium, which took place in a most agreeable and constructive atmosphere. 32 invited talks were given and more than thirty posters were presented. Altogether, the symposium was attended by 85 speakers and participants.

The present volume contains the contributions of the invited speakers. This written account is not intended to be a conventional set of conference proceedings. Each speaker was requested to prepare, and deliver before the start of the symposium, a written contribution that should be no more than four pages long. The speakers were asked to provide a crisp, stimulating, and preferably provocative contribution, which should be a statement that described the speaker's current picture of the biological membrane based on his or hers own work and prejudices. The entire set of contributions was collected and sent to all speakers in time to be studied before the symposium. After the symposium, the speakers were encouraged to amend their contribution in light of the discussion during the symposium.

The contributions were submitted electronically. They were organized and typeset by Helle Hjorting, who also acted as Symposium secretary. Bernd Dammann, PhD designed the LaTeX-format for the typesetting and provided invaluable help in expediting the typesetting. Ove Broo Sørensen of the Technical University of Denmark and Bruce Paul Gaber at the Navel Research Laboratory produced the color membrane cartoons which are reproduced in the introductory chapter.

The funding for the Symposium was provided by generous contributions from a number of private and public sponsors. It is with deep gratitude we extend our thanks to these sponsors: the Niels Bohr Legat under the Royal Danish Academy of Sciences and Letters, the Carlsberg Foundation, the Canadian Institute for Advanced Research, the Danish Medical Research Council, the Danish Natural Science Research Council, the Thomas B. Thrige Foundation, the Otto Mønsted Foundation, and Avanti Polar Lipids.

Copenhagen 1997
Ole G. Mouritsen

New York 1997
Olaf Sparre Andersen

Do We Need a New Biomembrane Model?

OLE G. MOURITSEN *and* OLAF S. ANDERSEN

Department of Chemistry
Technical University of Denmark
DK-2800 Lyngby, Denmark
e-mail: ogm@kemi.dtu.dk

Department of Physiology and Biophysics
Cornell University Medical College
New York, NY 10021, USA
e-mail: sparre@mail.med.cornell.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:7–12

For 25 years the Singer-Nicolson fluid mosaic model of biological membranes (Singer and Nicolson, 1972) has been a central paradigm in membrane science. The simple, yet powerful conceptual framework it provided continues to have an enormous impact on the field of biomembranes. As a key property, the Singer-Nicolson model assigned to the membrane's lipid bilayer component a certain degree of "fluidity". The fluidity concept was meant to characterize the lipid bilayer as a kind of pseudo-two-dimensional liquid in which both lipids and membrane-associated proteins display sufficient lateral mobility to allow for function. The overall random appearance of this lipid-protein fluid composite made the membrane look like a mosaic. Except in cases where sterols or unsaturated lipid acyl chains might alter the bilayer "fluidity", the conspicuous diversity in the chemical structures of lipids, which is actively maintained by cells, had little significance in the model. This lipid diversity, together with the varying (but characteristic) lipid composition of different types of cells and organelles, have become an increasing puzzle, which was exacerbated by the enhanced understanding of the variation in physical properties among different lipids and lipid assemblies (Gennis, 1989; Kinnunen, 1991; Bloom et al., 1991).

When Singer and Nicolson proposed the fluid-mosaic model in 1972, membrane modeling already had come a long way, see Fig. 1. The first important step was taken by Gorter and Grendel (1925), who showed experimentally that the membrane is very thin, being a bimolecular layer (Fig. 1a). The association of membrane proteins with the lipid bilayer was introduced in the Danielli-Davson model (1935), as a spread on the lipid polar head groups at the two sides of the lipid bilayer (Fig. 1b). A related version of membrane organization appears in Robertson's unit membrane model (1966) in which the proteins are pictured as stratified layers sandwiching the lipid bilayer (Fig. 1c). In the Singer-Nicolson fluid-mosaic model (1972) shown in Fig 1d, the proteins are grouped in two classes: integral membrane proteins, which traverse the bilayer and primarily interact with the bilayer through hydrophobic forces; and peripheral membrane proteins, that are peripherally associated with the lipid bilayer and primarily interact with the bilayer through polar (electrostatic and hydrogen bond) interactions. In either case, the proteins "float" in a fluid sea. Refinements of the fluid mosaic model

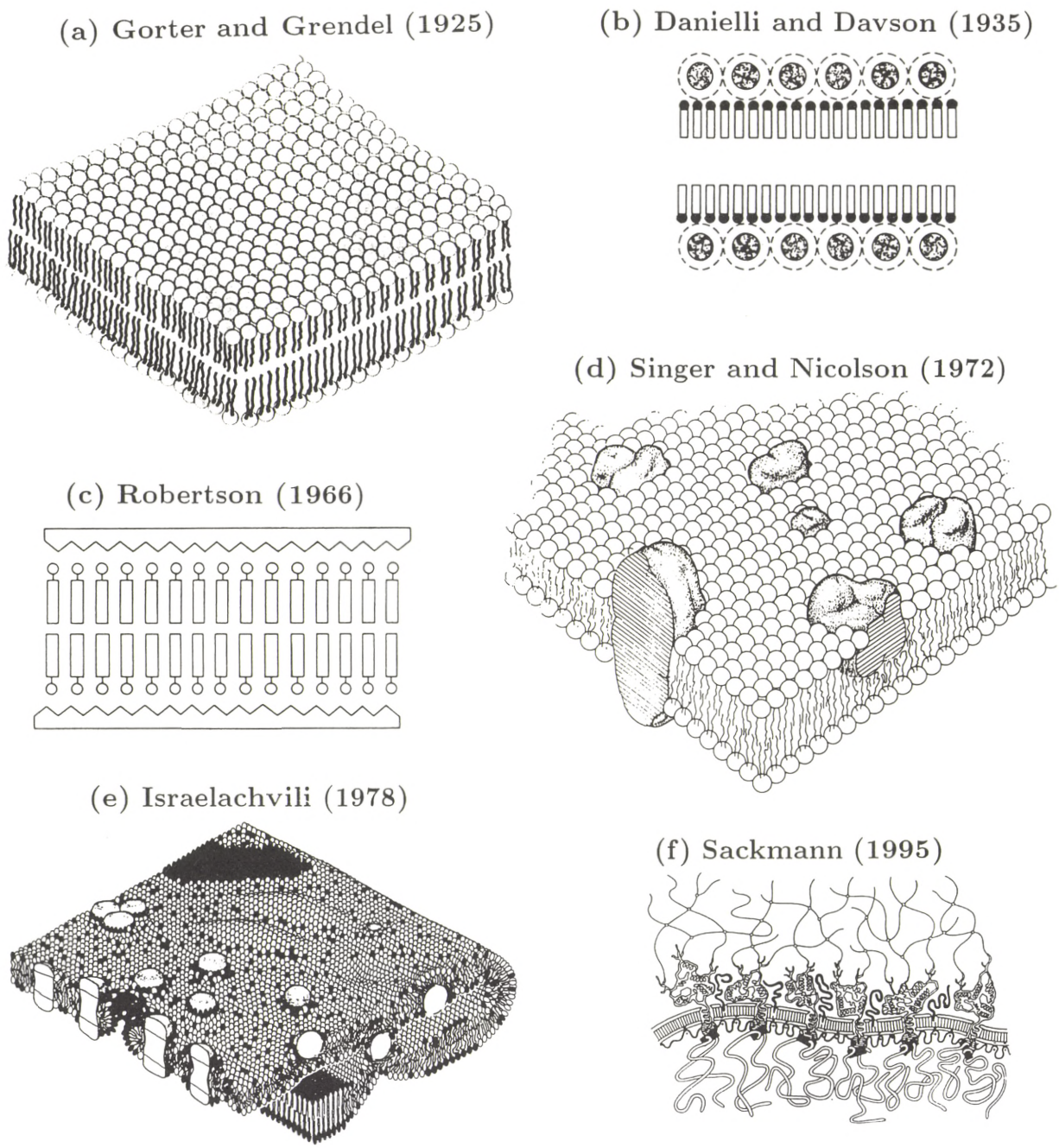


Figure 1. Historic picture gallery of membrane modeling.

have been suggested from time to time, usually inspired by new insights obtained by focusing on some specific, or specialized, membrane feature. One example is the model by Israelachvili (1977; 1978), who refined the Singer-Nicolson model to account for the need for membrane proteins and lipids to adjust to each other, and also incorporated membrane folding, pore formation, and thickness variations as well some degree of heterogeneity (Fig. 1e). Another elaboration of the Singer-Nicolson model, which emphasized the importance of the cytoskeleton and glycocalyx, was developed by Sackmann (Fig. 1f).

The notion of membrane fluidity, which was embodied in the Singer-Nicolson fluid-mosaic model (Fig. 1d), was important because it served to emphasize that membranes are dynamic structures. Unfortunately, many subsequent investigators assumed, explicitly or implicitly, that fluidity implies randomness. This assumption neglects that fluids may be structured on length scales between the molecular scale and scales that are accessible by the microscopic, spectroscopic, or scattering methods commonly used to study the lateral organization of membranes. Also, structuring in time, in particular the correlated dynamical phenomena characteristic of liquid crystals, were not appreciated as being important for membrane function. Importantly, however, such lively dynamics is perhaps the most conspicuous feature of a fluid membrane. In fact, the many-body nature inherent in the molecular assembly of a membrane insures that local order and structure develop naturally from an initially disordered fluid. Finally, the fluid-mosaic model in Fig. 1d pictured the membrane as a flat, pseudo-two-dimensional layer. This may be an artistic simplification; but it nevertheless de-emphasizes the transverse dynamical modes of individual lipid molecules as well as the existence of large-scale excursions into the third dimension with the ensuing curvature-stress fields, instabilities toward non-lamellar symmetries, and coupling between internal membrane structure and molecular organization on the one hand and membrane shape and shape transformations on the other (Lipowsky and Sackmann, 1995).

It is now recognized that the randomness implied in the fluid-mosaic membrane model does not exist. This recognition builds on a wealth of experimental results, which show that the lateral distribution of molecular components in membranes is heterogeneous, both statically and dynamically - corresponding to an organization into compositionally distinct domains and compartments. In addition to immobilization and domain formation due to interactions between the cytoskeleton or the extracellular matrix and the membrane, several physical principles generate dynamic lateral heterogeneity of both lipids and proteins in fluid (liquid-crystalline) membranes. This non-random organization imposed by the fluid membrane means that biomembrane functions do not need to depend on random collisions and interactions among reactants, but may be steered in a well-defined manner that allows for a considerable mobility of the individual constituents. This dynamic organization of the membrane makes it sensitive to perturbations by both physical (e.g. temperature and pressure) as well as chemical (e.g. drugs and metabolites) factors, which thus provides an exceptional vehicle for biological triggering and signaling processes. Specifically, local changes in organization, brought about by physiological perturbants, are likely to evolve and result in relevant changes in the function of the entire system.

The principles underlying these novel features of membrane organization are well known to scientists familiar with the physical properties of membranes, but they are only beginning to be appreciated by scientists more familiar with the function of biological membranes. Similarly, physical scientists often are unfamiliar with the functional properties of biological membranes. The aim of the present volume is to discuss and focus attention on these issues in order to bridge the gap between the different physical and biological approaches to biomembranes and their functions. To the extent this is accomplished, it will have implications for the future progress in biomembrane research.

In retrospect, one may ask why a more refined model, which incorporated the dynamic order and fluctuations of the biological membrane,

was not formulated long ago. Paradoxically, this may be due to the tremendous success of modern structural molecular biology and its focus on structure-function relationships that involve well-defined static (crystal) structures of proteins, nucleic acids, and lipids. From a static structure point of view, once the stable lipid bilayer has been established, the assembly of the lipid molecules in the membrane may appear featureless - like that of a passive solvent. The physical properties of a fluid membrane are not coded for directly by the genes, which together with the legitimate preoccupation with static structures, may have hampered the acceptance of membranes as dynamic entities in which lipids play active and important roles for structure, function, and regulation.

A key element in the formulation of a model is to find the proper balance between general principles and specific detail - or to balance the sometimes conflicting demands for truth and clarity. On the one hand, too many details will render the model applicable only to specific cases, and the details may obscure the generic underlying principles of organization. On the other hand, a too general model may provide little mechanistic insight, which makes the model less useful for the design of new critical experiments. Moreover, the important elements of a model are likely to depend on which length- and time-scales that are relevant for describing the problem of interest. This becomes a particular difficulty when building membrane models because many membrane properties are controlled by phenomena that take place over a range of scales, which are mutually coupled. Given the current, rather advanced, state of knowledge in the field it this is likely that one will be best served by working with a set of membrane models, chosen according to the particular type of question under consideration - and which time- and length scales that are likely to be relevant. This is not an easy task. Two examples of such models are illustrated in Fig. 2.

Figure 2. Cartoons illustrating different aspects of biomembrane structure and dynamics.

(a): A plasma membrane model that highlights the membrane as a stratified composite involving the central lipid bilayer, which is sandwiched between a rubber-like cytoskeleton, attached to the cytoplasmatic inner surface, and the glycocalyx carbohydrate network on the outer surface. The membrane displays undulations; the lipid bilayer displays lateral heterogeneity, lipid domain formation, and thickness variations - close to the integral proteins. Whereas the lipid molecules in this representation are given with some structural details, the membrane-associated proteins remain fairly featureless. In order to capture many different features in the same illustration, the different membrane components are not drawn to scale. The picture was drawn without consideration of time scales and can best be considered as an instantaneous snapshot. (Illustration by Ove Broo Sørensen, Technical University of Denmark.)

(b): A membrane model that highlights the lipid bilayer component and details of the molecular structure of a membrane protein (bacteriorhodopsin). The picture is drawn to scale and it reflects averaging over fast dynamical modes. A 200×200Å slap of a 50Å thick lipid bilayer is shown. The time scale of view is in the range of 10^{-3} to 10^{-6} seconds. On this scale most molecular processes will appear blurred, but not totally indiscernible. For example, the very rapidly moving chains seen on the edges of the lipid bilayer are indicated by subtle texturing parallel to the chain axis. The scale of the texture is on the order of the lipid chains, but the chains themselves are not seen. The membrane edge shading is based on information obtained from X-ray and neutron scattering. The shading used on the headgroup surfaces suggest the presence of small lipid domains. The lipid bilayer displays large-scale bending fluctuations. The transmembrane proteins are modeled by use of the X-ray coordinates for bacteriorhodopsin. Consistent with the slow time scale characterizing this picture, the protein surfaces have been slightly blurred. (Illustration and text by Bruce Paul Gaber, Laboratory for Molecular Interfacial Interactions, Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington, DC 20375.)

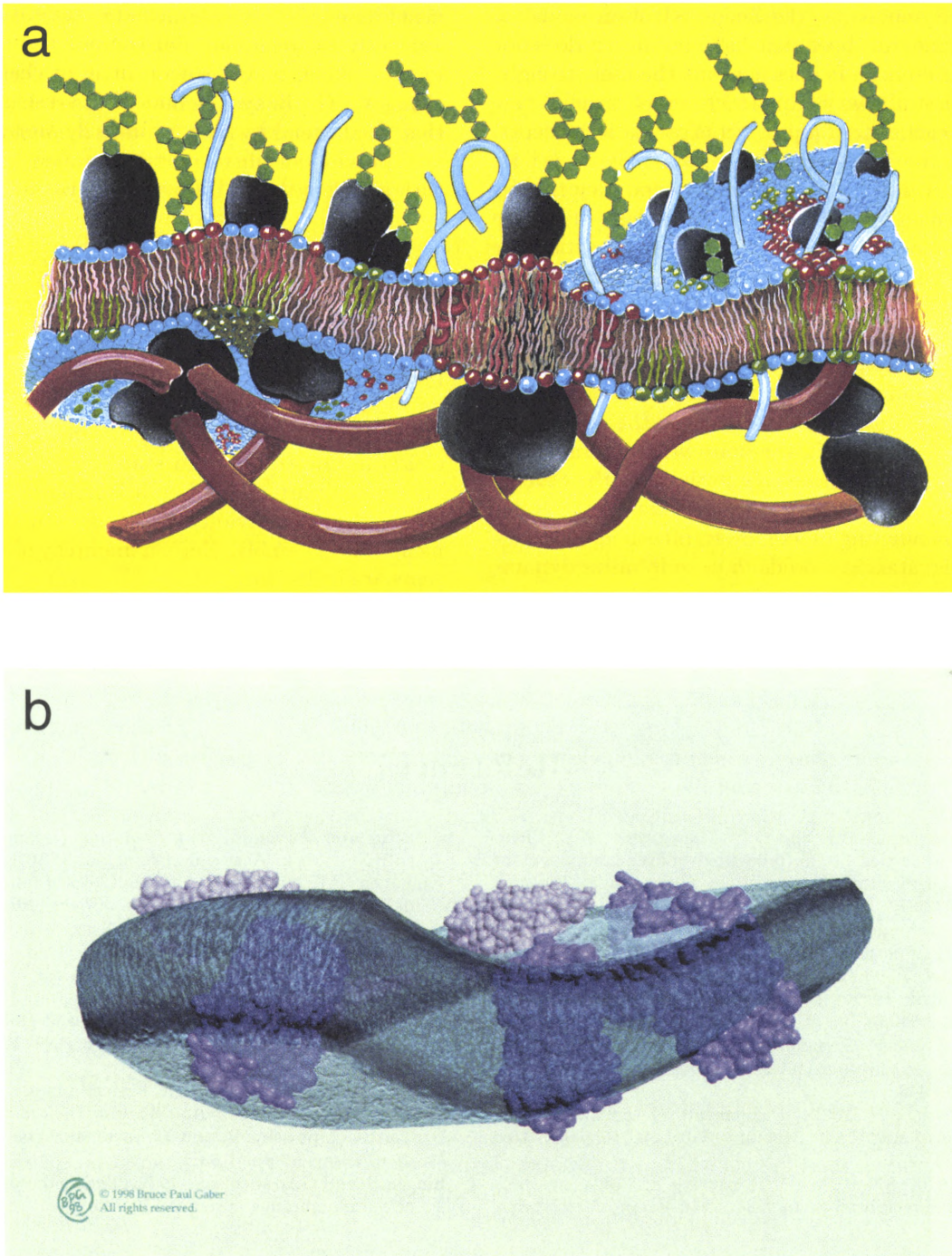


Figure 2.

As was brought out during the final discussions at the Symposium, the Singer-Nicolson model of membranes has been successful because it does not say (too) much. It does not bias the user strongly, and hence allows for broad interpretations of new experimental data and novel theoretical concepts. This is the strength of the model. It is also its weakness, as it in many cases is not very helpful when asking questions about membrane structure and, in particular, about membrane function. For these purposes the model is too generic - in part because it provides too little, or no, insight into membrane protein assembly, lipid bilayer heterogeneity, monolayer or bilayer curvature, and bilayer bending and thickness fluctuations. (One should not forget, however, that the membrane model(s) we use in discussions with our colleagues tend to be even more schematic than the Singer-Nicolson model.)

Moreover, the model, by emphasizing (thermodynamic) stability, tends to de-emphasize dynamics: it does not address the issues relating to conformational transitions in membrane proteins and, just as importantly, the model does not address the conflict between the need for bilayer stability (the membrane must be a permeability barrier and consequently be relatively defect-free) and the need for the bilayer to adapt to protein conformational changes . The bilayer cannot be too stable because that would tend to limit protein dynamics, which may provide insights into the prevalence of lipids with a propensity to form non-bilayer structures.

Finally, a major problem in membrane modeling is how to deal with phenomena that take place far from thermodynamic equilibrium. Non-equilibrium properties are not captured by models like those shown in Fig. 1. Still, non-equilibrium phenomena are the rule, rather than the exception, for biological membranes. The principles of membrane organization, and emergent membrane properties, in non-equilibrium states are basically unknown – in particular when it is important to consider not only individual molecules but the whole membrane assembly. Here, a hierarchy of new concepts are called for.

The answer to the question posed in the title will have to be both a "no" and a "yes".

References

Bloom, M., E. Evans, and O.G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Quart. Rev. Biophys.* 24:293-397.

Danielli, J.F. and H. Davson. 1935. A contribution to the theory of permeability of thin films. *J. Cellular Comp. Physiol.* 7:393-408.

Gennis, R.B. 1989. *Biomembranes.* London: Springer-Verlag. 530pp.

Gorter, E. and F. Grendel. 1925. On bimolecular layers of lipoids on chromatocytes of blood. *J. Exp. Medicine* 41:439-443.

Israelachvili, J.N. 1977. Refinements of the fluid-mosaic model of membrane structure. *Biochim. Biophys. Acta* 469:221-225.

Israelachvili, J.N. 1978. The packing of lipids and proteins in membranes. In *Light Transducing Membranes: Structure, Function, and Evolution* (Deamer, D.W., ed.) New York: Academic Press. pp. 91-107.

Kinnunen, P.K.J. 1991. On the principles of functional ordering in biological membranes. *Chem. Phys. Lipids* 57:375-399.

Lipowsky, R. and E. Sackmann (eds.) 1995. Structure and dynamics of membranes. In *Handbook of Biological Physics. Vol.1A&B* Amsterdam: Elsevier. 1020pp.

Robertson, J.D. 1966. Granulo-fibrillar and globular substructure in unit membranes. *Ann. NY Acad. Sci.* 137:421-440.

Sackmann, E. 1995. Biological membranes. Architecture and function. In *Handbook of Biological Physics. Vol.1A* (Lipowsky, R. and E. Sackmann, eds.) Amsterdam: Elsevier. pp. 1-63.

Singer, S. and G.L. Nicolson. 1972. The fluid mosaic model of cell membranes. *Science* 172:720-730.

Evolution of Membranes from a Physics Perspective

Myer Bloom

Abstract

Several examples are given of biological systems whose physical properties have been optimized via evolutionary processes. It is proposed that the systematic use of an "optimization of physics" hypothesis could lead to the discovery of new and unusual physical properties of biological systems. A speculative example involving membranes is presented.

Myer Bloom
CIAR Program on the Science of Soft Surfaces and Interfaces
Department of Physics and Astronomy
University of British Columbia
Vancouver, B.C., Canada V6T 1Z1
e-mail: bloom@physics.ubc.ca

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:13–17

Introduction

When physical properties of biological materials are carefully studied, it is invariably found that they have been optimized, often in subtle ways. This is presumably a manifestation of evolutionary processes. I will give some well established examples of this "naive Darwinism" in regard to the optimization of *fundamental physical processes* and the *physical properties of biological materials.* Explicit and systematic use of the Optimization of Physics Hypothesis (OPH) might ultimately be helpful in finding unconventional questions leading, with insight, to new discoveries in biology and/or physics. Of course many of these types of questions might be considered to be extremely speculative, stimulating but unlikely to be productive, and I give an example of one currently being discussed (Penrose, 1995). I will discuss various, reasonably well established, aspects of membrane fluidity in terms of the OPH including the role of cholesterol in eukaryotic cells (Bloom et al., 1991; Bloom and Mouritsen, 1995). Finally, I close with some speculations on where "really new Physics" associated with the properties of membranes might be found.

Examples of Optimization of Physics in Biological Systems

Examples from the physical properties of bacteria

Some of the most striking illustrations of the validity of the OPH are drawn from the myriad of phenomena occurring on the cellular length scale governing the function of individual prokaryotic cells. I expand a little on one of these below, *chemotaxis*, in order to illustrate the complexity involved in simultaneously optimizing several physical processes. *Magnetotaxis* is an instructive example of a different type. As described in detail elsewhere (Blakemore and Frenkel, 1981; Bloom and Mouritsen, 1995), *magnetotaxis* illustrates the optimum utilization of the material properties of a magnetic solid, *Magnetite* (Fe_3O_4), in a manner that exploits subtle aspects of magnetism.

Examples from neuroscience

Another class of phenomena, which I do not review because of lack of space, involves how the human brain collects and makes use of information associated with the sensors of our physical surroundings such as touch and other mechanical interactions (the somatic system), hearing (the ear), vision (the eye) and our manner of keeping track of motions and position of the head and body (the vestibular system) (Augustine et al., 1997).

A speculative example involving putative quantum mechanical aspects of consciousness

A stimulating example of a more speculative use of the OPH that has aroused much discussion and controversy in some circles is that some aspects of quantum mechanics and gravitational theory that are poorly understood at the present time may underlie the physical principles governing *consciousness* and how the brain works. Penrose (1995) suggests that quantum mechanical coherence on a macroscopic length scale, originating from the properties of microtubules, may underlie brain function. Although the theoretical basis for his proposal is weak, it should be possible to subject it to an experimental test as discussed briefly at the end of this paper.

Chemotaxis: an example from the physical properties of bacteria illustrating the simultaneous optimization of several fundamental physical processes

Although not necessarily essential for survival, the capability of swimming up or down chemical gradients can provide bacteria with a significant biological advantage in obtaining food or escaping from poisons. The "apparatus" required to achieve such an advantage includes three distinct processes (Purcell, 1977; Berg and Purcell, 1977; Berg, 1988):

(i) a mechanism for swimming as fast as possible under conditions of extremely low Reynolds number (i.e. high viscous drag); this includes development of remarkable rotary motors that drive flagella of ingenious design leading to a swimming speed $v_s \approx 30\mu$m/s $\approx$ 30 body lengths/s.

(ii) an efficient detector of the local density of specific types of molecules; Nature has discovered that 50% efficiency in detection of molecules striking the bacterial surface under diffusively governed conditions requires that only 0.1% of the surface need be covered by detectors.

(iii) a strategy for swimming up or down chemical gradients; optimum conditions for "directed random walk" up and down chemical gradients are obtained by swimming in a straight line for a time $\tau_s > D/(v_s)^2 \approx 1$ s, sufficiently long to "out-swim" the diffusion of the food molecules, which is characterized by a diffusion constant D. At the end of each swimming period, the molecular motor is reversed which, because of the peculiar structure of the flagella, leads to a type of cellular gyration that randomizes the cellular orientation before the next swimming period. The time τ_s is influenced by the measured counting rate for the detected

food molecules during a given swimming period as compared with the previous period. An increase (decrease) in the counting rate leads to a longer (shorter) period of uninterrupted swimming. This "directed random walk" may be shown to be an extremely efficient way of swimming up or down chemical gradients.

Fluid Membranes and the OPH

Biological membranes are fluid under physiological conditions

Although I have never seen this point discussed in detail, the evolutionary driving force behind the ubiquity of membrane fluidity must surely be the important role played by integral membrane proteins in mediating the flow of matter and energy between the inside and outside of cells and organelles, and that conformational freedom is a requirement for protein function. Nature's strategy of building materials using an assembly of "bags" bounded by a fluid has resulted in many natural materials having ultra-soft mechanical properties (Bloom et al., 1991), the "engineering" of which poses problems of a different type than normally encountered by physical scientists and engineers.

Mixtures of lipids mediate the physical properties of membranes

An important control parameter for membrane fluidity is the degree of unsaturation of the fatty acyl chains comprising the lipid molecules that define the membranes; unsaturated bonds favour membrane fluidity at a given temperature and much has been written about that sort of thing in the spirit of the OPH. In a similar vein, there has been much discussion of the potential influence on physical properties of the myriad of lipid molecules found in biological membranes. My own point of view is that most of the different lipid mixtures found in membranes are not there by accident, but have evolved in response to a need to optimize physical and chemical processes.

Cholesterol produces stronger and more impermeable plasma membranes in Eukaryotes

As an example, consider the high concentrations of cholesterol, or equivalent sterols, found in the plasma membranes of almost all eukaryotic cells, but not in prokaryotes. We believe the reason for this to be that cholesterol acts as an alloying agent with phospholipids to produce strong and relatively impermeable lipid bilayers, which is an important feature of plasma membranes of eukaryotic cells (Bloom et al., 1991; Bloom and Mouritsen, 1995).

Hydrophobic matching of lipids and proteins in membranes

Another manifestation of the validity of the OPH is the nature of the interactions between integral membrane proteins and phospholipid molecules, both of which are amphiphilic. The observed matching of the hydrophobic regions of the proteins and the lipid bilayer (in the fluid but not the gel phase!) leads to a strain-free membrane (Mouritsen and Bloom, 1993) which is desirable for efficient membrane function.

Poly-unsaturated Lipids in the Central Nervous System

As stated by Dratz and Holte (1992), see also (Brown, 1994), "retinal rod membranes have a very interesting fatty acid composition, containing 40-50% docosahexaenoic acid (22:6ω-3), DHA. However, an understanding of the role of DHA in this and any other membrane is still in its infancy." This statement is still true even though DHA is known from nutritional studies (Crawford and Marsh, 1989) to be of crucial importance in the development of human brain function. This seems to me an example of a situation in which the OPH might lead to some new questions concerning the physics underlying cell biology. I hope that the following brief discussion of DHA from the perspective of the OPH will stimulate some such fruitful questions.

A mechanical role for DHA in the visual process

This question has implicitly been addressed by Dratz and Holte (1992) and Brown and his coworkers (Brown, 1994), who have arrived at similar conclusions in considering the possible role of DHA in stabilizing the MII (metarhodopsin II) conformation of rhodopsin in the biochemically-well-characterized cascade of molecular forms that follow the absorption of a photon in rod outer segment disk membranes. MII plays a crucial catalytic role in transducing the influence of the photon so as to close Na^+ channels in the plasma membrane and ultimately generate an electrical signal to the brain. In particular, Michael Brown has studied carefully the influence of a variety of phospholipids of different head groups, acyl chain lengths and degrees of poly-unsaturation on the stability of MII in relation to MI (metarhodopsin I). He concludes that an important influence of DHA on the visual process is to promote mechanical conditions for MII stability in conjunction with other lipids that generate negative spontaneous curvature, i.e. H_{II} - formers. Personally, I find Brown's proposals to be plausible and sensible, i.e. it does appear that an important role of DHA is to help stabilize the metastable MII form of rhodopsin long enough to catalyse important steps in the visual process via its coupling to the G-protein *transducin*. I suppose that one can argue that the role of DHA in the gray matter of the brain, where it is also present in large concentrations, could be to activate G-proteins there, but am not convinced that this is the whole story. There are so many disadvantages to this bizarre, difficult-to-make, easily-oxidized molecule that I believe there to be another role for DHA in the visual and central nervous systems that cannot be readily duplicated by other lipids.

Conjecture of an electrical role for DHA in vision and in the brain

In the spirit of the OPH, our group at the University of British Columbia (Miroslava Cuperlovic, Frank Linseisen, James Lloyd-Smith, and myself) has looked for loose ends in the characterization of the physics associated with the photoreception process in which DHA might be playing a crucial and as yet undiscovered role. We are in the process of examining theoretical aspects of the electrical polarizability of poly-unsaturated fatty acyl chains and, with the help of Professor Walter Hardy, we are developing a technique and instrumentation for examining the electrical response under physiological conditions of DHA-containing lipids over a frequency range from radio- ($\approx$ 10 MHz) to microwave- ($\approx$ 10 GHz) frequencies. Our conjecture is that DHA might be playing an as yet undiscovered signaling role in the visual and central nervous systems of mammalian systems. It must be admitted that the basis of our conjecture is somewhat tenuous; it is that, following the beautifully characterized enzymatic effects of MII (Brown, 1994), there seems to be a gap in the explicit theoretical description of the transfer, to the *rod inner segment*, of the electrical signal associated with the closing of Na^+ channels in the plasma membrane of the *rod outer segment*. We speculate that DHA may give rise to unusual elec-

trical properties which speed up and improve the efficiency of signaling over the length of the photoreceptor cell. In making this conjecture, we have been stimulated by the enthusiasm and insights of Professor Michael Crawford, conveyed via both personal communications and his book (Crawford and Marsh, 1989). Based on the striking nutritional requirements for the essential fatty acids in the developing fetus, Professor Crawford has drawn attention to the potential role of DHA in the electrical activity required for brain function.

Finally, the instrumentation under development (see above) will allow us to check some possible experimental manifestations of the proposal by Penrose (1995) that important aspects of brain function and consciousness may require macroscopic quantum coherence to be associated with materials that comprise the brain. Though physicists and biologists have reacted to Penrose's proposal with understandable skepticism, his proposal does have the virtue of being capable of experimental verification.

References

Augustine, G.J., et al.. 1997. *Neuroscience.* Textbook by 15 authors from the Department of Neurology, Duke University. Ed. D. Purves *et al.* (Sinauer Associates).

Berg, H.C. 1988. A physicist looks at bacterial chemotaxis. *In:* Cold Spring Harbor Symposia on Quantitative Biology. LIII, 1-9.

Berg, H.C., and E.M. Purcell. 1977. Physics of Chemoreception. *Biophysics J.* 20:193-219.

Blakemore, R.P., and R.B. Frenkel. 1981. Magnetic navigation in bacteria. *Scientific American (December)* pp. 58-65.

Bloom, M., E. Evans, and O.G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Q. Rev. Biophys.* 24: 293-297.

Bloom, M., and O.G. Mouritsen. 1995. The evolution of membranes. Handbook of Biological Physics. Series editor: A.J. Hoff Volume 1, Structure and Dynamics of Membranes, Eds: R. Lipowsky and E. Sackmann. (Elsevier Publishing Company, Amsterdam, 1995), pp. 65-95.

Brown, M.F. 1994. Modulation of rhodopsin function by properties of the membrane bilayer. *Chem. Phys. Lipids* 73:159-180.

Crawford, M.A., and D.E. Marsh. 1989. The Driving Force (Heinemann, London).

Dratz, E.A., and L. Holte. 1992. The molecular spring model for the function of Docosahexaenoic Acid (22:6ω-3) in biological membranes. *In:* Essential Fatty Acids and Eicosanoids. Eds. A. Sinclair and R. Gibson, Am. Oil Chem. Soc. Champaign, IL, pp. 122-127.

Mouritsen, O.G., and M. Bloom. 1993. Models of lipid-protein interactions in membranes. *Annu. Rev. Biophys. Biomol. Struct.* 22:145-171.

Penrose, R. 1995. Shadows of the Mind. (Vintage, London).

Purcell, E.M. 1977. Life at low Reynolds number. *Am. J. Physics* 45:3-11.

Defining and Imaging Membrane Domains

Michael Edidin

Abstract

Though commonly conceived of as "fluid mosaics" cell plasma membranes are in fact patchy on many size and time scales. These patches, or domains, are detected by many different experimental techniques. They are as likely to arise as the consequence of vesicle traffic and barriers to lateral diffusion as they are to arise as a consequence of specific lateral interactions between their constiutent molecules.

Michael Edidin
Biology Department
The Johns Hopkins University
Baltimore, MD 21218, USA
e-mail: edidin@jhu.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:19–21

Background

Membrane domains broadly speaking are defined as lateral heterogeneities in the composition or state of a liposome or cell membrane. These heterogeneities are usually detected by measuring the behavior of some probe, for example the fluorescence of a lipid analog, mobility of an ESR probe, or the lateral diffusion of antibody-labeled membrane proteins. Evidence for membrane lipid domains has been recently reviewed (Tocanne et al., 1994; Edidin, 1997), as well as evidence for membrane proteins in domains (Edidin, 1996). All these reviews make it clear that the definition of a domain is operational, depending on the time and spatial scales of the techniques used to probe membranes. Indeed, the resolution of the technique used to detect lateral heterogeneities may give a false impression of the size of the domains detected. When discussing membrane domains we need to make sure that all involved are describing domains on the same scale.

Different views of membrane domains also arise from differences in the membranes studied. From the biologist's point of view liposomes are static membranes with well-defined compositions, and constant physical properties; cell plasma membranes are complex mixtures which in many (though not all) cells change rapidly as membrane vesicles arrive at, or leave, the cell surface. Most work on domains in liposomes emphasizes lateral heterogeneity of lipid distribution, lipid domains. Much of the work on cell plasma membranes emphasizes lateral heterogeneity of protein distribution, protein-enriched membrane domains. Again, the sizes of domains and mechanisms of their for-

mation may be quite different in the two types of membrane. The energetics of lipid/lipid interactions may dominate lipid domain formation in liposomes, while the organization of the membrane skeleton seems to be most important for domain formation in cell plasma membranes. Indeed, I have recently argued that lipid domains in the cell surface and endomembranes of actively metabolizing cells are stabilized by proteins (Edidin, 1997).

Experiments

Most of our experiments measure lateral diffusion in order to detect lateral heterogeneities in the membranes. The diffusion measurement is made by labeling the membrane of interest with a fluorescent tag, then bleaching a small spot, $\sim 1\ \mu m^2$, in the fluorescence and following recovery of fluorescence after photobleaching, FRAP. A lateral diffusion coefficient, D, is estimated from the halftime for recovery of fluorescence, and the fraction of mobile label, R, is estimated from the extent of recovery. We expect that the halftime of recovery will depend upon the area bleached, but R is expected to be independent of this area, for areas << than that of the entire labeled surface. Instead, we find that both D and R depend upon on the area bleached. R falls as this area increases over a 50-fold range and D increases. This is the case for both membrane proteins labeled with antibody fragments, and for a lipid analog NBD-PC, but not for another lipid analog, diI (Yechiel and Edidin, 1987). It is likely that the NBD lipid, whose rather polar fluorophore is on an acyl chain, associates with membrane proteins, while the diI lipid analog, whose hydrocarbon chains are unmodified, more directly reports on lipid organization in the membrane.

We interpret the results for R to indicate the confinement of proteins and lipids to domains which are often smaller than the area bleached in our experiment. The increase in D with increasing spot size is probably due to the fact that we are sampling different populations of labeled molecules with large bleaching spots than with small. Rapidly diffusing molecules may not be detected with small spots since the half-time for their recovery is not resolved. Increasing the spot size increases the half-time for recovery of fluorescence and so resolves these species, while slowly diffusing molecules do not contribute to the recovery and instead contribute to the immobile fraction. We have modeled this effect in 3D using mixtures of free fluorescein (mw $\sim$ 300) and fluorescein IgM (mw $\sim$ 900,000) in glycerol. D for the mixture depends on the size of the bleaching spot used in the FRAP measurement, and on the proportions of low mw and high mw molecules in the sample.

This raises the possibility that the domains inferred from the FRAP measurements are artifacts of the resolution of the method; low mobile fractions would simply be due to small D. However, low D in turn suggests that the diffusing species is interacting with some other molecules on the surface and we are led back to the idea that transient lateral associations or confinements contribute to the observed D and R in the FRAP experiment. Indeed, measurements of lateral confinement of membrane proteins, using a laser trap to drag small groups of labeled molecules across the cell surface, define the spatial frequency of obstacles to unhindered lateral mobility (Edidin et al., 1991). These obstacles occur on about the same scale as we use for FRAP measurements and appear to be located in the cytoplasm (the membrane skeleton) rather than in the membrane bilayer or extracellular domain (Edidin et al., 1994).

As just noted, the results from laser trap experiments speak to the statistics of barriers to lateral mobility. They imply, but do not define, the scale of lateral heterogeneities. We have used near-field scanning optical microscopy to image these heterogeneities, resolving membrane organization on a scale of 10's to 100's of nm. At this stage most of our images are of fixed and dried cell membranes,

but the patchiness of fluorescence seen in the images is of the same scale as we found in all of our other experiments. Recently we have imaged proteins in wet cell membranes and these also appear to be distributed in patches.

Even the heterogeneities that we see in cell membranes must be transient. We know from a number of studies tracking Brownian motion of membrane proteins (labeled with nm-size gold beads) that the barriers to lateral mobility are fluctuating (reviewed by Saxton and Jacobson, 1997). If this is so, then in a static membrane, neither budding, nor receiving membrane vesicles, diffusion should randomize the distribution of membrane proteins. However, continued membrane traffic, for example endocytosis of surface membrane and fusion of transport vesicles with this membrane, will constantly create new lateral inhomogeneities, domains, and disrupt existing domains. Indeed, this traffic must be an important factor in disrupting lipid domains which have segregated due to weak interactions between lipid molecules. It seems significant to me that the only good examples of lipid domains detected by FRAP or other methods are in gametes, sperm and eggs, cells whose surface membranes are quiescent for relatively long times (Wolf, 1992).

Of course, our model of membrane domains as transients created by vesicle fusion or vesicle budding begs the question, where in biosynthesis and transport of membranes to the surface are local concentrations of proteins and lipids created? We know very little about the lateral organization of endomembranes, the Golgi complex and the endoplasmic reticulum. However, recent work on the lateral diffusion of endogenous proteins of the Golgi complex (genetically labeled with the green fluorescent protein, GFP) suggests that there are no barriers to lateral diffusion in Golgi membranes (Cole et al., 1996). This observation limits models for selective retention of proteins in the Golgi complex, as well as limiting mechanisms for segregation of proteins into transport vesicles budding from the complex.

References

Cole, N., C. Smith, N. Sciaky, M. Terasaki, M. Edidin, and J. Lippincott-Schwartz. 1996. Diffusional mobility of Golgi proteins in membranes of living cells. *Science* 273: 797-801.

Edidin, M., S.C. Kuo, and M. Sheetz. 1991. Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. *Science* 254:1379- 1382.

Edidin, M., M.C. Zuniga, and M.P. Sheetz. 1994. Truncation Mutants Define and Locate Cytoplasmic Barriers to Lateral Mobility of Membrane Glycoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 91: 3378-3382.

Edidin, M. Getting there is only half the fun. 1996. *Current Topics in Membranes*, 43:1-13.

Edidin, M. 1997. Lipid microdomains in cell surface membranes. *Current Opinion in Structural Biology* 7:528-532.

Saxton, M.J. and K. Jacobson. 1997. Single-particle tracking: applications to membrane dynamics. *Annu. Rev. Biophys. Biomolec. Struct.* 26:373-399.

Tocanne, J-F., L. Cezanne, A. Lopez, B. Piknova, V. Schram, J.F. Tournier, and M. Welby. 1994. Lipid domains and lipid/protein interactions in biological membranes. *Chem. Phys. Lipids* 73:139-158.

Wolf, D.E. 1992. Lipid domains: The parable of the blind men and the elephant. *Comments Mol. Cell Biophys.* 8:83-95.

Yechiel, E. and M. Edidin. 1987. Micrometer Scale Domains in Fibroblast Plasma Membranes. *J. Cell Biol.* 105:755-760.

Membrane Organisation and Assembly - Perspectives From Spin Label ESR (and Other Biophysical Methods)

Derek Marsh

Abstract

Biological membranes are diverse and therefore any new membrane model should be minimalistic, in the spirit of the original Singer and Nicolson model. On this core, any specific features of particular membranes may then be draped. A suitable core model is an extensively proteolysed membrane, which has the advantage that it is amenable to direct experiment, an excellent example being the trypsinised Na,K-ATPase. Various new global features of membrane assembly that must be added to the standard membrane model are discussed here. These include: membrane surfaces as an organising principle for peripherally associated proteins, the selectivity of lipid-protein interactions and possible domain formation, and the intramembranous assembly and association of integral proteins.

Derek Marsh
Abteilung für Spektroskopie
Max-Planck-Institut für Biophysikalische Chemie
D-37070 Göttingen, Germany
e-mail: dmarsh@gwdg.de

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:23–29

In the search for a new membrane model, account must be taken of the functional implications of the thermodynamic and structural self-assembly as an organising principle. Three main aspects are considered here: 1) the effect of membrane surfaces on the organisation and assembly of peripheral membrane proteins; 2) the formation of spatially separated membrane domains by the mutual interaction of the membrane lipid and protein components; 3) the intramembranous assembly of integral membrane proteins and their component transmembrane segments. All the above features also will contribute to or modify both the tendency of the membrane layers towards spontaneous curvature and the macroscopic long-range elastic bending fluctuations that are excited thermally.

The (fluid) lipid bilayer is, of course, taken as read, but even here a couple of remarks are appropriate. (i) Kinks ($g^{\pm}tg^{\mp}$) are not highly favoured over other chain configurations containing *gauche* conformations, according to MD simulations. Nev-

ertheless, they definitely are present because they are seen by IR spectroscopy (Mendelsohn and Snyder, 1996). *Gauche* chain disorder is apparently accompanied by a high degree of intermolecular cooperativity, relative to intrachain cooperativity. (ii) Analysis of a large number of solid-state NMR restraints has arrived at a consensus core structure for the polar headgroup and glycerol backbone of the phospholipid molecules in fluid bilayers (Hong et al., 1996). The phosphatidylcholine headgroup is bent down over the *sn*-2 chain, but differs significantly from the crystal conformation (Marsh, 1997a).

Peripheral Protein Association: Membrane Surfaces as an Organising Principle

The relatively non-specific electrostatic association with negatively charged membrane surfaces can have a profound effect on the structure of peripherally bound membrane proteins.

(A) For precursors and other proteins that are unfolded in solution, membrane association has a structuring effect, causing formation of secondary structure. Examples are the precursor protein apocytochrome *c* (De Jongh et al., 1992; Snel et al., 1994a), and the myelin basic protein (Surewicz et al., 1987). The α-helical structure adopted by apocytochrome *c* constitutes part of the precursor folding process and also presents the protein in a form suitable for membrane translocation and subsequent attachment of the haem group (Snel and Marsh, 1994; Snel et al., 1994b). The structure containing β-sheet that is adopted by the myelin basic protein contributes to the stability of compact nerve myelin. In both cases, the negatively charged membrane surface acts as a chaperone-like organising principle, but also with a well-defined functional role.

(B) Globular cytochrome *c* retains its secondary structure on binding to negatively charged membrane surfaces, but loses much of its native tertiary structure, forming a molten globule-like state (De Jongh et al., 1992; Heimburg and Marsh, 1993). This loosening of the cytochrome *c* structure is also characterised by a reduction of 25-30°C in the temperature for thermal denaturation, upon membrane binding (Muga et al., 1991; Heimburg and Marsh, 1993). Membrane binding of cytochrome *c* also shifts the conformational equilibrium between states with differing redox potentials and haem coordination (Heimburg et al., 1991). This functionally significant conformational change is strongly dependent on the membrane lipid composition and phase state.

(C) There are therefore extensive conformational similarities between the precursor protein apocytochrome *c* and the native holoprotein cytochrome *c*, in their membrane-bound forms, in spite of their totally different folding patterns in solution (De Jongh et al., 1992). Both proteins are structured but are conformationally flexible, on the membrane, which has functional advantages, as already indicated. Molten-globule like, low-pH conformations are also a feature of the membrane interactions and translocation of diphtheria-like toxins and of α-lactalbumin (Montich et al., 1995; Montich and Marsh, 1995).

(D) Biological membrane lipids, in addition to any specific role they may have, provide a general organising structural substrate for the interaction with surface-binding proteins. This stabilises secondary structure giving rise to well-defined interactions, but at the same time labilises any tertiary fold, allowing optimisation of further structural and/or functional interactions. A final property of the membrane surface that has to be considered is its amphiphilic nature, because it constitutes the interface between the polar and apolar regions of the membrane. This immediately offers a further organising principle for the interaction with amphiphilic peptides and proteins, melittin being a classic example (e.g. Kleinschmidt et al., 1997).

Specificity of Lipid-Protein Interactions: Potential for Domain Formation

The specific interaction of a particular lipid species with a particular membrane protein immediately leads to an inhomogeneous distribution of lipids laterally within the membrane. Whether such interactions lead to macroscopic domain formation or phase separation depends on the propensity of the proteins and lipids themselves for mutual interaction (Marsh, 1995a; Heimburg and Marsh, 1996).

(A) There is now an extensive amount of data on the selectivity of interaction of different negatively charged lipid species with integral transmembrane proteins (Marsh, 1995b). This is mostly collected from ESR measurements with different spin-labelled lipids at probe concentrations, and therefore corresponds to a concentration locally at the lipid-protein interface and a corresponding depletion in the fluid lipid pool. The implication is that some basic amino acid side chains are located in the vicinity of the phospholipid headgroups, at the polar/apolar interface of the membrane (Marsh, 1993). Some of these first-neighbour lipid-protein interactions have been found to be of direct functional significance (Marsh, 1987; 1995b). Particularly of note are the aminated local anaesthetics that act as non-competitive blockers of the nicotinic acetylcholine receptor ion channel, at the lipid-protein interface (Horváth et al., 1990). Further, cardiolipin, which is unique to the inner mitochondrial membrane in eukaryotes and displays a selectivity for cytochrome oxidase, stimulates the activity of this latter enzyme in a way that depends on the detailed lipid structure (Abramovitch et al., 1990). Propagation of lipid-protein associations beyond the first boundary shell may be possible, if specific lipid-lipid interactions promote this and the first-shell population is sufficiently high. Spin label experiments have shown that cardiolipin exhibits a generalised selectivity for all lipid sites on cytochrome oxidase, rather than just a single highly specific cardiolipin binding site, and therefore favours the latter condition (Powell et al., 1985). It is notable that fluid lipid may be present, even at very high protein packing densities, although its mobility may be considerably perturbed (Marsh et al., 1978; Páli et al., 1995). Recent high-resolution structures of cytochrome oxidase and bacteriorhodopsin are beginning to reveal the configuration of the lipids next to the protein (Tsukihara et al., 1996; Grigorieff et al., 1996).

(B) For surface binding of basic membrane proteins to negatively charged lipids in a neutral lipid background host, such as phosphatidylcholine, it can be expected that lipids exhibiting a specificity for the protein will be recruited into the region of the bound protein (Sankaram and Marsh, 1993; Marsh, 1995b; Heimburg and Marsh, 1996). This will manifest itself in an increased apparent association constant for binding of the protein to the membrane, above that predicted for binding to a membrane with a homogeneous distribution of the specific charged lipid (Heimburg and Marsh, 1996). The lipid redistribution causes an augmentation of the protein binding. For specific lipids with a strong intrinsic tendency for self-association, this may lead to domain formation and even to a complete lipid phase separation, in which case the protein binding will be maximal.

(C) The relative equilibrium constants, K_r, for selective association of lipids with both integral and peripheral membrane proteins generally are relatively modest and therefore can be modulated by energetically rather weak interactions. However, because of the high effective concentrations relative to bulk solution that are caused by the reduction in dimensionality upon localisation to the membrane, the occupancy at the protein surface by the selective lipid can be quite high (Brotherus et al., 1981; Mosior and McLaughlin, 1992).

(D) The special case of specific interactions of proteins with lipids at, or close to, a chain-melting phase transition has been considered previously (Marsh, 1995a). This propagates preferential interactions with the gel or fluid lipids, thus enhancing domain formation by one of the two

lipid phases. A specific illustration, not involving a phase transition *per se*, is the propagation of a decreased lipid mobility by Ca-ATPase/lipid interactions in sarcoplasmic reticulum membranes at reduced temperatures, which is further enhanced by membrane binding of melittin (Mahaney et al., 1992). Very recently, it has also been found that the first shell of motionally restricted lipid surrounding cytochrome oxidase in negatively charged lipid membranes is augmented further on membrane binding of the peripheral protein cytochrome *c* (Kleinschmidt, J., Powell, G. L., and Marsh, D., unpublished). It therefore seems quite possible that integral proteins and their associated lipid may act as nucleation sites for formation of at least small-scale lipid domains. There are, additionally, also recent spin label ESR data on the effect of integral peptides or proteins on domain formation and domain size in two-phase, two-component lipid mixtures (Sankaram et al., 1994; Piknová et al., 1997). Whether such effects are operative in natural membranes is less clear, because of the complex lipid composition. The potential functional advantages, however, have long been recognised.

Integral Proteins: Intramembranous Assembly

There are at least three aspects that must be considered with regard to the intramembranous structure of integral proteins: 1) the association of transmembrane α-helices; 2) the presence of β-sheet structures; 3) the anchoring effects of interfacial tryptophan residues.

(A) Recent analysis of the stoichiometry of the first lipid shell surrounding integral membrane proteins, which is obtained from spin label ESR measurements, reveals those proteins that do, and those that do not, correspond to the helical sandwich paradigm (Marsh, 1997b, 1993). It is well known that certain transmembrane helices can assemble independently in lipid bilayers. The intramembranous assembly of the Na,K-ATPase is preserved upon removal of the extramembranous portion by extensive trypsinisation (Esmann et al., 1994). Of the different transmembrane segments, the occlusion capacity for Rb^+ is lost on further splitting a 19-kD (4-helix) fragment. Polytopic proteins with multiple transmembrane segments may undergo further intramembranous association to higher (channel) assemblies (e.g. Holzenburg et al., 1993).

(B) In addition to the outer membrane porins, there is increasing evidence for the presence of β-sheet structures in the intramembranous sections of integral proteins, such as the nicotinic acetylcholine receptor, certain P-type ATPases, and models proposed for the pore regions of voltage-gated ion channels (e.g. Marsh, 1996a; 1997c; Heimburg et al., 1997). Characterisation of the lipid-protein interactions and membrane assembly of β-sheet proteins and peptides, either as transmembrane hairpins or as highly tilted strands, has been carried out recently (Horváth et al., 1995; Aggeli et al., 1996; Wolfs et al., 1989).

(C) The role of tryptophan residues in anchoring integral proteins at the membrane polar-apolar interface has been proposed on the basis of high resolution structures, particularly of porins and of gramicidin. Whilst obviously related to issues of hydrophobic matching that can give rise to the formation of protein-rich domains (e.g. Ryba and Marsh, 1992), this aspect deserves further attention with respect to the effects that these residues may have both on helix assembly and on lipid-protein interactions (Killian et al., 1996).

Spontaneous Curvature and Bending Fluctuations: Global Membrane Parameters

Two of the intrinsic membrane properties that are required to complete any functional description of a membrane model are the lateral stress profile and the thermal fluctuations (Marsh, 1996b).

(A) In a net tension-free resting membrane, there may nonetheless exist a non-uniform transbilayer distribution of compensating membrane stresses that locally can achieve quite appreciable values. For a single asymmetric membrane layer, this would correspond to a tendency to spontaneous curvature, R_o^{-1}. A clear illustration of the possible functional implications of a non-uniform stress is afforded by the variation in population of alamethicin channel conductance levels with membrane lipid compositions of varying R_o^{-1} (Keller et al., 1993). These changes exactly mirrored those obtained in corresponding experiments (Opsahl and Webb, 1994) on membranes of constant composition with uniform net tensions (see Marsh, 1996b, for discussion). A further example is the activation of cytochrome oxidase by cardiolipin analogues with different propensities for H_{II}-phase formation (Abramovitch et al., 1990).

(B) Whereas density fluctuations are relatively unimportant for fluid lipid membranes, thermal excitation of large-amplitude bending fluctuations takes place readily. Such fluctuations can be of crucial importance for membrane adhesion and membrane-membrane interactions generally, and have recently taken on considerable significance with respect to molecular dynamics simulations of membrane models and the associated renormalisation of the membrane tension (Feller and Pastor, 1996; Marsh, 1997d).

Conclusion: A New Membrane Model?

The true picture of a biological membrane is a perturbed fluid mosaic with some lateral structure that admits of specific high local packing densities. Proteins are either transmembrane, interfacial, or superficially associated. Partial membrane penetration corresponds to a functional state, as in protein translocation. Sharp gel-to-fluid lipid phase transitions are to be avoided, as is crystalline-state lipid. Non-lamellar lipid phases are likewise to be avoided, although transient embryonic non-lamellar structures are relevant to membrane fusion, and the tendency to spontaneous curvature of the component lipid monolayers can be a functionally significant global membrane parameter. Experimental evidence for these assertions is not necessarily entirely compelling. The membrane has now become the site of intense lipid biochemical activity in the production of lipid second messengers, agonists and responses to stress.

What is most required is a **core** membrane model that is generic for both plasma membranes and intracellular membranes, and contains as much common representative molecular structural and dynamic features as are currently available. On this core, surface elaborations and cartoons depicting particular specialised aspects may be draped, and constrained realistically by the core structure. A strong candidate for such a **core model** is an extensively trypsinized membrane, which is not only accessible to theoretical definition but also realisable experimentally, and has been found in several practical cases to retain the intramembranous assembly of the parent native membrane (e.g., Esmann et al., 1994).

References

Abramovitch, D.A., D. Marsh, and G.L. Powell. 1990. Activation of beef heart cytochrome oxidase by cardiolipin and analogues of cardiolipin. *Biochim. Biophys. Acta* 1020:34-42.

Aggeli, A., N. Boden, Y.-L. Cheng, J.B.C. Findlay, P.F. Knowles, P. Kovatchev, P.J.H. Turnbull, L.I. Horvath, and D. Marsh. 1996. Peptides modelled on the transmembrane region of the slow-voltage-gated IsK potassium channel: structural characterization of peptide assemblies in the β-strand conformation. *Biochemistry* 35:16213-16221.

Brotherus, J.K., O.H. Griffith, M.O. Brotherus, P.C. Jost, J.R. Silvius, and L.E. Hokin. 1981. Lipid-protein multiple binding equilibria in membranes. *Biochemistry* 20:5261-6267.

De Jongh, H.H.J., J.A. Killian, and B. de Kruijff. 1992. A water-lipid interface induces a highly dynamic folded state in apocytochrome *c* and cytochrome *c* which may represent a common folding intermediate. *Biochemistry* 31:1636-1643.

Esmann, M., S.J.D. Karlish, L. Sottrup-Jensen, and D. Marsh. 1994. Structural integrity of the membrane domains in extensively trypsinized Na,K-ATPase from shark rectal glands. *Biochemistry* 33:8044-8050.

Feller, S.E., and R.W. Pastor. 1996. On simulating lipid bilayers with an applied surface tension: periodic boundary conditions and undulations. *Biophys. J.*71:1350-1355.

Grigorieff, N., T.A. Ceska, K.H. Downing, J.M. Baldwin, and R. Henderson. 1996. Electron-crystallographic refinement of the structure of bacteriorhodopsin. *J. Mol. Biol.* 259:393-421.

Heimburg, T., and D. Marsh. 1993. Investigation of secondary and tertiary structural changes of cytochrome *c* in complexes with anionic lipids using amide hydrogen exchange measurements: an FTIR study. *Biophys. J.*65:2408-2417.

Heimburg, T., and D. Marsh. 1996. Thermodynamics of the interaction of proteins with lipid membranes. *In* Biological Membranes. A Molecular Perspective from Computation and Experiment. K. M. Merz Jr., and B. Roux, editors. Birkhäuser, Boston. 405-462.

Heimburg, T., P. Hildebrandt, and D. Marsh. 1991. Cytochrome *c*-lipid interactions studied by resonance Raman and ^{31}P NMR spectroscopy. Correlation between the conformational changes of the protein and the lipid bilayer. *Biochemistry* 30:9084-9089.

Heimburg, T., M. Esmann, and D. Marsh. 1997. Characterization of the Secondary Structure and Assembly of the Transmembrane Domains of Trypsinized Na,K-ATPase by Fourier Transform Infrared Spectroscopy. *J. Biol. Chem.* 272:25685-25692.

Holzenburg, A., P.C. Jones, T. Franklin, T. Páli, T. Heimburg, D. Marsh, J.B.C. Findlay, and M.E. Finbow. 1993. Evidence for a common structure for a class of membrane channels. *Eur. J. Biochem.* 213:21-30.

Hong, M., K. Schmidt-Rohr, and H. Zimmermann. 1996. Conformational constraints on the headgroup and *sn*-2 chain of bilayer DMPC from NMR dipolar couplings. *Biochemistry* 35:8335-8341.

Horváth, L.I., H.R. Arias, H.O. Hankovszky, K. Hideg, F.J. Barrantes, and D. Marsh. 1990. Association of spin-labeled local anaesthetics at the hydrophobic surface of acetylcholine receptor in native membranes from *Torpedo marmorata*. *Biochemistry* 29:8707-8713.

Horváth, L.I., T. Heimburg, P., Kovatchev, J.B.C. Findlay, K. Hideg, and D. Marsh. 1995. Integration of a K^+ channel-associated peptide in a lipid bilayer: conformation, lipid-protein interactions, and rotational diffusion. *Biochemistry* 34:3893-3898.

Keller, S.L., S.M. Bezrukov, S.M. Gruner, M.W. Tate, I. Vodyanoy, and V.A. Parsegian. 1993. Probability of alamethicin conductance states varies with nonlamellar tendency of bilayer phospholipids. *Biophys. J.*65:23-27.

Kleinschmidt, J.H., J.E. Mahaney, D.D. Thomas, and D. Marsh. 1997. Interaction of bee venom melittin with zwitterionic and negatively charged phospholipid bilayers: a spin-label electron spin resonance study. *Biophys. J.*72:767-778.

Killian, J.A., I. Salemink, M. de Planque, G. Lindblom, R.E. Koeppe II, and D.V. Greathouse. 1996. Induction of non-bilayer structures in diacylphosphatidylcholine model membranes by transmembrane α-helical peptides. Importance of hydrophobic mismatch and propose role of tryptophans. *Biochemistry* 35:1037-1045.

Mahaney, J.E., J. Kleinschmidt, D. Marsh, and D.D. Thomas. 1992. Effects of melittin on lipid-protein interactions in sarcoplasmic reticulum membranes. *Biophys. J.*63:1513-1522.

Marsh, D. 1987. Selectivity of lipid-protein interactions. *J. Bioenerg. Biomembr.* 19:677-689.

Marsh, D. 1993. The nature of the lipid-protein interface and the influence of protein structure on protein-lipid interactions. *In* New Comprehensive Biochemistry, Vol. 25. Protein-Lipid Interactions. A. Watts, editor. Elsevier, Amsterdam. 41-66.

Marsh, D. 1995a. Lipid-protein interactions and heterogeneous lipid distribution in membranes. *Mol. Membr. Biol.* 12:59-64.

Marsh, D. 1995b. Specificity of lipid-protein interactions. *In* Biomembranes. Vol. 1. A. G. Lee, editor. JAI Press, Greenwich, CT. 137-186.

Marsh, D. 1996a. Peptide models for membrane channels. *Biochem. J.* 315:345-361.

Marsh, D. 1996b. Components of the lateral pressure in lipid bilayers deduced from H_{II} phase dimensions. *Biochim. Biophys. Acta* 1286:183-223.

Marsh, D. 1997a. Magnetic resonance of lipids and proteins in membranes. *Curr. Opin. Colloid Interface Sci.* 2:4-14.

Marsh, D. 1997b. Stoichiometry of lipid-protein interaction and integral membrane protein structure. *Eur. Bio-*

*phys. J.*26:203-208.

Marsh, D. 1997c. Dichroic ratios in polarized Fourier transform infrared for nonaxial symmetry of β-sheet structures. *Biophys. J.*72:2710-2718.

Marsh, D. 1997d. Renormalization of the tension and area expansion modulus in fluid membranes. *Biophys. J.*73:865-869.

Marsh, D., A. Watts, W. Maschke, and P.F. Knowles. 1978. Protein-immobilized lipid in dimyristoylphosphatidylcholine-substituted cytochrome oxidase: evidence for both boundary and trapped-bilayer lipid. *Biochem. Biophys. Res. Commun.* 81:403-409.

Mendelsohn, R., and R.G. Snyder. 1996. Infrared spectroscopic determination of conformational disorder and microphase separation in phospholipid acyl chains. *In* Biological Membranes. A Molecular Perspective from Computation and Experiment. K. M. Merz Jr., and B. Roux, editors. Birkhäuser, Boston. 145-174.

Montich, G.G., and D. Marsh. 1995. Interaction of α-lactalbumin with phosphatidylglycerol. Influence of protein binding on the lipid phase transition and lipid acyl chain mobility. *Biochemistry* 34:13139-13145.

Montich, G.G., C. Montecucco, E. Papini, and D. Marsh. 1995. Insertion of diphtheria toxin in lipid bilayers studied by spin label ESR. *Biochemistry* 34:11561-11567.

Mosior, M., and S. McLaughlin. 1992. Electrostatics and reduction of dimensionality produce apparent cooperativity when basic peptides bind to acidic lipids in membranes. *Biochim. Biophys. Acta* 1105:185-187.

Muga, A., H.H. Mantsch, and W.K. Surewicz. 1991. Membrane binding induces destabilization of cytochrome *c* structure. *Biochemistry* 30:7219-7224.

Opsahl, L.R., and W.W. Webb. 1994. Transduction of membrane tension by the ion channel alamethicin. *Biophys. J.*66:71-74.

Páli, T., M.E. Finbow, A. Holzenburg, J.B.C. Findlay, and D. Marsh. 1995. Lipid-protein interactions and assembly of the 16-kDa channel polypeptide from *Nephrops norvegicus*. Studies with spin-label electron spin resonance spectroscopy and electron microscopy. *Biochemistry* 34:9211-9218.

Piknová, B., D. Marsh, and T.E. Thompson. 1997. Fluorescence quenching and electron spin resonance study of percolation in a two-phase lipid bilayer containing bacteriorhodopsin. *Biophys. J.*72:2660-2668.

Powell, G.L., P.F. Knowles, and D. Marsh. 1985. Association of spin-labelled cardiolipin with dimyristoylphosphatidylcholine-substituted bovine heart cytochrome *c* oxidase. A generalized specificity increase rather than highly specific binding sites. *Biochim. Biophys. Acta* 816:191-194/ 821, 507.

Ryba, N.J.P., and D. Marsh. 1992. Protein rotational diffusion and lipid/protein interactions in recombinants of bovine rhodopsin with saturated diacylphosphatidylcholines of different chain lengths studied by conventional and saturation transfer electron spin resonance. *Biochemistry* 31:7511-7518.

Sankaram, M.B., and D. Marsh. 1993. Protein-lipid interactions with peripheral membrane proteins. *In* New Comprehensive Biochemistry, Vol. 25. Protein-Lipid Interactions. A. Watts, editor. Elsevier, Amsterdam. 127-162.

Sankaram, M.B., D. Marsh, L.M. Gierasch, and T.E. Thompson. 1994. Reorganization of lipid domain structure in membranes by a transmembrane peptide: an ESR spin label study on the effect of the *Escherichia coli* outer membrane protein A signal peptide on the fluid lipid domain connectivity in binary mixtures of dimyristoyl phosphatidylcholine and distearoyl phosphatidylcholine. *Biophys. J.*66:1959-1968.

Snel, M.M.E., and D. Marsh. 1994. Membrane location of apocytochrome *c* and cytochrome *c* determined from lipid-protein spin exchange interactions by continuous wave saturation electron spin resonance. *Biophys. J.*67:737-745.

Snel, M.M.E., B. de Kruijff, and D. Marsh. 1994a. Interaction of spin-labeled apocytochrome *c* and spin-labeled cytochrome *c* with negatively charged lipids studied by electron spin resonance. *Biochemistry* 33:7146-7156.

Snel, M.M.E., B. de Kruijff, and D. Marsh. 1994b. Membrane location of spin-labeled apocytochrome *c* and cytochrome *c* determined by paramagnetic relaxation agents. *Biochemistry* 33:11150-11157

Surewicz, W.A., M.A. Moscarello, and H.H. Mantsch. 1987. Fourier transform infrared spectroscopic investigation of the interaction between myelin basic protein and dimyristoyl phosphatidylglycerol bilayers. *Biochemistry* 26:3881-3886.

Tsukihara, T., H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, and S. Yoshikawa. 1996. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å E. *Science* 272:1136-1144.

Wolfs, C.J.A.M., L.I. Horváth, D. Marsh, A. Watts, and M.A. Hemminga. 1989. Spin-label ESR of bacteriophage M13 coat protein in mixed lipid bilayers. Characterization of molecular selectivity of charged phospholipids for the bacteriophage coat protein in lipid bilayers. *Biochemistry* 28:9995-10001.

The Bending Elasticity Concept of Biomembrane Function

Erich Sackmann

Abstract

Cell plasma membranes are composite shells composed of two semiflexible layers: the (smectic liquid crystalline) lipid/protein bilayer and the actin based macromolecular network, which is coupled to the bilayer in a controllable way. The bilayer shell is an open system owing to its coupling to microbuds and vesicles. The important role of these coupled shells for the control of cell adhesion is discussed.

Erich Sackmann
Physik Department E22 (Biophysics Laboratory)
Technische Universität München
D-85747 Garching, Germany
e-mail: sackmann@physik.tu-muenchen.de

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:31–34

From a phenomenological point of view biomembranes are two-dimensional smectic liquid crystals which has two important consequences. They exhibit bending elasticity (Evans, 1974) and their lateral organization and local curvature can be sensitively regulated by adsorption of macromolecules. The bending elasticity plays a ubiquitous role for many membrane processes (Sackmann, 1994). Examples are (1) the budding of vesicles by the adsorption of clathrin coats to the lipid bilayer during endocytosis or intracellular trafficking by vesicles (where budding is induced in a controlled way by local bending moments), (2) the control of shapes of cells and intracellular compartments, and (3) the control of adhesion of cells.

A distinct feature of the lipid-protein bilayer is its extremely low bending modulus (κ) which can be reduced to a few k_BT by addition of small amounts of solutes (in particular amphiphiles). Owing to this small bending modulus, membranes exhibit pronounced thermally excited bending undulations which lead to entropy driven repulsion forces between membranes of cells or cell membranes and solids (Helfrich and Harbich, 1984; Lipowsky and Seifert, 1991)). The remarkably strong repulsive potential of the undulation forces of tension free membranes exhibits the same distance (h) dependenc as the van der Waals potential: namely $V_{rep} \approx (k_BT)^2/\kappa h^2$ and can largely compensate the van der Waals attraction between membranes and solids (Rädler et al., 1995). It

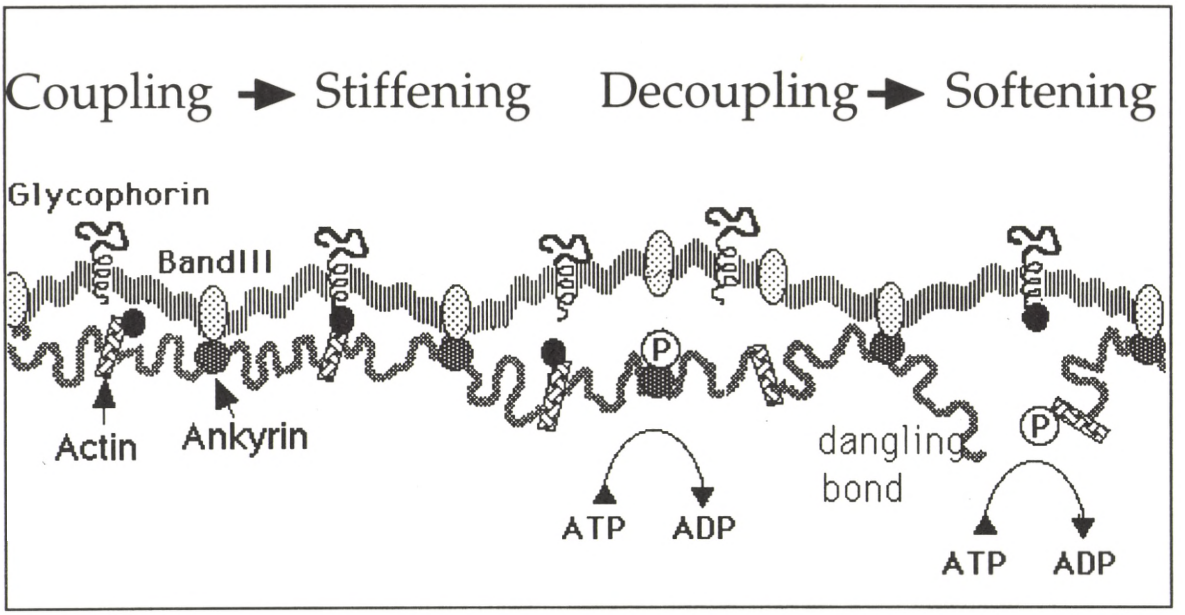


Figure 1. Present view of composite plasma membrane of erythrocytes (viewed parallel to the membrane). The quasi-two-dimensional macromolecular mesh exhibiting roughly a triangular network is composed of flexible spectrin tetramers (end-to-end distances ~80nm) interconnected by actin oligomers. The network is coupled to integral proteins of the bilayer through coupling proteins (ankyrin and band 4.1). However, direct interaction of spectrin with the negatively charged inner leaflet of the bilayer is also possible (as suggested by neutron reflectivity studies of model membrane systems).

may help to prevent the fall of cells into the van der Waals hole.

The development of eukaryotic cells became possible only after Nature found the trick with the composite membrane by combining the hyperelastic bilayers with quasi two dimensional macromolecular networks (Häckl et al., 1998). The red cell membrane is the simplest and most prominent example (Fig. 1).

In most cells the membrane associated macromolecular network consists of a thin shell of the actin based cytoskeleton ($\sim$ 0.2μm thick): the actin cortex. The coupling of the network to the bilayer introduces two new features. The membrane exhibits shear elasticity and the flexural rigidity of the composite membranes can be varied enormously through the degree of coupling of the two shells. Erythrocyte membranes for example are extremely soft (bending $\kappa \sim 10 k_B T$) which is attributed to continuous ATP-driven coupling and decouplings of the coupling protein (ankyrin and band 4.1) to their respective membrane proteins resulting in transient dangling bonds (Sackmann, 1996). The cells exhibit very strong bending fluctuations (so-called flickering) which may be even driven by ATP-dependent chemical fluctuating forces (cf. Sackmann, 1996). The much thicker actin cortex of other cells is coupled to the bilayer through the coupling proteins vinculin plus talin to receptors of the integrin family or through band 4.1-like molecules to receptors for extracellular matrix proteins such as for hyaluronic acid. In this case the composite membrane is much stiffer ($\kappa \sim 1000 k_B T$). Uncoupling the two shells by mutagenic removal of talin, for instance, results in the reduction of the membrane bending modulus from $1000 k_B T$ to $100 k_B T$, the value characteristic for bilayers containing about 50mole% of cholesterol.

The control of the membrane bending elasticity through the bilayer-cytoskeleton coupling plays a key role for the regulation of cell adhesion. This

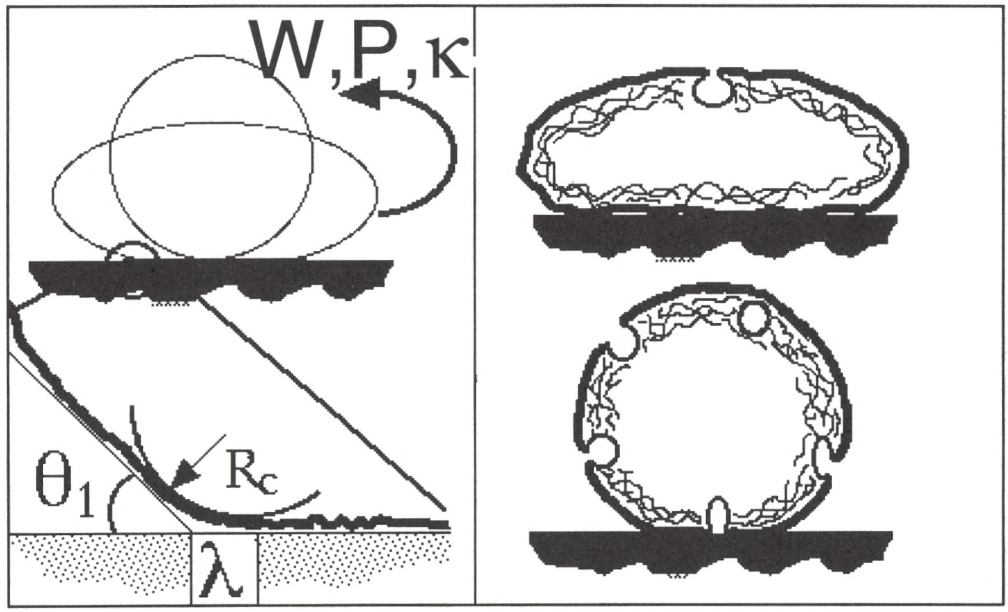


Figure 2. a) Illustration that shell may detach from the substrate by decrease of the adhesion energy (W), by rounding through osmotic swelling (P) and by membrane stiffening (κ). The lower part shows the enlarged contour near the surface. It is determined by the elastic boundary conditions which result in defined values of the contact angle and the contact curvature. b) Demonstration that composite membranes may decrease the effective area-to-volume ratio and unbind by contraction of the cytoskeleton. The excess area of the bilayer can be stored in undulations or in buds or can be released by vesicle fission.

most fascinating phenomenon is controlled by the interplay of specific receptor mediated lock-and-key forces and a manifold of (nonspecific) universal forces (including electrostatic, van der Waals and undulation forces (Sackmann, 1996)); but also by the membrane bending elasticity due to adhesion induced shape changes. The adhesion therefore is described by the general free energy

$$\Delta G = \int pdV - WA_c + \gamma \int dA + \frac{1}{2}\kappa \int \left(\frac{1}{R_1} + \frac{1}{R_2} - C_o\right)^2 dS$$

A_c is the contact area, γ is the membrane tension, R_1 and R_2 are the principal radii of curvature of the soft elastic shell and C_o is the spontaneous curvature. The first three terms correspond to the energy associated with the osmotic pressure, the membrane tension and the adhesion energy, respectively. These contributions describe completely also wetting by fluid droplets. The last term is unique for soft elastic shells and accounts for the bending energy (Evans, 1974) associated with the adhesion induced shape changes.

The adhesion induces tension causing flattening of the membrane near the substrate (which allows the definition of a contact angle θ_1) while the bending elasticity enforces rounding at the contact line (characterized by a contact curvature R_c), cf. Fig. 2. The shape near the surface is essentially determined by the elastic boundary condition which allows quantitative analysis of adhesion solely by considering the shape near the surface by application of microinterferometry (Rädler et al., 1996).

Adhesion induced domain formation is a nucleation process, as illustrated in Fig. 3. The interplay of receptor mediated short range attraction and long range repulsion (e.g. by steric repulsion between macromolecules of glycocalyx and/or undulation forces) leads to the spontaneous forma-

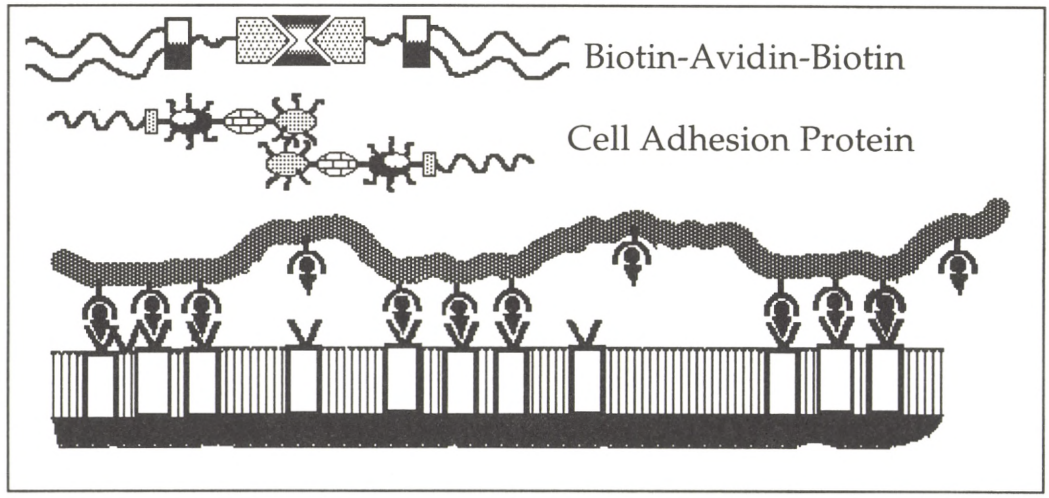


Figure 3. Adhesion induced domain formation. Model system (giant vesicle adhering to supported membrane = phantom cell) showing adhesion plaque formation. The glycocalyx is mimicked by lipopolymers. The specific forces are generated by contact site A cell adhesion molecules or by biotin-streptavidin-biotin linkage. Adhesion plaques are formed as a consequence of interplay of short range receptor mediated forces and long range (polymer-induced electrostactic or undulation-induced) forces favouring different equilibrium distances (after Sackmann (1996)).

tion of tight adhesion domains. The origin of the spontaneous formation of tight adhesion plaques is a consequence of the fact that the attraction and repulsion forces favour different equilibrium distances. In order to form tight bonds, work has to be performed against the repulsive force (e.g. undulation force or any other long range repulsion force). Single bonds are therefore extremely unstable.

The local bending of the bilayer at the transition between the domains is associated with an elastic line tension τ. The line tension must be overcompensated by the gain in adhesion energy, ΔW, which requires a minimum radius of the adhesion cluster $\rho \geq 2\tau/\Delta W$. τ is of the order $\kappa d/R_c^2$ where R_c is the contact curvature (defined in Fig. 2) and d is the height difference between the two types of adhesion domains. Typically $R_c \cong$ 100nm, $d = 100$nm, $W \sim 10^{-6}\mathrm{J/m}^2$ and one finds critical radii of the order of 1μm.

References

Evans, E.A. 1974. Bending resistance and chemically induced moments in membrane bilayers. *Biophys. J.* 14: 923-931.

Helfrich, W., and W. Harbich. 1984. Undulation, Steric Interaction and Cohesion of Fluid Membranes. *Il Nuovo Ciminto* 3D: 137-151.

Häckl, W., M. Bärmann, and E. Sackmann. 1998. Shape changes of self-assembled actin bilayer composite membranes. *Phys. Rev. Letters* in press.

Lipowsky, R., and U. Seifert. 1991. Adhesion of Membranes: A Theoretical Perspective. *Langmuir* 7: 1867-1873.

Rädler, J.O., T.J. Feder, H. Strey, and E. Sackmann. 1995. Fluctuation analysis of tension controlled undulation forces between giant vesicles and solid substrates. *Phys. Rev. E* 51: 4526-4536.

Sackmann, E. 1994. Membrane Bending Energy Concept of Vesicle- and Cell- Shapes and Shape Transitions. *FEBS Letters* 346: 3-16

Sackmann, E. 1996. Supported Membranes: Scientific and Practical Applications. *Science* 271: 43-48.

Membranes are Fluid Mosaics With Transient Tiles (Dynamic Domains) Defined by Curvature, Traffic and Tension

Michael P. Sheetz

Abstract

Cellular plasma membranes are fluid mosaics (Singer and Nicolson, 1972) that contain clearly differentiated domains or tiles, which are submicron to micron-sized areas of membrane containing a five to twenty-fold concentration of membrane proteins. Although many domains are produced by strong bonds between the membrane glycoproteins and the cytoskeleton resulting from ligand or extracellular matrix binding to the glycoprotein, there are other domains on free membranes that do not involve glycoprotein-cytoskeleton bonds. We have experimental evidence that freely diffusing membrane glycoproteins involved in cell motility are concentrated by membrane curvature (0.1-0.3 micron radius of curvature). This raises the possibility of controlling domain distributions and sizes by altering cell shape and the balance of the bilayer couple. We speculate on the basis of known membrane exchange rates and membrane structural studies that membrane traffic and membrane thickness can produce domains in flat membrane regions. Traffic and thickness domains could be controlled by altering membrane traffic and membrane tension which we have shown are linked. Our working model of the plasma membrane emphasizes the dynamic domain structures defined by membrane curvature, thickness and trafficking under the control of the intensive variables of membrane tension and bilayer couple imbalance.

Michael P. Sheetz
Department of Cell Biology
Duke University Medical Center
Durham, NC 27710, USA
e-mail: m.sheetz@cellbio.duke.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:35–39

Membrane Morphology and Dynamics

The fluid nature of plasma membrane lipids allows the bilayer to follow the contours of the cytoskeleton and enables glycoproteins to diffuse laterally over many microns. Membrane bilayers lack structural integrity and generally can not hold non-spherical shapes in the presence of external forces.

Although membranes do not have significant shear elasticity, they do have a significant bending modulus and can generate significant bilayer couple forces (Sheetz and Singer, 1974). Bending stiffness and bilayer couple forces are major components in determining red cell shape although rigidification of the membrane skeleton can block shape changes. In cells with a rigid cytoskeleton, the bending stiffness and bilayer couple forces appear to play only a secondary role in overall cell shape since the forces needed to alter the cytoskeleton are greater than those needed to alter membrane shape. It is unknown whether or not bending and bilayer couple forces play a significant role in membrane trafficking or other cellular functions. Recent experimental evidence indicates that the tension within the plasma membrane is a major factor in many cellular functions, basically defining endocytosis rate and motility (Sheetz and Dai, 1996). Diffusion in plasma membranes is relatively rapid with typical diffusion coefficients in the range of 10^{-9} to 10^{-10} cm^2/sec. (Sheetz, 1993). There are, however, barriers to lateral diffusion (corrals (Sheetz, 1983)) that have been observed experimentally with single particle tracking (Sheetz et al., 1989) or with membrane drag experiments (Edidin et al., 1991). The decreased rate of glycoprotein diffusion in biological membranes compared with model bilayers is the result of viscous drag and not the corrals in the membrane except perhaps in the case of the erythrocyte (see review Sheetz (1993)). In some membranes the size of diffusional corrals can be several microns or more indicating that the attachments between the membrane and the cytoskeleton need not be numerous or strong (Kucik et al., 1990; 1991). Thus diffusion studies support a model in which the membrane is draped over the cytoskeleton raising the question of how the two structures are bonded.

Curvature-Dependent Domains

Some membrane glycoproteins are concentrated ten to twenty fold in curved regions of plasma membranes. Concentrations were observed in positively curved regions at the leading edges of lamellipodia and along filopodia in diffusion measurements of integrins (Schmidt et al., 1993) and the embryonic membrane antigen of mouse cortical neurons (Sheetz et al., 1990). Integrins are involved in motility on specific extracellular matrix molecules and their concentration at leading edges is important because those are the first regions to reach new matrix sites. The function of the embryonic membrane antigen is unknown but its localization to the leading portions of growth cones put it in a logical location to participate in pathfinding and other signaling functions. To better understand which portion of the molecule is involved in localizing the integrin ($\alpha\beta$ heterodimer) to curved regions, we observed movement of $\beta 1$ integrin with and without its cytoplasmic tail and found no difference (Schmidt et al., 1993). Concentration in positively (outward) curved regions could be a property of the external domain. It is logical to postulate that other proteins may partition to inwardly curved regions such as those in clathrin or caveolar pits. Many of the seven-transmembrane spanning receptors do partition to clathrin pits upon ligand binding and get endocytosed. Although adaptin binding to the cytoplasmic surface of the receptors may hold them in clathrin pits, they may initially partition into pits because of the negative curvature. Membranes are naturally curved when they conform to the thin regions of lamellipodia and filopodia and the energy for curving the membranes comes from the membrane-cytoskeleton interaction and tension in the membrane. Contributions from imbalances in the bilayer couple will contribute but they will be a secondary factor except in cases like the erythrocyte or where there is an extreme imbalance in the bilayer couple.

Membrane Traffic and Domains

Because many plasma membranes turn over every 30 minutes, it is possible to maintain specialized domains of the plasma membrane based upon membrane dynamics (see review by Sheetz (1993)). There are several studies of the primary exocytic and endocytic sites that show them to be localized on fibroblasts. This raises the possibility that specific glycoproteins could be added preferentially in one place and endocytosed preferentially in another. This need not represent a bulk flow of membrane, which has not been observed in fibroblasts (Kucik et al., 1990) but the flow of membrane seen in growing axons constitutes an extreme example (Dai and Sheetz, 1995). The membrane corrals would favor diffusional domain formation by increasing dramatically the time needed for membrane proteins to equilibrate over the surface by diffusion.

Membrane Thickness and Domains

It is known that bilayer thickness changes dramatically with changes in lipid state from fluid to gel. There is little reason, however, to believe that biological membrane lipids under physiological conditions will go into a gel state. However, there is an increasing interest in membrane rafts wherein proteins of similar properties but not biochemically bonded codistribute on the cell surface. One possibility is that the proteins promote a change in bilayer thickness that causes a phase separation in the plane of the membrane. Since aggregation often stimulates the coalescence of the components in the membrane, there is likely a cooperative component to the process. Although there may be mechanisms for achieving separation in the water phase, it is attractive to think of how the membrane phase could cause coalescence and membrane thickness is a property that could produce a phase separation of proteins and some lipids. Since many of the raft effects are related to cholesterol and blocked by cholesterol depletion, a mechanism based on the lipid phase is preferred.

Membrane Tension Effects on Endocytosis and Motility and Possibly Domains

A critical element in our understanding of membrane domains that has been often overlooked is tension in the plane of the membrane. For example, in the case of curvature-dependent domains, it is evident that high membrane tensions will decrease the amount of highly curved membrane. We have found that extension of lamella and filopodia is decreased dramatically at high membrane tensions resulting in a loss of those structures and consequently of curved membrane surfaces. In addition, we have found that high membrane tensions dramatically inhibit endocytosis (Dai et al., 1997) which would result in the loss of any domains produced by membrane exchange. Finally, any domain that relied upon a physical change in the bilayer such as its thickness would be sensitive to changes in membrane tension. In the case of thickness, increased tension would favor the formation of a thinner bilayer phase. Even in the case of domains formed by cytoskeletal corrals, membrane tension is a critical parameter for holding the membrane and cytoskeleton in contact and preventing bleb formation.

Membrane-Cytoskeleton Interaction and Domains

Separation of the membrane from the cytoskeleton results in bleb formation. Blebs are essentially membranous bubbles of cytoplasm without cytoskeletal support. Glycoprotein diffusion rates are increased in blebs. Disruption of actin filament organization with cytochalasin B or D stimulates blebbing which indicates that actin or actin-associated proteins such as spectrin or actin-binding protein (ABP) are important for membrane cytoskeleton adhesion. Indeed, the loss of ABP results in spontaneous bleb formation and similar behavior is seen after spectrin depletion in anti-sense experiments. The dynamic interaction between membrane and cytoskeleton contribute to many kinds of transient domains in the plasma membrane. Many different types of experiments indicate that adhesion between membrane and cytoskeleton is a result of multiple weak bonds that are dynamic. Thus, the membrane and cytoskeleton adhere nearly continuously along their interface without many high affinity bonds except at specialized adhesive contacts.

Summary

Our working model of plasma membrane structure has evolved from that of a fluid mosaic in which the membrane and the cytoskeleton are extensively bonded through multiple membrane protein-cytoskeleton linkages (which is indeed the case for the erythrocyte membrane). We now believe that a tension produced by the endocytic machinery holds the membrane on the cytoskeleton where weak, non-bonding interactions reinforce the complex. Strong membrane-cytoskeleton bonds depend upon activation of membrane proteins by ligand binding or other signals. Domains of protein concentration without cytoskeletal attachment are clearly formed by positive membrane curvature and can result from membrane dynamics with or without diffusional corrals. We speculate that negative curvature and membrane thickness can also produce membrane domains. Membrane tension is a critical intensive variable that can modulate domains either indirectly through its effects on cell motility (shape) and endocytosis or directly in the case of thickness domains.

References

Dai, J., P. Beall, and M.P. Sheetz. 1997. The secretion-coupled endocytosis correlates with tension changes in RBL 2H3 cells. *J. Gen. Physiol.* 110:1-10.

Dai, J., and M.P. Sheetz. 1995. Axon membrane flows from the growth cone to the cell body. *Cell* 83: 693-701.

Edidin, M., S.C. Kuo, and M.P. Sheetz. 1991. Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. *Science* 254: 1379-82.

Kucik, D.F., E.L. Elson, and M.P. Sheetz. 1990. Cell migration does not produce membrane flow. *J. Cell Biol.* 111: 1617-22.

Kucik, D.F., S.C. Kuo, E.L. Elson, and M.P. Sheetz. 1991. Preferential attachment of membrane glycoproteins to the cytoskeleton at the leading edge of lamella. *J. Cell Biol.* 114: 1029-36.

Schmidt, C.E., A.F. Horwitz, D.A. Lauffenburger, and M.P. Sheetz. 1993. Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell Biol.* 123: 977-91.

Sheetz, M.P. 1983. Membrane skeletal dynamics: role in modulation of red cell deformability, mobility of transmembrane proteins, and shape. *Semin. Hematol.* 20: 175-88.

Sheetz, M.P. 1993. Glycoprotein motility and dynamic domains in fluid plasma membranes. *Annu. Rev. Biophys. Biomol. Struct.* 22: 417-31.

Sheetz, M.P., N.L. Baumrind, D.B. Wayne, and A.L. Pearlman. 1990. Concentration of membrane antigens by forward transport and trapping in neuronal growth cones. *Cell* 61: 231-41.

Sheetz, M.P., and J. Dai. 1996. Modulation of membrane dynamics and cell motility by membrane tension. *Trends Cell Biol.* 6: 85-89.

Sheetz, M.P., and S.J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA* 71: 4457-61.

Sheetz, M.P., S. Turney, H. Qian, and E.L. Elson. 1989. Nanometre-level analysis demonstrates that lipid flow does not drive membrane glycoprotein movements. *Nature* 340: 284-8.

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of cell membranes. *Science* 175:720-731.

Dynamic Heterogeneities of the Lipid Matrix of Biological Membranes and Associated Protein Function

Rodney L. Biltonen

Abstract

This report will focus on the physical basis for lateral heterogeneity in the lipid matrix of the bilayer membrane and how the associated properties can play a role in the function of membrane-associated proteins. The propensity for lateral heterogeneity can provide sites of lateral density fluctuations, enhance both lipid and protein domain formation and lead to local and global structural changes in the bilayer. The activation of phospholipase A_2 and protein kinase C will be used as two examples demonstrating the correlation between lateral heterogeneity and protein function.

Rodney L. Biltonen
Departments of Biochemistry and Pharmacology
University of Virginia Health Sciences Center
Charlottesville, VA 22908, USA
e-mail: rlb1t@uva.pcmail.virginia.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:41–45

The lipid matrix of the biological membrane is a heterogenous mixture of lipids existing in varying degrees of individual disorder, the distribution of which is continuously fluctuating with time. Both compositional fluctuations and thermal fluctuations can lead to local and global structural changes. In addition, the composition of the membrane can be altered by the action of enzymes such as the lipases producing fatty acids, lysophospholipids and diacylglycerols. These lipid metabolites can act as protein effectors and also produce alterations in lipid distribution and membrane structure. It is these heterogenieties in composition and structure and alterations therein which could play major roles in the activation and modulation of membrane-associated protein function (Biltonen, 1990; Kinnunen, 1991; Mouritsen and Biltonen, 1993; Mouritsen and Jørgensen, 1994).

In single component systems, fluctuations in the degree of order or structure of any individual lipid are dictated by the energies and degeneracies of the accessible structural states and, to the

first approximation, the various nearest-neighbor interactions. Long range correlations in structure may exist depending upon the differences in the nearest-neighbor interactions among the various accessible states. For example, the parameter determining the extent of long-range correlation in a system in which a lipid can exist in only one of two states is

$$W = E_{a,b} - 1/2(E_{a,a} + E_{b,b})$$

where $E_{i,j}$ is the interaction energy between two neighboring lipids in states i, j. If $W = 0$ the distribution of lipid in the two structural states over the membrane lattice will be random, whereas if $W = \infty$ the lattice of any single membrane will be found completely in either state a or b. Between these two extremes the membrane lattice will exist in some nonrandom lipid organization producing a distribution of clusters of varying size. The cluster distribution will depend upon the intrinsic probabilities of the lipid existing in a or b and the magnitude of the interaction term, W (Heimburg and Biltonen, 1996; Jerala et al., 1996).

The probability of the lipid existing in state a or b in a single component system is a function of the free energy change associated with a structural transition. In a two component system this probability is also related to the composition of the system. Even if the matrix is in one phase (e.g. gel) compositional heterogeneities will exist. In this case W will be defined by the interactions between the chemically distinct species, defined by such aspects of the system as acyl-chain mismatch (Mouritsen and Jørgensen, 1994). In more complex systems, the situation becomes more difficult to define, but the basic features remain: The bilayer will possess heterogeneities in structure and composition which could result in distinct cluster or domain formation which, in turn, could strongly influence membrane-associated protein function.

Domain formation within the lipid matrix of the biological membrane can influence function in several ways. For example, density fluctuations at the interface between distinct domains could ease the penetration of peptide sequences into the hydrophobic interior of the membrane or for the lipid hydrolases, these regions could be the location for optimal interaction between the enzyme and substrate. The existence of clusters could enhance the probability of protein aggregation on the lipid surface by promoting regions of high local concentrations of protein (Sperotto and Mouritsen, 1993; Tocanne et al., 1994; Heimburg and Biltonen, 1996). Here it should be noted that the clusters or domains do not need to preexist, but protein localization will occur if only there is a propensity for lipid domain existence. Proteins can induce domain formations via thermodynamic coupling between lipid-lipid interactions and protein-lipid interactions. Finally, lipid domains and protein/lipid domains can alter the kinetics of processes requiring diffusion in the plane of the membrane (Thompson et al., 1995).

If domain formation, rather than the existence of a particular phase, is functionally important then it is expected that an extremum in certain functional behaviors (e.g. enzyme activation) will be observed in regions of compositional or temperature variation where domain coexistence is maximal. This is indeed observed for the activation of phospholipase A_2 as a function of temperature (Hønger et al., 1996) and the activation of protein kinase C as a function of diacylglycerol mole fraction (Dibble et al., 1996). Although the two examples to be discussed are related to lipid systems in the gel state or the gel-liquid coexistence region, regions of compositional heterogeneity in binary mixtures in the liquid state have been suggested by Pedersen et al. (1996) using fluorescence energy transfer and Lehtonen et al. (1996) using pyrene excimer fluorescence.

Phospholipase A_2 exhibits a long lag period until maximal activity is observed when large unilamellar vesicles composed of saturated phosphatidylcholine vesicles are used as the substrate. This lag period is a function of temperature, substrate and calcium concentration. This lag period exhibits a minimum very close to the gel-liquid phase transition temperature, a temperature at which phase coexistence occurs (Romero et al., 1987) . Recently, Hønger et al. (1996) using a ten-state model in a Monte Carlo simulation, demonstrated that the inverse of the lag period was correlated with the calculated amount of interfacial area between gel and liquid domains.

It has previously been shown that the onset of rapid hydrolysis occurred when a precise amount of reaction product (fatty acid and lysophospholipid) were produced (Bell and Biltonen, 1992). Burack, et al. (1995) showed that this onset was not the result of enhanced binding because of the presence of the negatively charged fatty acid. Rather, they demonstrated with a pyrene-labelled fatty acid that lateral phase separation of the reaction products from the bulk lipid occurred at that point (Burack and Biltonen, 1994). This compositional demixing then leads to a change in morphology of the aggregated substrate (Hønger et al., 1995; Burack et al., 1997). While the details of this activation process have remained elusive, these studies clearly show that activation is intimately related to the structural and compositional heterogeneity of the lipid bilayer.

Protein kinase C is activated on membrane surfaces and requires the presence of phosphatidylserine and diacylglycerol. The negatively-charged lipid enhances binding of the protein to the surface and the diacylglycerol (Nakamura and Nishizuka, 1994; Newton and Keranen, 1994) is thought to be a specific activator, the mechanism of which is unclear. Working with the hypothesis that the structural aspects of the membrane (Sando et al., 1992; Sando and Chertihin, 1996), specifically lipid domain formation, could play a key role in the activation process, we undertook a study of a ternary system containing these components (Dibble et al., 1996; Hinderliter et al., 1997). The pseudo phase diagram of a 1:1 mixture of phosphatidylcholine/phosphatidylserine as a function of the concentration of various diacylglycerols was determined with differential scanning calorimetry. The results of these experiments indicated that a 1:1:1 compound formation occurred and that in the range of about 0.1 to 0.35 mole-fraction of the diacylglycerol two phases, one rich in diacylglycerol and one poor in diacylglycerol, coexisted in the gel state. The formation of the compound was verified by FTIR experiments which demonstrated that all three components (including the diacylglycerol which melts at a low temperature) melt concurrently in the vicinity of 25°C when the mole-fraction of diacylglycerol was sufficiently high.

What was most significant was that the activity of protein kinase C increased as the system in the gel state entered the putative compositional phase coexistence region, achieving a maximum near mole-fraction 0.2 and then falling as the diacylglycerol component was further increased. This observed increase in activity is consistent with domain formation playing a role in activation and could be related to lipid-induced clustering of the protein or the existence of domain interfaces. It is interesting to note that Glaser and coworkers have recently shown by fluorescence microscopy (Yang and Glaser, 1995; Glaser et al., 1996) that lipid and substrate clustering appear to be related to protein kinase C activation (see also McLaughlin, this volume.)

One aspect of compositional heterogeneity or phase separation that deserves special mention is that strong coupling between lateral phase separation or compositional differences in the two planes of the bilayer and membrane mechanical stress can exist. Such stress can lead to strong fluctuations in the plane normal to the membrane surface and could be important in many membrane processes as has been pointed out by Sackmann and Feder (1995). Lundbæk and Andersen (1994) have also suggested that changes in membrane deformation energy caused by certain membrane-modifying compounds such as lysophospholipids can modulate membrane channel function. In extreme cases it could lead to major changes in membrane morphology (Burack and Biltonen, 1994; Hønger et al., 1995; Burack et al., 1997). Finally, it should be emphasized that fluctuations in the structure of the lipid matrix of the biological membrane can occur over a very wide time scale. While some local fluctuations may be very rapid (nanoseconds to microseconds), relaxations in cooperative structure may occur on the time scale of milliseconds to hours (van Osdol et al., 1991; Biltonen and Ye, 1994; Jørgensen et al., 1996). The dynamic coupling of the bilayer fluctuations to protein processes is an area that is yet to be investigated in much detail. In any case, it seems clear that lipid domain formation is an important feature of membrane structure and that the existence of distinct domains and their time-dependent

variation may play significant roles in cellular function.

***

The work reported herein was supported by the National Science Foundation, The National Institutes of Health and The Danish Technical Research Council and is the result of many extensive collaborations including those with Drs. Thomas Hønger, Kent Jørgensen and Ole G. Mouritsen of the Technical University of Denmark and Drs. John D. Bell, W. Richard Burack, Andrew R. Dibble, Martha Gadd, Anne K. Hinderliter, Guilleromo Romero, Julianne J. Sando, Kim K. Thompson and Thomas E. Thompson of the University of Virginia. I am deeply indebted to them for their experimental contributions and many useful discussions.

References

Bell, J.D., and R.L. Biltonen. 1992. Molecular Details of the Activation of Soluble Phospholipase A_2 by Lipid Bilayer Substrates: Comparison of Computer Simulations with Experimental Results. *J. Biol. Chem.* 267: 11046-11056.

Biltonen, R.L. 1990. A Statistical Thermodynamic View of Cooperative Structural Changes in Phospholipid Bilayer Membranes: Their Potential role in Biological Function. *J. Chem. Therm.* 22:1-19.

Biltonen, R.L. and Q. Ye. 1994. Differential Scanning and dynamic calorimetric studies of cooperative phase transitions in phospholipid bilayer membranes. *Subcellular Biochemistry* 23:121-160.

Burack, W.R. and R.L. Biltonen. 1994. Lipid Bilayer Heterogeneities and Modulation of Phospholipase A_2 Activity. *Chem. Phys. Lipids* 73:209-222.

Burack, W.R., M.E. Gadd, and R.L. Biltonen. 1995. Modulation of Phospholipase A_2: Identification of an Inactive Membrane-bound State. *Biochemistry* 34:14819-14828.

Burack, W.R., A.R.G. Dibble, M.M. Allieta, and R.L. Biltonen. 1997. Changes in Vesicle Morphology Induced by Lateral Phase Separation Modulate Phospholipase A_2 Activity. *Biochemistry* 36:10551-10557.

Dibble, A.R.G., A. Hinderliter, J.J. Sando, and R.L. Biltonen. 1996. Lipid lateral heterogeneity within phosphatidylcholine/phosphotidylserine/diacylglycerol vesicles and its influence on protein kinase C activation. *Biophysical J.* 71:1877-1890.

Glaser, M., S. Wanaski, C.A. Buser, V. Boguslavsky, W. Rashidzada, A. Morris, M. Rebecchi, S.F. Scarlata, L.W. Runnels, G.D. Prestwich, J. Chen, A. Aderem, J. Ahn, and S. McLaughlin. 1996. Myristoylated alenine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. *J. Biol. Chem.* 271:26187-26193.

Heimburg, T., and R.L. Biltonen. 1996. A Monte-Carlo Simulation Study of Protein Induced-Heat Capacity Changes and Lipid-Induced Protein Clustering. *Biophysical J.* 70:84-96.

Hinderliter, A.K., A. Dibble, R.L. Biltonen, and J.J. Sando. 1997. Activation of Protein Kinase C by Coexisting Diacylglycerol- enriched and Diacylglycerol-Poor Lipid Domains. *Biochemistry* 36:6141-6148.

Hønger, T.E.R., A.R.G. Dibble, W.R. Burack, M.M. Allietta, and R.L. Biltonen. 1995. Vesicle Morphology Modulates Phospholipase A_2 Activity. *Biophys. J.* 68: A218.

Hønger, T., K. Jørgensen, R.L. Biltonen, and O.G. Mouritsen. 1996. Correlation between phospholipase A_2 activity and dynamic lipid bilayer micro-heterogeneity. *Biochemistry* 35:9003-9006.

Jerala, R., P.F.F. Almeida, and R.L. Biltonen. 1996. Simulation of the Gel-Fluid Transition in a Membrane Composed of Lipids with Two connected Acyl Chains: Application of a Dimer-move Step. *Biophysical Journal* 71:609-615.

Jørgensen, K., A. Klinger, M. Braiman, and R.L. Biltonen. 1996. Slow non-equilibrium dynamical rearrangement of the lateral structure of a lipid membrane. *J. Phys. Chem.* 100:2766-2769.

Kinnunen, P.K.J. 1991. On the Principles of Functional Ordering in Biological Membranes. *Chem. Phys. Lipids* 57:375-399.

Lehtonen, J.Y.A., J.M. Holopainen, and P.K.J. Kinnunen. 1996. Evidence for the Formation of Microdomains in Liquid Crystalline Large Unilamellar Vesicles Caused by Hydrophobic Mismatch of the Constituent Phospholipids. *Biophys. J.* 70:1753-1760.

Lundbæk, J.A. and O.S. Andersen. 1994. Lysophospholipids Modulate Channel Function by Altering the Mechanical Properties of Lipid Bilayers. *J. Gen. Physiol.* 104:645-673.

Mouritsen, O.G., and R.L. Biltonen. 1993. Protein-Lipid Interactions and Membrane Heterogeneity. The New Comprehensive Biochemistry: Protein-Lipid Interactions (A. Neuberger and L.L.M. Van Dennen, eds.) *Elsevier.* pp. 1-39.

Mouritsen, O.G., and K. Jørgensen. 1994. Dynamical Order and disorder in lipid bilayers. *Chem. Phys. Lipids* 73:3-25.

Nakamura, S.-I., and Y. Nishizuka. 1994. Lipid Mediators and Protein Kinase C Activation for the Intracellular Signaling Network. *J. Biochem.* 115:1029-1034.

Newton, A.C., and L.M. Keranen. 1994. Phosphatidyl-L-Serine is Necessary for Protein Kinase C s High-Affinity Interaction with Diacylglycerol-Containing Membranes. *Biochemistry* 33:6651-6658.

Pedersen, S., K. Jørgensen, T.R. Baekmark, and O.G. Mouritsen. 1996. Indirect Evidence for Lipid Domain Formation in the Transition Region of Phospholipid Bilayers for Two-Probe Fluorescence Energy Transfer. *Biophys. J.* 71:554-560.

Romero, G., K. Thompson, and R.L. Biltonen. 1987. The Activation of Procine Pancreatic Phospholipase A_2 by Dipalmitoylphosphatidylcholine Large Unilamellar Vesicles. *J. Biol. Chem.* 262: 13476-13480.

Sackmann, E., and T. Feder. 1995. Budding, fusion and domain formation in mixed lipid vesicles induced by later phase separation and macromolecular condensation. *Mol. Memb. Biol.* 30:4516:4521.

Sando, J.J., M.C. Maurer, E.J. Bolen, and C.M. Grisham. 1992. Role of Cofactors in Protein Kinase C Activation. *Cell. Signalling* 4:595-609.

Sando, J.J. and O.I. Chertihin. 1996. Activation of Protein Kinase C by Lysophosphatidic Acid: Dependence on Composition of Phospholipid Vesicles. *Biochem. J.* 317:583:588.

Sperotto, M.M., and O.G. Mouritsen. 1993. Lipid Enrichment and Selectivity of Integral Membrane Proteins in Two Component Lipid Bilayers. *Eur. Biophys. J.* 22:323-328.

Thompson, T.E., M.B. Sankaram, R.L. Biltonen, D. Marsh, and W.L.C. Vaz. 1995. Effects of domain structure on in-plane reactions and interactions. *Mol. Memb. Biol.* 12:157-162.

Tocanne, J.-F., L. Dupou-Cezanne, A. Lopez, E. Perochon, B. Piknova, V. Schram, J.-F. Tournier, and M. Welby. 1994. Lipid Domains and Lipid/Protein Interactions in Biological Membranes. *Chem. Phys. Lipids* 73:139-158.

van Osdol, W., Q. Ye, M.J. Johnson, and R.L. Biltonen. 1991. The Effect of Chainlength and Vesicle Size on the Dynamics of the Gel-Liquid Crystalline Transition of Lecithin Bilayers. *Biophysical J.* 59:775-785.

Yang, L. and M. Glaser. 1995. Membrane Domains Containing Phosphatidylserine and Substrate can be Important for the Activation of Protein Kinase C. *Biochemistry* 34:1500-1506.

Membranes Display Nano-scale Heterogeneity - Beating the Randomness of the Fluid Lipid Bilayer[1]

Ole G. Mouritsen

Abstract

Based on theoretical and experimental evidence obtained from model studies it is argued that lipid bilayer membranes display lateral heterogeneity in the nano-meter range. It is pointed out that this small-scale structure is important for various membrane functions.

Ole G. Mouritsen
CIAR Program on Science of Soft Surfaces and Interfaces
Department of Chemistry
Technical University of Denmark
DK-2800 Lyngby, Denmark
e-mail: ogm@kemi.dtu.dk

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:47–53

Introduction[2]

The conventional text-book picture of the fluid lipid-bilayer component of biological membranes as a fairly structure-less and disordered 'fluid mosaic' solvent needs modification (Mouritsen and Jørgensen, 1997). The lipid bilayer displays distinct static and dynamic structural organization on a small scale in the nano-meter range (1-100 nm), e.g. in terms of differentiated lipid domains. This scale is in between the molecular scale, accessible by most spectroscopic techniques as well as molecular dynamics calculations, and the micrometer scale which is accessible by scattering and various microscopy techniques. The lateral heterogeneity is an inescapable physical consequence of the fact that the lipid bilayer is a many-particle system, a structured fluid, controlled by cooperative molecular interactions. The question is not whether small-scale lipid domains and lateral heterogeneity is present in membranes, but rather on which time- and length-scales these phenomena occur. Indirect and some direct experimental and theoretical evidence is now becoming available in favor of small-scale lipid-domain formation. Furthermore, evidence is accumulating that small-scale lipid bilayer structure is of importance for the functioning of biological membranes, including trans-membrane permeation, activity of membrane-bound enzymes and receptors, as well as morphological changes at the cell surface. The small-scale structure of membranes may be a way

[1] An extended version of this contribution can be found in Mouritsen and Jørgensen (1997).
[2] Abbreviation: DC_nPC: di-acyl phosphatidylcholine with n carbon atoms in each acyl chain.

of beating the randomness of the fluid bilayer, creating specific differentiated regions or reaction compartments which can steer and possibly trigger the biochemical reactions of the membrane.

Small-Scale structure vs. Large-Scale Structure

Many cell membranes are known to be differentiated into lipid domains on the micrometer scale (Edidin, 1997; Sheetz, 1993; Tocanne, 1992; Bergelson et al., 1995) which seems to be important for gene expression (Norris and Madsen, 1995), for binding and activation of certain enzymes, e.g. protein kinase C (Glaser et al., 1996; Yang and Glaser, 1996), as well as membrane biogenesis and cell division (Welby et al., 1996). Domains of this size, which are visible in fluorescence microscopy, have long lifetimes and may be the result of equilibrium phase separation processes or coupling of the membrane proteins to the cytoskeleton (Kusumi and Yashushi, 1996; Felsenfeld at al., 1996). Indirect evidence exists of domain formation on a smaller, but less well-defined scale, in erythrocyte (More et al., 1996; 1997) and mitochondrial (Richelli et al., 1995) membranes.

Theoretical Evidence for Nano-scale Structure

It can been argued on general theoretical grounds, that lipid domains as well as differentiated regions near proteins should exist also on a much smaller scale, in the nano-meter range, due to the basic physical fact that the fluid lipid bilayer, in the capacity of being a many-particle system consisting of $10^9 - 10^{10}$ molecules, should behave as a structured liquid with a morphology and a lateral organization that reflect the underlying phase equilibria of the membrane (Mouritsen and Jørgensen, 1995; Mouritsen and Kinnunen, 1996). Membrane organization and lipid domains in the nano-meter range are much more difficult to investigate experimentally, and to date mostly indirect evidence of their existence is available. In this situation, computer-simulation calculations have proved useful to investigate, within simple model-membrane systems, under which conditions small-scale membrane structure arises, how it can be characterized, and how it can be modulated, e.g. by solutes, sterols, and integral membrane proteins. Examples of small-scale structure predicted by Monte Carlo computer-simulation calculations are shown in Fig. 1. It is not possible from these calculations to determine the lifetime of the domains.

The examples in Fig. 1 illustrate how order and compartments can arise from cooperative many-particle phenomena such as density fluctuations (Fig. 1a), compositional fluctuations (Fig. 1b), and phase separation processes (Fig. 1c).

The mutual relationship between nano-scale lipid bilayer heterogeneity as well as protein organization has also been studied by computer-simulation techniques (Mouritsen at al., 1996). It is found, that the proteins so to speak 'pick up' the small-scale structure which leads to the formation of differentiated regions around the protein of a structure and composition that is different from that of the bulk, cf. Fig. 1d. This type of cooperative lipid-protein interaction is important for protein organization in membranes, in particular two-dimensional crystallization, cf. Fig. 1e (Gil et al., 1997). Even more strikingly, special compartments can be formed if the integral proteins exhibit activity that drives the membrane assembly into a non-equilibrium steady state, cf. Fig. 1f (Sabra and Mouritsen, 1998).

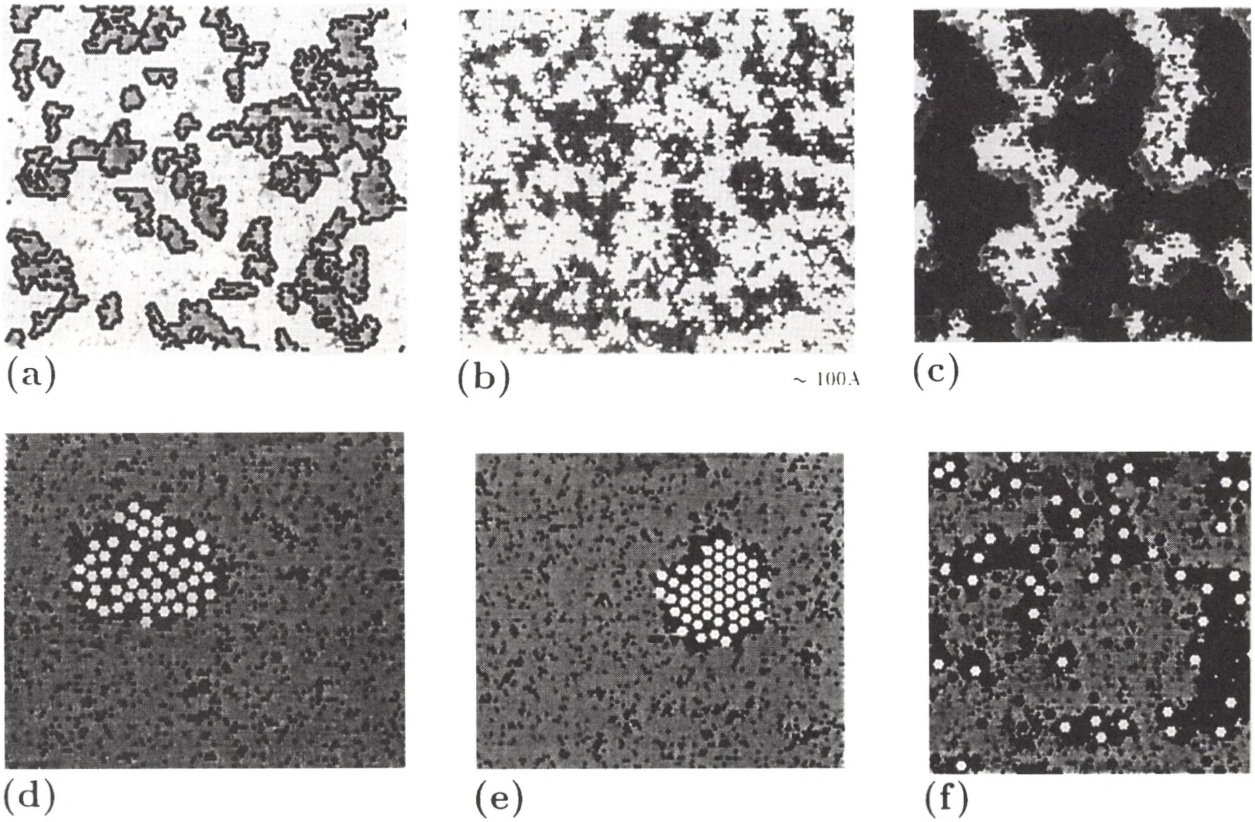


Figure 1. Computer simulation of lipid-bilayer nano-scale lateral organization. **(a):** Density fluctuations in fluid $DC_{14}PC$ bilayers. **(b):** Compositional fluctuations in fluid bilayer mixtures of $DC_{12}PC$ and $DC_{20}PC$. **(c):** Gel-fluid phase separation pattern in mixtures of $DC_{12}PC$ and $DC_{18}PC$. **(d):** A protein-rich domain formed as a capillary condensate in lipid bilayer mixtures of $DC_{14}PC$ and $DC_{18}PC$. **(e):** Two-dimensional protein crystallization induced by lipid phase structure in binary lipid bilayers. **(f):** Steady-state compartmentalization of lipid bilayer mixtures of $DC_{14}PC$ and $DC_{18}PC$ in the presence of active proteins.

Experimental Evidence for Nano-Scale Structure

In one-component lipid bilayers, fluorescence energy transfer techniques (Pedersen et al., 1996; Loura et al., 1996) and eximer-to-monomer fluorescence emission spectroscopy (Lehtonen et al., 1996) have been used to provide indirect evidence for lipid-domain formation, cf. Fig. 1a. These techniques are adequate to probe lateral structure in the range of about 10 nm. Evidence for lipid micro-domains consisting of less than fifty lipid molecules in the gel phase of lipid binary mixtures of phospholipids with different acyl-chain lengths comes from infrared spectroscopy (Mendelsohn et al., 1995). Although some evidence of fluid-fluid immiscibility has been reported for binary lipid mixtures with different polar head groups, phosphatidylserine and PC (Hinderliter et al., 1994), nano-scale lipid structure formation in the fluid phase of binaries, cf. Fig. 1b, has yet to be demonstrated experimentally.

In a series of experiments, T.E. Thompson and collaborators (Piknová et al., 1996; Schram et al., 1996) have over the last several years, by

different spectroscopic techniques, provided compelling evidence in favor of small-scale gel-phase domain formation and compartmentalization of binary mixtures of PC lipids with different acyl-chain lengths in the gel-fluid phase coexistence region, cf. Fig. 1c. The results have provided information on the diffusional characteristics of the probes which have been interpreted in terms of static percolation structures and domains of fractal structure. The sizes of the domains range up to several hundred molecules, depending on the degree of ideality of the mixture, the composition, and the temperature. It is difficult to reconcile the observation of static domains of finite size in the gel-fluid coexistence region of binary lipid mixtures with equilibrium thermodynamics and it is possible that these domains are consequences of slow non-equilibrium rearrangements (Jørgensen and Mouritsen, 1995; Jørgensen et al., 1996) or coupling to the bilayer curvature.

The binding of peripheral proteins, such as cytochrome c (Kinnunen et al., 1994) has been shown to be controlled by the formation of lipid domains of charged lipids leading to a local surface charge density that is larger that the average surface charge and hence locally promote binding. The binding, in turn, of the charged proteins leads to a stabilization of the small-scale lipid domain structure (Heimburg and Biltonen, 1996).

A number of investigations have been conducted on lipid bilayers reconstituted with integral membrane proteins in order to investigate the effect on lipid structure due to a hydrophobic mismatch between the lipid-bilayer thickness and the trans-membrane hydrophobic membrane domain of the protein (Mouritsen and Bloom, 1993; Mouritsen, 1998). A mismatch is theoretically predicted to not only influence the phase equilibria of the membrane but also to lead to a special lipid profile at the lipid-protein interface. In the case of lipid bilayers with more than one lipid species, this could lead to a physical lipid selectivity and sorting mechanism at the lipid-protein interface and the creation of a special local lipid structure that may be markedly different from the bulk, both with respect to molecular composition and lipid acyl-chain conformational order.

Studies of bacteriorhodopsin in binary lipid mixtures of $DC_{14}PC$-$DC_{18}PC$ lipids have indicated that the presence of the protein has a significant influence on the percolation properties and the topology of the domains in the gel-fluid phase coexistence region (Piknová et al., 1997; Schram and Thompson, 1997). It has been suggested, based on a combined fluorescence energy transfer experiment and Monte Carlo simulation on bacteriorhodopsin reconstituted into an even more non-ideal mixture of PC lipids, $DC_{12}PC$-$DC_{18}PC$, that the hydrophobic matching principle acts as to sort the two lipid species at the lipid-bacteriorhodopsin interface, such that there is an enrichment of that lipid species, which under the thermodynamic conditions given, most easily adapts to hydrophobic matching (Dumas et al., 1997). These results are clear manifestations of small-scale, nano-meter-range structural organization of lipid-protein assemblies.

Relationship Between Nano-Scale Membrane Structure and Function

The function of certain lipases active on lipids in aggregated form seems to be controlled by the nano-scale structure of the lipid bilayer. In the case of phospholipase A_2 it has been demonstrated that the activity, measured via the lag time for the onset of rapid hydrolysis, has a systematic dependence on temperature and acyl-chain length of the PC substrate which correlates closely with the degree of heterogeneity, i.e. small-scale bilayer structure (cf. Fig. 1a) (Hønger et al., 1996). The ac-

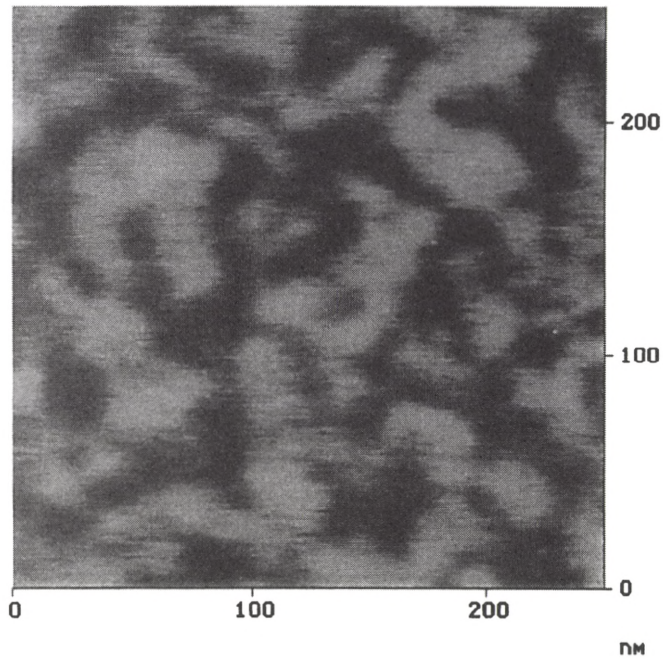


Figure 2. Image of a lipid monolayer 1:1 mixture of $DC_{14}PC$ and $DC_{18}PC$ on a solid support obtained by atomic force microscopy (L. Kildemark, T. Bjørnholm, and O. G. Mouritsen, unpublished).

tivity of phospholipase C has also been correlated with the small-scale lipid structure in terms of defects or lipid domains (Basanez et al., 1996).

Protein kinase C is an enzyme active at membranes that requires charged lipids, like phosphatidylserine, to bind to the membrane and small amounts of diacylglycerol to become activated. It has been argued (Dibble et al., 1996; Hinderliter et al., 1997) that a reason why small amounts of diacylglycerol may be sufficient is that, due to the small-scale structure of the lipid bilayer substrate, the diacylglycerol is not randomly distributed, but rather accumulates into domains and that the protein kinase C activity is controlled by the interface between regions enriched in and poor in diacylglycerol.

Several other membrane-bound proteins and receptors have been investigated in order to unravel the relationship between function and the physical properties of the lipid membrane, in particular the possible small-scale structure. Examples, where recent experimental data have been obtained and taken to support this relationship, include the activity of the nicotinic acetylcholine receptor channel (Zanello et al., 1996) and the activation by phosphatidylglycerol of diglucosyldiacylglycerol synthase from *Acholeplasma laidlawii* membranes (Karlsson et al., 1996), controlled by accumulation of the activator into domains that are formed in response to a hydrophobic mismatch between the acyl chains of the activator and the host bilayer.

Small-Scale Lipid Domains — are They Really There?

Using glancing incidence neutron scattering, Gliss et al. (1997) have recently found evidence for lipid domain formation on the scale of about 50Å in supported lipid bilayer mixtures of $DC_{14}PC$ and $DC_{18}PC$ in their gel-fluid coexistence region. Scattering studies provide information in reciprocal space. A direct visualization of nano-meter scale domains would have to use ultra-sensitive surface probe techniques, like atomic force microscopy. Preliminary studies applying such techniques to lipid monolayers, prepared in thermodynamic state equivalent to that of a bilayer and transferred to a solid support, have lead to real-space pictures like the one in Fig. 2 (L. Kildemark, T. Bjørnholm, and O. G. Mouritsen, unpublished). This picture shows the presence of a small-scale domain structure characterized by a length scale of about 100-200Å. — Nano-scale lipid domains undoubtedly exist in lipid bilayer membranes. The existence of this type of nano-scale heterogeneity may be a way of compartmentalizing the membrane thereby beating the randomness of the fluid lipid bilayer.

***

Members of the MEMPHYS group at the Technical University of Denmark are thanked for discussion on various aspects of the work described here.

References

Basanez, G., J.L. Nieva, M. Goñi, and A. Alonso. 1996. Origin of the lag period in the phospholipase C cleavage of phospholipids in membranes. Concomitant vesicle aggregation and enzyme activation. *Biochemistry* 35:15183-15187.

Bergelson, L.O., K. Gawrisch, J.A. Feretti, V. Blumenthal (eds.). 1995. Domain organization in biological membranes. Special issue of *Mol. Memb. Biol.* 12, 1-162.

Dibble, A.R.G., A.K. Hinderliter, J.J. Sando, and R.L. Biltonen. 1996. Lipid lateral heterogeneity in phosphatidylcholine/phosphatidylserine/diacylglycerol vesicles and its influence on protein kinase C activation. *Biophys. J.* 71:1877-1890.

Dumas, F., M.M. Sperotto, C. Lebrun, J.-F. Tocanne, and O.G. Mouritsen. 1997. Molecular sorting of lipids by bacteriorhodopsin in DLPC/DSPC lipid bilayers. *Biophys. J.* 73:1940-1953.

Edidin, M. 1997. Lipid microdomains in cell surface membranes. *Comm. Mol. Cell Biophys.* 8:73-95.

Felsenfeld, D.P., D. Choquet, and M.P. Sheetz. 1996. Ligand binding regulates the directed movement of $\beta 1$ integrins on fibroblasts. *Nature* 383:438-440.

Gil, T., M. Sabra, J.H. Ipsen and O.G. Mouritsen. 1997. Wetting and capillary condensation as means of protein organization in membranes. *Biophys. J.* 73:1728-1741.

Glaser, M., S. Wanaski, C.A. Buser, V. Boguslavsky, W. Rashidzada, A. Morris, M. Rebecchi, S.F. Scarlata, L.W. Runnels, G.D. Prestwich, L. Chen, A. Aderem, J. Ahn and S. McLaughlin. 1996. Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. *J. Biol. Chem.* 271:26187-26193.

Gliss, C., R. Günther, S. Odenbach, O. Randl, A. Meierhofer, and T.M. Bayerl. 1997. Direct detection of domains in phospholipid bilayers by glancing incidence difraction of neutrons. (preprint).

Heimburg, T. and R.L. Biltonen. 1996. A Monte Carlo simulation study of protein-induced heat capacity changes and lipid-induced protein clustering. *Biophys. J.* 70:84-96.

Hinderliter, A.K., A.R.G. Dibble, R.L. Biltonen, and J.J. Sando. 1997. Activation of protein kinase C by coexisting diacylglycerol/enriched and diacylglycerol-poor lipid domains. *Biochemistry* 36:6141-6148.

Hinderliter, A.K., J. Huang, and G.W. Feigenson. 1994. Detection of phase separation in fluid phosphatidylserine/phosphatidylcholine mixtures. *Biophys. J.* 67:1906-1911.

Hønger, T., K. Jørgensen, R.L. Biltonen, and O.G. Mouritsen. 1996. Systematic relationship between phospholipase A_2 activity and dynamic lipid bilayer heterogeneity. *Biochemistry* 35:9003-9006.

Jørgensen, K., A. Klinger, M. Braiman, and R.L. Biltonen. 1996. Slow nonequilibrium dynamical rearrangement of the lateral structure of a lipid membrane. *J. Phys. Chem.* 100:2766-2769.

Jørgensen, K. and O.G. Mouritsen. 1995. Phase separation dynamics and lateral organization of two-component lipid membranes. *Biophys. J.* 95:942-954.

Karlsson, O.P., M. Rytömaa, A. Dahlqvist, P.K.J. Kinnunen, and Å. Wieslander. 1996. Correlation between bilayer lipid dynamics and activity of the diglucosyldiacylglycerol synthase from *Acholeplasma laidlawii* membranes. *Biochemistry* 35:10094-10102.

Kinnunen, P.K.J., A. Kõiv, J.Y.A. Lehtonen, M. Rytsmaa, and P. Mustonen. 1994. Lipid dynamics and peripheral interactions of proteins with membrane surfaces. *Chem. Phys. Lipids* 73:181-207.

Kusumi, A. and S. Yashushi. 1996. Cell surface organization by the membrane skeleton. *Curr. Opin. Cell Biol.* 8:566-574.

Lehtonen, J.Y.A., J.M. Holopainen, and P.K.J. Kinnunen. 1996. Evidence for the formation of microdomains in liquid crystalline large unilamellar vesicles caused by hydrophobic mismatch of the constituent phospholipids. *Biophys. J.* 70:1753-1760.

Loura, L.M.S., A. Fedorov, and M. Prieto. 1996. Resonance energy transfer in a model system of membranes: Application to gel and liquid crystalline phases. *Biophys. J.* 71:1823-1836.

Mendelsohn, R., G.L. Liang, H.L. Strauss, and R.G. Snyder. 1995. IR spectroscopic determination of gel state miscibility in long-chain phosphatidylcholine mixtures. *Biophys. J.* 69:1987-1998.

Moore, D.J., R.H. Sills, and R. Mendelsohn. 1997. Conformational order of specific phospholipids in human erythrocytes: correlations with changes in cell shape. *Biochemistry* 36:660-664.

Moore, D.J., R.H. Sills, N. Patel, and R. Mendelsohn. 1996. Conformational order of phospholipids incorporated into human erythrocytes: and FTIR spectroscopic study. *Biochemistry* 35:229-235.

Mouritsen, O.G. 1998. Self-assembly and organization of lipid-protein membranes. *Curr. Opin. Colloid Int. Sci.* 3:78-87.

Mouritsen, O.G. and M. Bloom. 1993. Models of lipid-protein interactions in membranes, *Ann. Rev. Biophys. Biomol. Struct.* 22:145-171.

Mouritsen, O.G. and K. Jørgensen. 1995. Micro-, nano- and meso-scale heterogeneity af lipid bilayers and its influence on macroscopic membrane properties. *Mol. Memb. Biol.* 12:15-20.

Mouritsen, O.G. and P.K.J. Kinnunen. 1996. Role of lipid organization and dynamics for membrane functionality. In K. M. Merz Jr. and B. Roux B (eds.): *Biological membranes: A molecular perspective from computation and experiment.* Boston: Birkhäuser, pp.463-502.

Mouritsen, O.G., M.M. Sperotto, J. Risbo, Z. Zhang, and M.J. Zuckermann. 1996. Computational approach to lipid-protein interactions in membranes. *Adv. Comp. Biol.* 2:15-64.

Mouritsen, O.G. and K. Jørgensen. 1997. Small-scale lipid-membrane structure: simulation vs. experiment. *Curr. Opin. Struct. Biol.* 7:518-527.

Norris, V. and M.S. Madsen. 1995. Autocatalytic gene expression occurs via transertion and membrane domain formation and underlies differentiation in bacteria: A model. *J. Mol. Biol.* 253:739-748.

Pedersen, S., K. Jørgensen, T. Bækmark, and O.G. Mouritsen. 1996. Indirect evidence for lipid-domain formation in the transition region of phospholipid bilayers by two-probe fluorescence energy transfer. *Biophys. J.* 71:554-560.

Piknová, B., D. Marsh, and T.E. Thompson. 1996. Flourscence-quenching study of percolation and compartmentalization in two-phase lipid bilayers. *Biophys. J.* 71:892-897.

Piknová, B., D. Marsh, and T.E. Thompson. 1997. Flourescence quenching and ESR study of percolation in a two-phase lipid bilayer containing bacteriorhodopsin. *Biophys. J.* 72:2660-2668.

Richelli, F., S. Gobbo, G. Jori, C. Salet, and G. Moreno. 1995. Temperature-induced changes in fluorescence properties as a probe of porphyrin microenvironment in lipid membranes. *Eur. Biochem. J.* 233:165-170.

Sabra, M.C. and O.G. Mouritsen. 1998. Steady-state compartmentalization of lipid membranes by active proteins. *Biophys. J.* 74:745-752.

Sheetz, M. 1993. Glycoprotein motility and dynamic domains in fluid plasma membranes. Annu. Rev. Biophys. Biomol. Struct. 22:417-431.

Schram, V., H.N. Lin, and T.E. Thompson. 1996. Topology of gel-phase domains and lipid mixing properties in phase separated two-component phosphatidylcholine bilayers. *Biophys. J.* 71:1811-1822.

Schram, V. and T.E. Thompson. 1997. Influence of the intrinsic membrane protein bacteriorhodopsin on the gel phase domain topology in two-component phase-separated bilayers. *Biophys. J.* 72:2217-2225.

Tocanne, J.-F. 1992. Detection of lipid domains in biological membranes. Comm. Mol. Cell Biophys. 8:53-72.

Welby, M., Y. Poquet, and J.-F. Tocanne. 1996. The spatial distribution of phospholipids and glycolipids in the membrane of the bacterium *Micrococcus luteus* varies during the cell cycle. *FEBS Letters* 384:107-111.

Yang, L. and M. Glaser. 1996. Formation of membrane domains during the activation of protein kinase C. *Biochemistry* 35:13966-13974.

Zanello, L.P., E. Aztiria, S. Antollini, and F.J. Barrantes. 1996. Nicotinic acetylcholine receptor channels are influenced by the physical state of their membrane environment. *Biophys. J.* 70:2155-2164.

The Biological Membrane Structure Paradigm: One View of Its Current Condition

Thomas E. Thompson

Abstract

The biological membrane structure paradigm is alive and well, however many details, most of which are pecular to specific membranes or membrane-localized systems, need to be worked out at the molecular level.

Thomas E. Thompson
Department of Biochemistry
University of Virginia
Charlottesville, VA 22908, USA
e-mail: tet@avery.med.virginia.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:55–57

It has been known for many years that biological membranes separate the cell from its environment and compartmentalize the cell interior. The various membranes playing these vital roles are comprised of roughly equal weight percent protein and lipid, with carbohydrates constituting less than 10% in the plasma and nuclear membranes. Although there are many hundreds of molecular species present in any one membrane, the general organization of the generic components was established in the 1960's (Korn, 1969; Henn and Thompson, 1969). In this model, the lipids, all of which have their polar and nonpolar portions geographically segregated within each molecule, are arranged in a continuous bimolecular leaflet with the polar portions of the constituent molecules forming the two bilayer faces, and the nonpolar portions, the interior of the bilayer. This structure forms the permeability barrier for the essential, water soluble molecules and ions of the cell, and in so doing provides the basis of the compartmentalizing function of biological membranes. The protein components are of two kinds. Some are inserted into the bilayer and most traverse this structure. These so-called integral membrane proteins have amino acids with nonpolar side chains at the interface between the protein and the nonpolar central region of the lipid bilayer. Other proteins associated with the polar surfaces of the bilayer and with the intrinsic membrane proteins are termed peripheral. The carbohydrate component of those membranes that display it is covalently linked to either lipids or proteins.

The protein components confer on biological

membranes their physiological functions, which are particular for each type of membrane. The lipid component, apart from its critical barrier function, is, for the most part, physiologically silent, although derivatives of certain membrane lipids can serve as intracellular messengers. The most remarkable feature of this general structure is the fact that neither the lipid nor the protein components are covalently linked to one another or to each other. This structure formed by molecular associations only, is less than 100 Å in thickness, but many orders of magnitude larger in the other two orthogonal dimensions. Although it is mechanically surprisingly strong, it is fluid-like and as a dielectric can withstand field strengths up to 10^5 volts cm^{-2} without breakdown. Experimental and theoretical studies carried out over a period of many years have established the fact that these unusual properties are conferred on biological membranes by the lipid bilayer matrix (Henn and Thompson, 1969).

An important modification to this model of biological membrane structure was made in the early 1970's by Singer and Nicolson, who offered strong evidence that the molecules, both lipid and intrinsic proteins, comprising this structure undergo a variety of thermally-driven motions (Singer and Nicolson, 1972). Both types of molecules diffuse laterally, and rotate about an axis normal to the membrane plane. In addition, the lipids can flip from one face of the membrane bilayer to the other and adsorb/desorb from the bilayer at slow, but measurable rates. Although these latter motions are forbidden to intrinsic proteins, both lipids and proteins can to some degree exhibit limited bobbing motions normal to the membrane plane. As a corollary to this idea of a dynamic membrane structure, which came to be known as the fluid mosaic model, the notion rapidly developed that within the planar confines of the membrane, the molecular components formed a two-dimensional, anisotropic, stochastic system.

In recent years, the random character of the in-plane mix of the membrane molecules has given way to the realization that, although the dynamic aspect is correct, there is considerable order in the plane of the membrane. This order takes the form of a compositional mosaic of molecular association complexes in the membrane plane (Jacobson and Vaz, 1992). The plane of the biological membrane is thus compartmentalized by domain structures much as the three dimensional space of the cell is compartmentalized by the membranes themselves. The dimensional range of the domain mosaic runs from tens of microns to tens of nanometers over persistence intervals of minutes to nanoseconds. Because of these large ranges in time and space, experimental investigation of domain structure is limited by the spatial and dynamic ranges intrinsic to a particular method. This fact has given rise to much confusion and apparently conflicting information about membrane domain structure.

In addition to this in-plane domain structure, it has been known for some time that the two lipid monolayers comprising the membrane bilayer do not in many membranes have the same composition. This transbilayer lipid compositional asymmetry appears to be in large part stable in the absence of metabolic energy. Whether the stability is an equilibrium or a kinetically-trapped configuration is not known (Devaux, 1991). This lipid asymmetry and the fact that intrinsic membrane proteins do not rotate about an axis in the membrane plane, combine to make the two faces of the bilayer separate domains.

The membrane paradigm outlined above incorporates the features that are at present known to be common to most, if not all, biological membranes. Many questions remain unanswered, however. A large number are specific to individual membranes and their idiosyncratic functions. Some questions are more general. Important among these are the physical basis or bases of in-plane domain structure and its physiological consequences. The ultimate physical basis for domains in membranes must, of course, lie in the interactions between molecules. Interactions can not only give rise to association complexes of molecules that specifically interact with each other, but also to groups of molecules that do not specifically interact but are excluded from other association complexes and thus form domains by default. Interactions that are important in forming domain structures can occur between membrane proteins, or

between these molecules and proteins of the cytoskeleton and cytoplasm. Association domains can equally well be the result of interactions between membrane proteins and lipids or between different types of bilayer lipids. Obviously, in any membrane at any time these interactions can operate singly or in combination. The non-ideal mixing that is characteristic of membrane lipids can be used to understand domain structure arising from the coexistence of multiple lipid phases. The basis of domain structure formed by membrane proteins is more varied and is dependent on highly specific interactions between molecules. A unitary hypothesis applicable to all such protein-protein association complexes is probably impossible to formulate. Interactions between membrane proteins and lipids of the bilayer are more fully understood (Mouritsen and Bloom, 1993; Mouritsen et al., 1996).

We have suggested that modulation by the cell of the percolation properties of its membrane domain systems can be used to control the extent and rate of physiologically important in-plane molecular interactions (Thompson et al., 1992). This idea has been examined experimentally using simple two-component, two-phase phospholipid bilayers in which interactant molecules are confined to the fluid domains in temperature and composition ranges where both solid and fluid lipid phases co-exist. The results of the experimental studies agree well with computer simulations of such systems (Sankaram et al., 1992; Piknova et al., 1996; Schram et al., 1996; Schram and Thompson, 1997; Piknova et al., 1997). Taken together, this work provides strong support for the idea that domain structures in the plane of bilayer membranes can have a marked effect on the apparent equilibrium poise and effective rates of in-plane reactions. Although this work has been carried out on two-component lipid bilayers and utilizes the coexistence of solid and fluid domains as the interaction domain matrix, the conclusions drawn are applicable to any planar domain system regardless of its physical origin.

References

Devaux, P.F. 1991. Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* 30:1163-1173.

Henn F.A., and T.E. Thompson. 1969. Synthetic lipid bilayer membranes. *Ann. Rev. Biochem.* 38:241-262.

Jacobson, K., and W.L.C. Vaz (eds). 1992. *Comments on Molecular and Cellular Biophysics.* 1992. 8:1-114.

Korn, E.D. 1969. Cell membranes: structure and synthesis. *Ann. Rev. Biochem.* 38:263-288.

Mouritsen, O.G., and M. Bloom. 1993. Models of lipid-protein interactions in membranes. *Ann. Rev. Biophys. Biomol. Struct.* 22:145-171.

Mouritsen, O.G., M.M. Sperotto, J. Risbo, Z. Zhang, and M.J. Zuckermann. 1996. Computational approach to lipid-protein interactions in membranes. *Adv. Comp. Biol.* 2:15-64.

Piknova, B., D. Marsh, and T.E. Thompson. 1996. Fluorescence quenching study of percolation and compartmentalization in two-phase lipid bilayers. *Biophys. J.* 71:892-897.

Piknova, B., D. Marsh, and T.E. Thompson. 1997. Fluorescence quenching and ESR study of percolation in a two-phase lipid bilayer containing bacteriorhodopsin. *Biophys. J.* 72:2660-2668.

Sankaram, M.B., D. Marsh, and T.E. Thompson. 1992. Determination of fluid and gel domain sizes in two-phase lipid bilayers: an ESR spin label study. *Biophys. J.* 63:340-349.

Schram, V., H-N Lin, and T.E. Thompson. 1996. Topology of gel-phase domains and lipid mixing properties in phase-separated, two-component phosphatidylcholine bilayers. *Biophys. J.* 71:1811-1822.

Schram, V., and T.E. Thompson. 1997. Influence of the intrinsic membrane protein bacteriorhodopsin on the gel phase domain topology in two-component phase separated bilayers. *Biophys. J.* 72:2217-2225.

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720-731.

Thompson, T.E., M.B. Sankaram, and R.L. Biltonen. 1992. *Comments on Molecular and Cellular Biophysics* 8:1-15.

Phospholipid-bilayer Vesicle Shapes and Shape Transformations: Theory vs. Experiment

Michael Wortis

Abstract

Laboratory preparations of micron-scale fluid-phase phospholipid vesicles exhibit a rich fauna of vesicle shapes and a complex systematics of shape transformations induced by changing accessible control parameters. At the same time, there is a well-developed theory of vesicle shapes based on the concept of minimizing the elastic bending energy. This contribution describes progress that has recently been made in comparing theory and experiment in a quantitative manner.

Michael Wortis
Physics Department
Simon Fraser University,
Burnaby, BC, Canada V5A 1S6
e-mail: wortis@sfu.ca

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:59–63

Theory of Vesicle Shapes

Canham (Canham, 1970) and Helfrich (Helfrich, 1973) in the early 1970's were the first to propose that the shapes of fluid-phase phospholipid vesicles in aqueous solution are determined by simple minimization of an elastic bending energy. Deuling and Helfrich (1976) then produced a catalogue of minimum-energy shapes in good correspondence with the simple shape classes observed in the laboratory for both red blood cells (rbc's) and artificially prepared pure-lipid vesicles. Returning to this problem in the early 1990's with the aid of better computational facilities, we (Miao et al., 1991) and other groups (Seifert et al., 1991) began to map out the phase diagram of Helfrich's (Helfrich, 1973) model, showing the energy-minimizing shapes as a function of the control parameters. Two things became clear. First, we found that, for appropriate parameters, budded or vesiculated shapes, with one or more small (infinitesimal) necks, were minimizing shapes (Miao et al., 1991; Fourcade et al., 1994). Second, in comparing the calculated phase diagram with experiments in which shape transitions were induced by smoothly varying the control parameters (e.g., by varying the temperature), it became clear that the original Helfrich model was systematically inconsistent with experiment.

Several groups (Seifert et al., 1991; Miao et al., 1994; Svetina and Zeks, 1989) worked in the early 1990's to understand these discrepancies based

on ideas originally put forward earlier by Evans (1974; 1980), Helfrich (1974), and others. The physics behind these studies was the observation that the rate of flip-flop—the exchange of phospholipid molecules between the two leaves of the bilayer— is slow on mechanical time scales. Thus, the number of molecules on the inside and outside leaves of the bilayer—and, therefore, the relaxed area difference ΔA_0 between the two bilayer leaves—are conserved quantities. The upshot of this work (Miao et al., 1994) was the so-called area-difference elasticity (ADE) model, defined by the Hamiltonian,

$$H_{ADE}[S] = \frac{1}{2}\kappa_b\left[\oint dA\big(C_1(\mathbf{r}) + C_2(\mathbf{r}) - C_0\big)^2 + \frac{\alpha\pi}{AD^2}\big(\Delta A[S] - \Delta A_0\big)^2\right]. \tag{1}$$

In this expression, the first term is the original Helfrich bending energy (Helfrich, 1973), where $C_1(\mathbf{r})$ and $C_2(\mathbf{r})$ are the two local principal curvatures at the point $\mathbf{r}$ on the vesicle surface and C_0 is the so-called spontaneous curvature, which reflects any intrinsic asymmetry between the inner and outer bilayer leaves. The second term is the area-difference contribution, which measures the elastic energy cost of forcing the actual area difference ΔA (between inner and outer leaves) to differ from the relaxed area difference ΔA_0. The actual area difference ΔA depends on the shape $[S]$ of the vesicle via the relation,

$$\Delta A[S] = D\oint dA\big(C_1(\mathbf{r}) + C_2(\mathbf{r})\big), \tag{2}$$

where D is the interleaf separation (assumed fixed), A is the vesicle area, α is a dimensionless material constant (generically of order unity), and κ_b is the Helfrich bending rigidity. The fact that κ_b is 10—20 times the thermal energy k_BT for typical phospholipids near room temperature means that the shape problem is a low-temperature, purely mechanical problem to first approximation. The stable mechanical shapes are predicted to be the local minima of Eq. (1) subject to constraints of fixed volume V and area A. Because of the particular form of Eq. (2), it turns out that these shapes are precisely those of the Deuling-Helfrich catalogue (Deuling and Helfrich, 1976), only with a self-consistently determined effective value of the spontaneous curvature C_0.

Equation (1) is at present qualitatively consistent with experimental observations (in a way that the Helfrich Hamiltonian (Helfrich, 1973) was not); however, until recently, it had never been directly tested. This may seem surprising; but, the reasons are rather simple. A direct test would be to measure the control parameters of a particular experimental vesicle, A, V, C_0, and ΔA_0 (κ_b, α, and D may be assumed known), and then to compare the observed and predicted shapes. There are two difficulties with this scenario. First, the control parameters cannot be simply measured: A and V must somehow be inferred from 2D phase-contrast microscope images, while C_0 and ΔA_0, not being geometrical, cannot be inferred from the microscope images. Second, thermal shape fluctuations are not really small (indeed, near shape instabilities they can be quite large!), so what one measures in the laboratory for a given vesicle is a time-sequence of shapes, constituting a thermal shape ensemble. One must find some way to infer from this shape ensemble the corresponding zero-temperature, mechanical shape, which is the object most directly predicted by the theory. We have developed (Döbereiner et al., 1997) a procedure, combining theory with observation of micron-scale vesicles by phase-contrast microscopy, which allows these difficulties to be circumvented and provides what we believe to be the first direct quantitative confrontation of theory with experiment. It is not, so far, a very extensive test, nor is it at all precise. On the other hand, it remains, in our view, the only one available. Theory appears to pass the test (Döbereiner et al., 1997).

As a consequence, we have at this point what appears to be a viable theory of single-component fluid-bilayer vesicle shapes and, thereby, an understanding of the influence on these shapes of a (small) set of control parameters—at the level of a fairly extensive but still incomplete shape/phase diagram. By systematically manipulating these control parameters, we know how to move vesi-

cles across instability boundaries (spinodals, in the language of phase transitions) so as to produce systematically in the laboratory shape transitions like budding and vesiculation, the discocyte-to-stomatocyte transition, etc.

What does this have to do with biological membranes? To first approximation, the honest answer may be "not much"; but, let me make a few remarks, anyway. The main issue here is that biological membranes, even those as simple as the rbc membrane, are much more complicated than one-component lipid bilayers.

Effect of the Cytoskeleton on the Red Blood Cell Shape

The rbc cytoskeleton is a tethered network of protein polymers anchored to the interior side of the plasma membrane (but not, as in many cells, extending into the cytosol). Unlike the (fluid) plasma membrane, the cytoskeleton has a well defined shape and its shape mechanics cannot be described by an energy functional of the form of Eq. (1) but must include local dilation and shear elasticities (in addition to bending). In situations where these contributions are important (as they certainly are at high deformation), an approach to rbc shapes based on Eq. (1) alone (and on the control parameters we have identified above) is bound to fail. However, there is some evidence that the cytoskeletal contribution to the energy is rather small for weak deformations of the normal discocyte, so that the discocyte and shapes not too far from it (including those accessed by thermal flickering) can probably be effectively described by Eq. (1). In this spirit, it may be argued that echinocyte shapes (which do not show up in the Deuling-Helfrich catalogue (Deuling and Helfrich, 1976) occur when the plasma membrane by itself would like to form multiple outward buds (i.e., when it has extra material in the outer leaf of the bilayer). Such buds require large shear deformations near the narrow necks and are thus suppressed by the cytoskeleton, resulting in the smooth crenellations characteristic of echinocytes. This hypothesis remains untested, as far as I know.

Effect of Lipid Mixtures

Typical biological membranes contain a mixture of many different lipids plus important proteins, etc. As long as this mixture remains spatially homogeneous, it can be characterized at long wavelength by appropriate (average) values of the parameters, κ_b, α, C_0, and D. On the other hand, as soon as inhomogeneities occur, in the form of either spatial fluctuations or full phase separation, then modification of Eq. (1) is required. (And, indeed, an additional term involving the Gaussian curvature cannot be ignored, as we have done above.) These modifications are not hard to incorporate and a few calculations have been carried out (Lipowsky, 1995). The issue is interesting when the compositional degrees of freedom are coupled to the geometric ones (e.g., different components having different κ_b's and/or different C_0's) and it is further complicated by the fact that compositional fluctuations at the same point on the bilayer but in different leaves may be coupled. When full phase separation takes place, the phase boundary acts as a line under tension, which under appropriate conditions can promote bud formation (just as an elastic band might pull closed the open neck of a bag)—a process called domain-induced budding (Lipowsky, 1992; Jülicher and Lipowsky, 1996).

Another possible consequence is the aggregation of species favoring large Gaussian curvature in a neck region, thus lowering the energy of neck formation. Such effects may also be associated with the experimental observation that buds, once formed, tend to fission spontaneously in some systems but not in others (Döbereiner et al., 1993).

Budding Mechanisms

It is well established that the mechanisms described above, based on Eq. (1) and its associated control parameters, can and do produce budding (and other shape instabilities) in artificial vesicles and in rbc's. It is also known that many instances of cellular and intracellular budding are driven by specific, energy-consuming processes involving proteins such as clathrins. I cannot point to any cases where it has been established that a particular biological budding processes proceeds via (biochemical) manipulation of the control parameters of Eq. (1); however, there are many budding processes the mechanisms of which remain to be understood. What is clear in any case is that, to initiate budding, nature must control the energy landscape set by Eq. (1) or its generalization in the case of lipid mixtures and/or cytoskeletal involvement. In this connection, it is interesting to note that estimates of the closure energy of clathrin cages come in just about an order of magnitude larger than the energy scale κ_b of typical membrane lipids. This is probably not an accident. One may speculate as to whether at early stages in cellular evolution, before the development of clathrin and clathrin-like mechanisms, nature made more general use of the "physical" control parameters whose action we have studied.

Summary

In summary, the significance of understanding the shape mechanics of one-component lipid bilayer membranes probably does not lie principally in any direct or immediate application to cellular processes. Such applications will usually have to incorporate elaborations of the simple theory discussed here to account for the richer biochemical environment of the working cell. What our work (and that of our many collaborators and competitors) has shown is that we are at least on our way to understanding at a quantitative, predictive level the mechanical properties of one of the simplest biological materials, the fluid-phase phospholipid bilayer. Such bilayers (in the many cellular contexts where they occur) form a substrate for much important cellular machinery. And, it is increasingly being appreciated that this material is far from passive but modulates in crucial ways the structure and behavior of the proteins and other biomolecules that use it as a substrate.

***

This research was supported by the Natural Sciences and Engineering Research Council of Canada.

References

Canham, P.B. 1970. The minimum energy of bending as a possible explanation of the biconcave shape of the human red blood cell. *J. Theor. Biol.*, 26, 61–81.

Deuling, H.J., and W. Helfrich. 1976. The curvature elasticity of fluid membranes: A catalogue of vesicle shapes. *J. Physique (Paris)*, 37, 1335–1345.

Döbereiner, H.-G., E.A. Evans, M. Kraus, U. Seifert, and M. Wortis. 1997. Mapping vesicle shapes into the phase diagram: A comparison of experiment and theory. *Phys. Rev. E* , 55, 4458–4474.

Döbereiner, H.-G., J. Käs, D. Noppl, I. Sprenger, and E. Sackmann. 1993. Budding and fission of vesicles. *Biophys. J.*, 65, 1396–1403.

Evans, E.A. 1974. Bending resistance and chemically induced moments in membrane bilayers. *Biophys. J.*, 14, 923–931.

Evans, E.A. 1980. Minimum energy analysis of membrane deformation applied to pipette aspiration and surface adhesion of red blood cells. *Biophys. J.*, 30, 265–284.

Fourcade, B., L. Maio, M. Rao, M. Wortis, and R.K.P. Zia. 1994. Scaling analysis of narrow necks in curvature models of fluid lipid-bilayer vesicles. *Phys. Rev. E*, 49, 5276–5286.

Helfrich, W. 1973. Elastic properties of lipid bilayers: Theory and possible experiments. *Z. Naturforsch.*, 28c, 693–703.

Helfrich, W. 1974. Blocked lipid exchanges in bilayers and its possible influence on the shape of vesicles. *Z. Naturforsch.*, 29c, 510–515.

Jülicher, F., and R. Lipowsky. 1996. Shape transformations of vesicles with intramembrane domains. *Phys. Rev. E*, 53, 2670–2683.

Lipowsky, R. 1992. Budding of membranes induced by intramembrane domains. *J. Phys. II France*, 2, 1825–1840.

Lipowsky, R. 1995. The morphology of lipid membranes. *Curr. Opin. Struct. Biol.*, 5, 531–540. See also references contained therein.

Miao, L., B. Fourcade, M. Rao, M. Wortis, and R.K.P. Zia. 1991. Equilibrium budding and vesiculation in the curvature model of fluid lipid vesicles. *Phys. Rev. A*, 43, 6843–6856.

Miao, L., U. Seifert, M. Wortis, and H.-G. Döbereiner. 1994. Budding transitions of fluid-bilayer vesicles: The effect of area-difference elasticity. *Phys. Rev.*, 49, 5389-5407.

Seifert, U., K. Berndl, and R. Lipowsky. 1991. Shape transformations of vesicles: Phase diagrams for spontaneous-curvature and bilayer-coupling models. *Phys. Rev. A*, 44, 1182–1202.

Svetina, S., and B. Zeks. 1989. Membrane bending energy and shape determination of phospholipid vesicles and red blood cells. *Eur. Biophys. J.*, 17, 101–111. This article contains earlier references.

The Role of Minimal Models in the Context of a New Biomembrane Model[1]

Martin J. Zuckermann

Abstract

In recent years there have been performed a considerable number of excellent molecular dynamics (MD) simulations with realistic potentials which have made detailed predictions for the structure of lipid mono- and bilayers. However these simulations are restricted to a relatively small number of lipid molecules and it is therefore difficult to use such simulations to predict the detailed phase behaviour of lipid membranes. Here we discuss the role of minimal models in making such predictions. Furthermore we classify the models into two types and give one example for each of type of model. In the context of the type II model presented here, we describe some detailed results for the phase behaviour of lipid-sterol bilayers. This is followed by a discussion of the role of minimal models in the construction of a new biomembrane model.

Martin J. Zuckermann
Department of Physics
McGill University
Montréal, Québec, Canada H3A 2T8.
e-mail: martin@physics.mcgill.ca

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:65–74

Introduction

The increase in speed and memory and the use of parallel computing in recent decades has given us the possibility of understanding the fundamental interactions between biomembrane components and at the same time allowed us to visualize their microscopic behavior in terms of real space snapshots. The improvement in computer power has gone hand-in-hand with the development of several software packages for molecular modeling based on molecular dynamics (MD) techniques and has often led to an analysis not just of structure but also of function of membrane proteins (Humphrey et al., 1995, Schulten, 1997). The inclusion of lipid and water molecules in the simulation of membrane proteins and peptides has been pioneered by Benoit Roux and his research group in particular with regard to gramicidin, melittin and Pf1 coat proteins (Roux and Woolf, 1995). Furthermore MD simulations were carried out by Benoit Roux, Jim Davis (1997) and colleagues and Shen et al.

[1]This article is a shorter version of a contribution to the fall (1997) edition of 'Physics in Canada'.

(1997) for the behavior of single α-helix intrinsic polypeptides which span a lipid bilayer, and the numerical results were used to understand related nuclear magnetic resonance (NMR) data by Davis. Clearly MD simulations based on detailed molecular interactions have been shown to be extremely successful for the understanding of structure and, in some cases, function of proteins at the molecular level.

A typical MD sample investigated by molecular dynamics is one gramicidin molecule surrounded by fifty lipid molecules plus water molecules in a hexagonal cell subject to periodic boundary conditions with a maximum time scale of 1 ns (Woolf and Roux, 1994). There now exist many results from MD simulations of lipid bilayers and monolayers at the molecular level (Zhou and Schulten, 1995; Pastor and Feller, 1996; Berger et al., 1997) for small samples for a maximum time of 1 ns. For example Zhou and Schulten (1995) performed a 200ps MD simulation for a dipalmitoylphosphatidylethanolamine (DPPE) bilayer composed of 202 lipid molecules and 8108 water molecules. They obtained data for the water polarization profile, the membrane dipole moment and the susceptibility profile. Recently Berger et al. (1997) used MD to simulate a fluid dipalmitoylphosphatidylcholine (DPPC) bilayer at full hydration and constant pressure and temperature. The bilayer comprised 64 DPPC molecules with 23 water molecules per lipid. As with previous simulations of this kind, they were able to examine the structure of the bilayer at the lipid-water interface and they obtained excellent agreement with experiment for the NMR order parameter along the acyl chains of the lipid molecules.

Even though the speed of numerical computation is increasing rapidly as we approach the second millennium, it will be some time before an analysis of collective behavior such as self-assembly, phase separation, clustering, etc. is possible at the molecular level since this requires a number of molecules of the order of a thousand at least. Such numerical studies at present require considerable simplification of the model. These simplifications could include for example the use of systems of 'monomers' in which each 'monomer' represents a complex molecular or sub-molecular component of the system whose details are not important to the processes under investigation. This is called a 'coarse grained' model where we simplify the interactions between the 'monomers'. The first step towards "minimal" modeling is to simulate such systems using MD without including full molecular details. A very successful example of such modeling is the work of Grest and Murat (1995) on many polymer systems using a 'ball and string' model for each polymer in conjunction with MD simulations. Consider a homopolymer brush (i.e. a flat plane containing a relatively dense system of end-grafted homopolymers) in a good solvent. If the solvent is changed from good to poor, the brush makes a transition from a homogeneous system to a lattice of pinned micelles (Soga et al., 1995) each of which is a collapsed cluster of many polymers. An MD simulation using the 'ball-and-string' model will at best result in the formation of a single cluster but there is no chance of simulating the collective nature of the system. This implies that collective behavior requires further 'simplifications' in the model, dare we say more 'minimality' in the model. The group at McGill is at present applying the minimal model for-end grafted polymer systems of reference (Soga et al, 1995) to a study of the properties of lipid vesicles containing lipopolymers i.e. homopolymers covalently bonded to the polar heads of some of the lipid molecules in the vesicle's bilayer (Rex at al., 1998).

The purpose of this introduction was to present the main concepts underlying minimal modeling in complex fluids. We now turn our attention to the minimal modeling of lipid bilayer systems.

Minimal Models for Lipid Phase Behaviour

There are at present several minimal models for the large scale behavior of lipid bilayers and they are based on a well tried philosophy. We could first simulate all possible configurations of a single lipid acyl chain and then model the interactions between chains in terms of a self-consistent field (SCF) which removes fluctuation effects. Let me call this a type I model. A second approach, which has been our philosophy, is to use multistate interacting lattice models typical of statistical physics to model lipid bilayers and their various phases in conjunction with Monte Carlo simulations. We were able to include the effects of cholesterol, hydration, drug molecules and proteins on such phases via specific interactions (Mouritsen, 1990). For these models, we used and still use a two-dimensional triangular lattice, the sites of the lattice represent single lipid acyl chains. The details of the lipid chain conformations (lateral area/chain, internal energy, degeneracy) are treated as state parameters. We will refer to these models as type II models. We have recently extended this philosophy to the off-lattice case (see below).

Type I Models

Some of the most recent models of type I were simulated by Szleifer et al. (1987; 1990). The lipid configurations of a single lipid acyl chain were evaluated by a Monte Carlo simulation using the rotational isomeric (RIS) model in which each C-C bond has three possible states, one *trans* and two *gauche*. The bilayer is divided into layers and the number of carbon atoms per layer is counted. The incompressibility condition is imposed via an osmotic pressure and the resulting equations are solved self-consistently in the sense of mean-field theory. The ends of the chains are pegged onto a flat or curved surface and properties of the liquid crystalline (fluid phase) of the bilayer are calculated including the elastic constant for the bending energy. This model has very recently been extended by Müller and Schick (1996) by first removing the constraint of a "pegging" surface and then adding a rigid polar head to the chain. Interactions between the acyl chain and the polar head and between the chain and water were taken as repulsive and were described by a single parameter. The volumes of the polar head and chain segments completed the parameterization of the model and again the system is constrained by an incompressibility condition. Müller and Schick used this model to calculate the phase diagram of monoacyl glycerol-water systems which exhibited lamellar, hexagonal, inverted hexagonal and Ia3d cubic phases. Agreement with experiment for one phase transition was found and detailed composition profiles were calculated. This example shows clearly the great advantage of minimal modeling. We have here three basic parameters plus the RIS model which represent the fundamental physics of the problem and these are then used to calculate and understand an extremely complex phase diagram which would have been virtually impossible to obtain via MD methods for a model containing the full molecular details. Müller and Schick plan to extend this method to the phase behavior of membrane lipids.

The mechanistic, thermodynamic explanation of general anesthesia proposed by Cantor (1997) represents a direct application of minimal modeling using a type I model. On the basis of lattice SCF calculations, Cantor predicts the incorporation of interfacially active solutes in bilayers to perturb the distribution of local lateral stresses, with a large pressure increase near the aqueous interface compensated by a decrease distributed through the bilayer interior. Although this perturbation is typically relatively small at clinical anesthetic concentrations, it is large in absolute magnitude since the lateral pressures are themselves enormous. Suppose, as is generally accepted, that general anesthesia involves a shift in the conformational equilibrium of an ion-channel receptor protein. If the opening of the channel is accompanied by a nonuniform change in protein cross-sectional area, then the mechanical work of channel opening will be altered by the anesthetic-induced re-

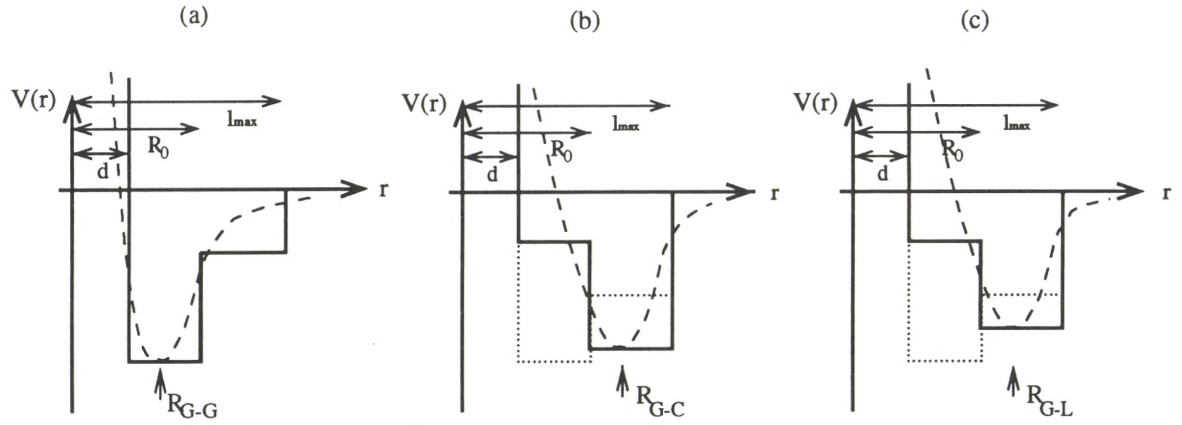


Figure 1. Schematic illustration of the interaction potential in the off-lattice model. The potential consists of a sum of a hard-disk potential and two square-well potentials. The hard-disk radius is d and the range and strength of the square-well potentials are $(l_{\max}, R_0)$ and (V_1, V_2), respectively. The dashed line illustrates a Lennard-Jones-like potential, to which the model potential is an approximation. (a) Interaction potential between two lipid chains in the gel state. (b) Interaction potential between a lipid chain in the gel state and a cholesterol molecule. (c) Interaction potential between a lipid chain in the gel state and a "lanosterol" molecule. R_{G-G}, R_{G-C} and R_{G-L} give the position of the minimum of the interaction potential for the gel-gel, gel-cholesterol and gel-lanosterol pairs. We chose $\frac{R_{G-G}}{R_{G-G}} \approx 1.3$ corresponding to the values of the lipid-cholesterol system. Interactions between lipid chains in the fluid state are weak and not shown in this figure.

distribution of lateral pressures, causing a shift in the equilibrium between the closed and open protein states. Calculations yield qualitative agreement with anesthetic potency at clinical anesthetic membrane concentrations, and predict the anomalously low potencies of long-chain alcohols and of strongly hydrophobic molecules with little or no attraction for the aqueous interface, such as perfluorocarbons. Clearly, there are many other processes involving conformational changes in membrane proteins whose sensitivity to altered membrane composition may also result from changes in the pressure profile. Cantor speculates that the homeostatic response of membranes may serve to restore the pressure profile, and thus reestablish the proper protein conformational distribution.

Lipid-Sterol Systems

We will now describe some recent work performed at the MEMPHYS group at the Technical University of Denmark (DTU) in collaboration with our group at McGill using a type II model (Zuckermann et al., 1993). We have worked for a considerable time using multi-state lattice models to describe the generic phase behavior of lipid-cholesterol bilayers. The physical principles defining the model were simply that cholesterol is an "ice breaker" which disrupts the gel phase of lipid bilayers but which is able at the same time to rigidify the acyl chains. This led to the classification of a phase with high cholesterol concentration which we called the **lo** (liquid ordered) phase. This is basically a 2d-fluid phase with relatively rigid chains and corresponds to the experimental β-phase of Vist and Davis (Davis, 1993). Using again very few additional parameters for the inclusion of cholesterol in our model for the main phase transition of lipid bilayers, we employed mean field theory to calculate the phase diagram of DPPC-cholesterol bilayers and it agreed with that of Vist and Davis. Since we had values for the fitted parameters from the phase diagram, it was possible to calculate a variety of physical properties, e.g. the specific heat and the hydrophobic thickness of the bilayer as function of temperature and concentration of cholesterol, which gave

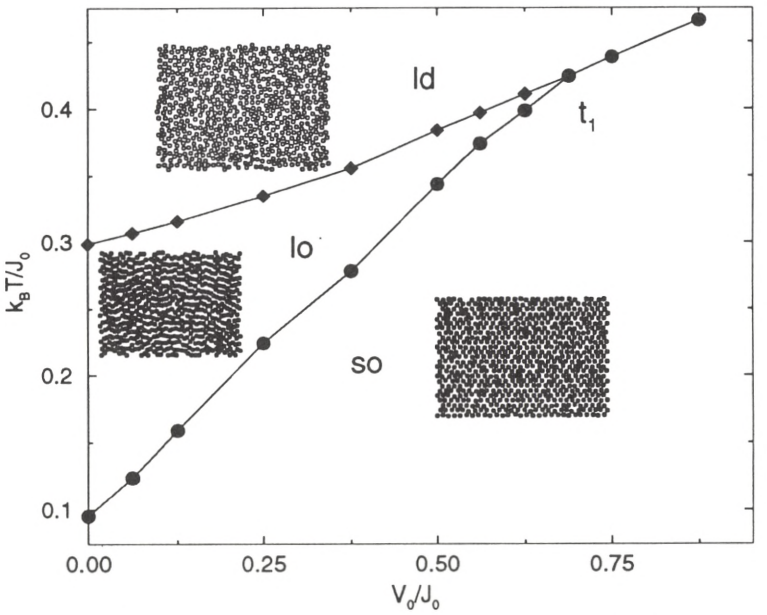


Figure 2. Phase diagram for the off-lattice model. All three phase boundaries are first-order phase boundaries. The insets show snapshots of typical micro-configurations for the three different phases labeled **so** (solid-ordered), **ld** (liquid-disordered), and **lo** (liquid-ordered). Chains in the disordered state are plotted as (○) and chains in the ordered chain state as (●). The three snapshots are not given to scale. t_1 is the triple point described in the text.

excellent quantitative agreement with experiment. Cruzeiro-Hansen and Mouritsen (Zuckermann et al., 1993) proposed and simulated (using Monte Carlo methods) a simplified version of this model which led to the understanding of the effect of cholesterol on the main phase transition and the ionic permeability at low cholesterol concentration (Zuckermann et al., 1993). One very basic problem with these models was that the **lo** phase was examined using a lattice model and there was therefore no qualitative difference between the **lo** phase and the **so** (solid ordered or gel) phase. Again in collaboration with the MEMPHYS group we have recently constructed the following minimal model to overcome this problem. This work is part of the Ph.D. thesis of Morten Nielsen, a graduate student of the McGill Physics Department.

The new model (Nielsen et al., 1996) is an off-lattice spin-$\frac{1}{2}$ Ising model in which the translational and internal (spin) degrees of freedom are coupled via microscopic interactions. The two internal (spin) degrees of freedom represent gel phase (rigid) and fluid phase acyl chain configurations respectively with the 'fluid phase' configuration being highly degenerate (Doniach, 1978). The translational degrees of freedom are unconventionally described in terms of a random lattice, which is structured by dynamically triangulating the spatial configurations. There is a 'hard core' interaction between all spin states and an attractive interaction between the 'gel' spin states only as shown in Fig. 1(a). This model leads to the phase diagram of Fig. 2 in which three phases are represented; an **so** phase ('gel' spin 2d-'solid'), an **ld** or liquid disordered phase ('fluid' spin, 2d-liquid), an **lo** ('gel' spin, 2d-liquid) and a triple point, t_1. We chose the interaction strength such that the main phase transition which lies on the **so-ld** phase line is located just beyond the triple point (see Fig. 2).

The next question is: how can we include lipid-cholesterol interactions into this minimal model with the least number of parameters, but in such a way that it retains its well-known properties

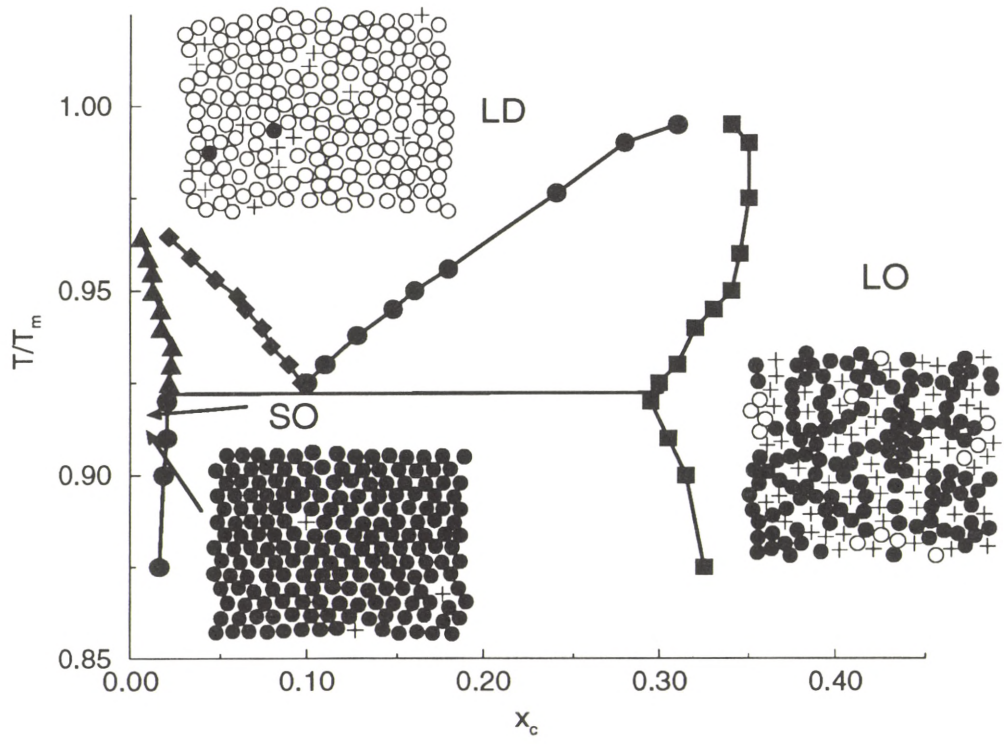


Figure 3. Lipid-cholesterol phase diagram. The insets show snapshots of micro configurations for the three different phases labeled **so** (solid-ordered), **ld** (liquid-disordered) and **lo** (liquid-ordered). The lipid chain conformational states are plotted as in the previous figure. Cholesterol molecules are plotted as +. The three snapshots are not given to scale. The concentration of cholesterol in each snapshot is: $c_{so} = 0.015$, $c_{ld} = 0.08$ and $c_{lo} = 0.31$. The x-axis gives the concentration of cholesterol, the y-axis the temperature measured in terms of T_m, the transition temperature for the off-lattice model.

of ice breaker and chain 'rigidifier'? The lipid-cholesterol interaction potential is shown in 1(b) where it can be seen that the basic difference is that the position of the minimum in the attractive interaction between 'gel' acyl chains and cholesterol is further out than that of the 'gel'-'gel' interaction in Fig. 1a. This potential brings one new variable parameter in the formalism, this being the depth of the potential well. Fig. 3 gives the resultant phase diagram for lipid-cholesterol systems and shows that, providing the position of the minimum of the 'gel'-cholesterol interaction has been correctly chosen, it exhibits the same generic behavior as the experimental phase diagram for DPPC-cholesterol bilayers. What do we gain from this?

This is where the philosophy of minimal models and their great advantage in terms of the description of the properties of lipid systems comes into play. Suppose we look at lipid bilayers containing sterols other than cholesterol. This is the subject of the work by Konrad Bloch and its interpretation by Bloom and Mouritsen (1991) These works deal with the evolution of eucaryotic membranes based on the hypothesis of Bloch that Nature optimizes membrane components and that cholesterol is optimized vis-a-vis precursor membrane sterols taking part in the evolutionary process with respect to its physical properties. Bloch pointed out that fluorescence experiments show that cholesterol gives the greatest decrease in fluidity in lipid bilayers. Bloom and Mouritsen interpreted this in terms of the increase in order of the bilayer, implying that cholesterol rigidifies neighboring chains more

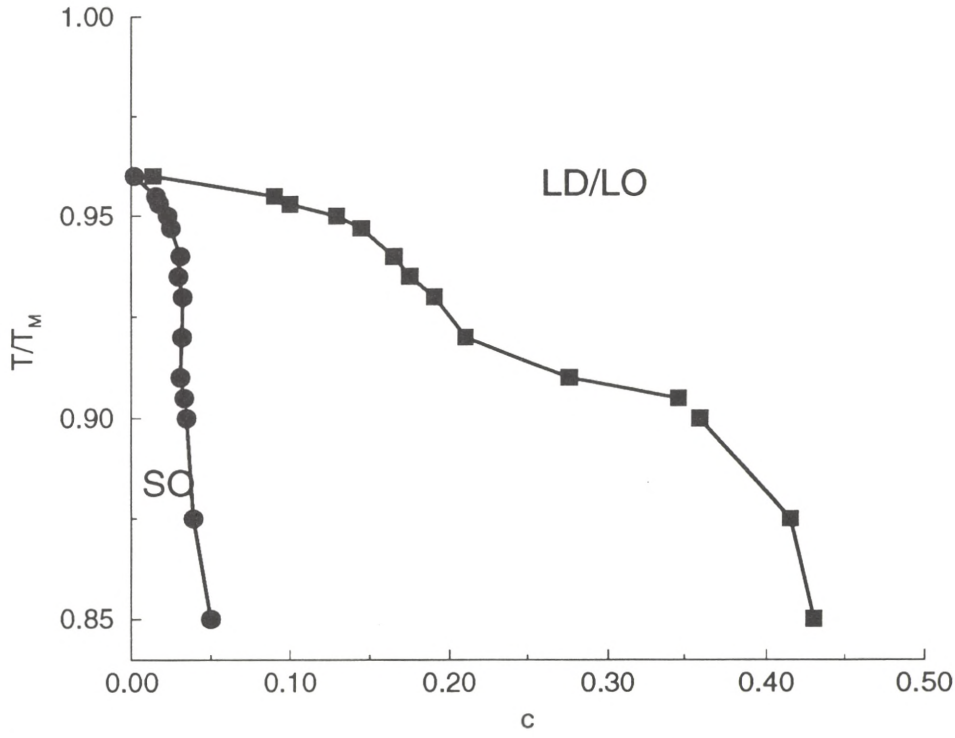


Figure 4. Lipid-lanosterol phase diagram. The x-axis gives the concentration of cholesterol, the y-axis gives the temperature measured in terms of T_m, theoretical transition temperature for the off-lattice model.

strongly than the other sterols in the evolutionary process. What does this mean in terms of our minimal model? Let us now consider lanosterol, which causes a smaller increase in chain order than cholesterol but larger than 'simpler' sterols. To explain this effect we appropriately adjusted the depth of the well of the lipid-cholesterol interaction shown in Fig. 1(b). By sufficiently reducing the depth of the well, we obtained the interaction used for lipid-lanosterol shown in Fig. 1(c) and the related phase diagram given in Fig. 4. It was quite pleasing to us that this phase diagram is qualitatively very similar to the experimental phase diagram for DPPC-lanosterol obtained from NMR experiments by Thewalt and Bloom (1997) even though the model is minimal. It should be mentioned that our theoretical phase diagram was found from Monte Carlo simulations which took time and effort on the part of Morten Nielsen. An article on our theoretical work on lipid-sterol phase diagrams is in preparation (Nielsen et al., in preparation).

Discussion and Conclusion

The following questions can now be posed:

- What information can we obtain from these simulations?
- What is the relation between numerical simulations and analytic methods for lipid bilayer properties?

- How does the information obtained from simulations lead to further improvements in the problem and in our knowledge of lipid systems in the context of a new biomembrane model?

We can calculate phase diagrams for precursor sterols between lanosterol and cholesterol. We can also (and are) calculating the specific heat and the hydrophobic thickness as a function of temperature and concentration for a qualitative comparison with experiment particularly for the case of DPPC-lanosterol bilayers. The calculations could be improved by increasing the number of conformations (states) per acyl chain and softening the potentials, which will allow quantitative comparison with experiment.

However the main aim of the simulations is considerably broader. We need to identify trends which will encourage new experiments. A specific example of this is the completely new picture of the main gel-fluid phase transition in terms of fluctuating gel (fluid) clusters in fluid (gel) phases above (below) the phase transition due to Ole Mouritsen (Mouritsen, 1990) This was only possible using a type II model since MD simulation would at most capture the interior of one cluster. Recent work in Paavo Kinnunen's group at Helsinki University has led to the experimental identification of this phenomenon. In our case of lipid-lanosterol systems, we hope that the phenomenological microscopic identification of the lipid-lanosterol interaction will encourage experimental workers to replace cholesterol by lanosterol in their samples. One example is the model system for stratum corneum (SC) of human skin which exhibits a low-temperature mixed phase with low hydration related to the fact that SC is impermeable to water. How would this phase and its characteristics change if cholesterol were replaced by lanosterol?

What is the connection between numerical simulations and analytic results for lipid bilayers? Using recent terminology, sterols can be viewed as small inclusions in lipid bilayers and they have a considerable perturbative effect. The most important inclusions biologically are proteins and the application of computational methods to the examination of lipid-protein interactions has been described in detail by Mouritsen et al. (1993) The proteins in this case are amphiphilic transmembrane proteins, i.e. their hydrophobic amino-acid residues have an affinity for the hydrophobic core of the lipid bilayer while their hydrophilic residues are screened from the hydrophobic core by being inside the protein or they lie in the aqueous medium surrounding the bilayer or else they are positioned in the polar head region of the bilayer. The interactions between lipids and proteins are given in terms of (i) direct hydrophobic interactions between neighbouring lipids and proteins and also between neighbouring proteins themselves as well as (ii) mismatch interactions between a protein and an adjacent lipid acyl chain. The mismatch interactions describe the effect of mismatch between the hydrophobic thickness of the lipid bilayer and the hydrophobic length of the transmembrane protein. The reader is referred to Mouritsen et al. (1993) for details. These interactions are basically phenomenological. The numerical simulations using these models have been very successful in predicting nanoscale organisation of lipid-protein bilayers, and protein selectivity.

The analytic theories are not able to give a full picture of lateral bilayer organisation and protein selectivity. Their advantage, however, is that they can give a detailed description of the interactions between proteins mediated by the lipid molecules. For example, the excellent paper by Aranda-Espinosa et al. (1996) is the latest in a series of papers by this group which builds on the seminal work of Huang (1986) on the deformation energy of lipid bilayers. These authors use analytic expressions for the bending stiffness and spontaneous curvature of fluid lipid bilayers to write an expression for the effective interaction between protein inclusions in the lipid bilayer. The local thickness itself is determined from an Euler-Lagrange equation which is solved using matching boundary conditions at the surface of the inclusion. The resultant interaction between inclusions is then used together with the Ornstein-Zernicke equation for liquid structure under Percus-Yevick closure to calculate the radial distribution function of the inclusions. They find that, when the spontaneous curvature is zero, the interaction be-

tween inclusions is similar to a hard core interaction. However for finite spontaneous curvature, a positive spontaneous curvature leads to conditions favorable for the adsorption of inclusions in the bilayer.

Clearly such an effective interaction between proteins induced by the bilayer can be used directly in simulations to determine the microscopic lateral organisation in the bilayer. Thus analytic methods and numerical simulation can often be regarded as giving complementary information. A recent example of this complementarity is the work of Gil et al. (1997) on wetting and capillary condensation as a means of protein organisation in membranes. In this case the analytic theories of Gil and Mikheev (1995) and Gil and Ipsen (1996) for wetting of proteins by one lipid component of a binary lipid mixture and the resultant effective interaction between proteins due to this wetting has led to the construction of a minimal microscopic model for wetting and protein induced phase equilibrium. Using this model in conjunction with Monte Carlo simulations, Gil et al. (1997) were able to examine the conditions for the formation of protein aggregates in lipid bilayers containing proteins.

How does all this relate to investigations for a new biomembrane model? Clearly minimal models for lipids alone are insufficient to result in such a new model directly. However such minimal models are essential in order to understand and describe collective behavior of molecules in lipid systems, both pure and mixed. It is our hypothesis that such behavior is biologically important in the context of (i) lipid protein interactions and (ii) systems where lipids alone play an important role such as the impermeability of the stratum corneum of mammalian skin to water. Thus minimal models for lipid systems must in general be used as 'sub-models' in the context of more complex models which describe biological phenomena. Minimal models of this type are then useful for such more complex models in the sense that they provide an understanding of the basic nature of the interactions between lipids (regarded as membrane components) important for collective behavior.

It should be pointed out that up to now we have in no sense exhausted the range of minimal models for lipid systems. For example, one vital area is the effect of the internal degrees of freedom of the lipid molecules on the phenomena arising from the curvature elasticity of lipid bilayers (Lemmich et al., 1994, Hansen, 1997) Consider for example the case of the phase separation of two lipids species in a curvature model of this type. Now include in the bilayer the presence of intrinsic proteins which strongly prefer one lipid species in its neighborhood over the other species. This will cause clustering of the proteins in the bilayer and such clustering may result in the collective (biological) activation of the proteins given the right external conditions. Clearly in order to understand the protein case, we must first understand the underlying lipid behavior. This is the reason for minimal modeling and its role in the development of new biomembrane models. A second area deals with minimal modeling of rotational isomerism and electrostatics in the polar head region (Belaya et al., 1994) of lipid bilayers and there are many more areas. The opportunities for development and application of such models are extremely wide, and the challenge is to find out using these models where the physical properties of lipid systems have biological significance.

***

The author wishes to thank Myer Bloom, Robert Cantor, Jim Davis, Ole Mouritsen, Morten Nielsen, David Pink, Michael Schick and Jenifer Thewalt for helpful discussions and Morten Nielsen for a critical reading of the manuscript. He particularly wishes to thank Sebastian Doniach for introducing him to the philosophy of minimal modeling in lipid systems and wishes to thank his colleagues and friends at the Technical University of Denmark, St. Francis Xavier University and McGill University for many years of exciting and fruitful collaboration and discussion in this field. The author also wishes to thank the NSERC of Canada for operating and equipment grants and le Fonds FCAR du Québec for Team and Centre grants. Finally the author is an Associate of the Canadian Institute for Advanced Research.

References

Aranda-Espinosa, H., A. Berman, N. Dan, P. Pincus, and S. Safran. 1996. Interaction between inclusions embedded in membranes. *Biophys. J.* 71:648–656 and references therein.

Belaya, M.L., V. Levadny, and D. Pink. 1994. Electric double layer near soft permeable interfaces. *Langmuir* 10:2010–2014.

Berger, O., O. Edholm, and F. Jähnig. 1997. Molecular dynamics simulations of a fluid bilayer of dipalmitoyl phosphatidyl choline at full hydration, constant pressure and constant temperature. *Biophys.J.* 72:2002–2013.

Bloom, M., and O.G. Mouritsen. 1995. The evolution of membranes. *In* Handbook of Biological Physics, Ed. Lipowsky R. and Sackmann E., (Elvisier Science B. V.) Vol 1, pp. 65–95.

Cantor, R.S. 1997. The lateral pressure profile: a physical mechanism of general anesthesia, Biochemistry 36:2339–2344; Lateral pressures in cell membranes: a mechanism for modulation of protein function. *J. Phys. Chem.* 101:1723–1725.

Davis, J.H. 1993. The molecular dynamics, orientational order, and thermodynamic phase equilibria of cholesterol–PC mixtures: ^{2}H Nuclear Magnetic Resonance. *In* Cholesterol in Membrane Models, Ed. Feingold L.X., (CRC Press, Boca Raton), pp. 67–135.

Davis, J.H. 1997. Private Communication.

Doniach, S. 1978. Thermodynamic Fluctuations in Lipid Bilayers. *J. Chem. Phys.* 68:4912–4916.

Gil, T., and J.H. Ipsen. 1997. Capillary condensation between disks in two dimensions. *Phys. Rev.* E55:1713–1721.

Gil, T., and L. Mikeev. 1995. Curvature controlled wetting in two dimensions. *Phys. Rev.* E52:772–778.

Gil, T., M.C. Sabra, J.H. Ipsen, and O.G. Mouritsen. 1997. Wetting and capillary condensation as means of protein organization in membranes. *Biophys. J.* 73:1728-1741.

Grest, G.S., and M. Murat. 1995. Computer Simulations of Tethered Membranes. *In* Monte Carlo and Molecular Dynamics Simulations, (Ed. Binder K., Oxford University Press) p. 476.

Hansen, P.L. 1997. Ph.D. Thesis, Technical University of Denmark and references therein.

Huang, H.W. 1986. Deformation free energy of bilayer membrane and its effects on gramicidin channel lifetime. *Biophys. J.* 50:1061–1070.

Humphrey, W., D. Xu, M. Sheves, and K. Schulten. 1995. Molecular dynamics studies of early intermediates in the bacteriorhodopsin photocycle. *J. Chem. Phys.* 99:14549–14560.

Lemmich, J., J.H. Ipsen, T. Hønger, K. Jørgensen, O.G. Mouritsen, K. Mortensen, and R. Bauer. 1994. Soft and repulsive. Relationship between lipid bilayer in–plane fluctuations, bending rigidity and repulsive undulation forces. *Modern Physics Letters* B29:1803–1814.

Mouritsen, O.G. 1990. Computer simulation of cooperative phenomena in lipid membranes. *In* Molecular Description of Biological Membrane Components by Computer Aided Conformational Analysis, Ed. Brasseur R., (CRC Press, Boca Raton), Vol. 1, pp. 3–83 and references therein.

Mouritsen, O.G., M.M. Sperotto, J. Risbo, Z. Zhang, and M.J. Zuckermann. 1993. Computational Approach to Lipid–Protein Interactions in Membranes. *In* Advances in Computational Biology (JAI Press Inc.), Volume 2, pp. 15–64 and references therein.

Nielsen, M., L. Miao, J.H. Ipsen, O.G. Mouritsen, and M.J. Zuckermann. 1996. Random lattice models and simulation algorithms for the phase equilibria of two dimensional condensed systems of particles with coupled internal and translational degrees of freedom. *Phys. Rev.* E54:6889–6905.

Pastor, R.W., and S.E. Feller. 1996. Time Scales of Lipid Dynamics and Molecular Dynamics. *In* Biological Membranes, (Ed. K.M. Merz Jr., and B. Roux, (Birkhäuser, Basel), pp. 3–29.

Rex, S., M. Lafleur, J. Silvius, and M.J. Zuckermann. 1998. *Biophys. J.* (Submitted).

Roux, B., and T.B. Woolf, Molecular dynamics of Pf1 coat Protein in a phospholipid bilayer. *In* Biological Membranes (Ed. K.M. Merz Jr., and B. Roux, (Birkhäuser, Basel, 1996), pp. 556–587.

Schick, M. 1997. Private Communication.

Schulten, K. 1997. How nature harvests sunlight. *Physics Today* (August Issue) pp. 28–34.

Shen, L., D. Bassolino, and T. Stouch. 1997. Transmembrane Helix Structures, dynamics and interactions. Multi-nanosecond molecular dynamics simulations. *Biophys.J.* 73:3–12.

Soga, K.G., M.J. Zuckermann, and H. Guo. 1995. Polymer brushes in a poor solvent. *Europhys. Lett.* 29:531–536 and references therein.

Szleifer, I., A. Ben–Shaul, and W.M. Gelbart. 1987. Statistical mechanics of molecular organisation in mixed micelles and bilayers. *J. Chem. Phys.* 86:7094-7110.

Szleifer, I., D. Kremer, A. Ben–Shaul, W.M. Gelbart, and S. Safran. 1990. Molecular theory of curvature elasticity in surfactant films. *J. Chem. Phys.* 92:6800–6817.

Thewalt, J. and M. Bloom. 1997. In preparation.

Woolf, T.B. and B. Roux. 1994. Molecular dynamics simulations of the gramicidin channel in a phospholipid bilayer. *Proc. Nat. Acad. Sci. USA.* 91:11631–11635.

Zhou, F., and K. Schulten. 1995. Molecular dynamics study of a membrane–water interface, *J. Phys. Chem.* 99:2194–2207.

Zuckermann, M.J., J.H. Ipsen, and O.G. Mouritsen. 1993. Theoretical studies of the phase behavior of lipid bilayers containing cholesterol. *In* Cholesterol in Membrane Models, Ed. Feingold L.X., (CRC Press, Boca Raton), pp. 223–257 and references therein.

Gramicidin Channels: Molecular Force Transducers in Lipid Bilayers

O. S. Andersen, C. Nielsen, A. M. Maer
J. A. Lundbæk, M. Goulian *and* R. E. Koeppe II

Abstract

The thermodynamic need to maximize hydrophobic interactions between integral membrane proteins and their host bilayer serves as one of the major guiding principles in models of biological membranes. These hydrophobic interactions govern not only the folding and membrane insertion of the proteins, they also affect membrane protein function. The control of protein function is due to the hydrophobic coupling between the membrane-spanning part of integral membrane proteins and the surrounding bilayer core. This coupling causes protein conformation changes that involve the protein/lipid interface to perturb the surrounding bilayer. The elastic membrane deformation energy associated with a protein conformational change thus will contribute to the overall free energy difference between different protein conformations. The importance of these membrane elastic deformations can be evaluated using ion channels, where measurements of the channel-mediated current allows for a direct measure of the (equilibrium) distribution among different protein conformations.

O. S. Andersen, C. Nielsen, A. M. Maer
Department of Physiology and Biophysics
Cornell University Medical College
New York, NY 10021, USA
e-mail: sparre@mail.med.cornell.edu

M. Goulian
Center for Studies in Physics and Biology
The Rockefeller University
New York, NY 10021, USA

J. A. Lundbæk
Neuropharmacology Unit
Novo-Nordisk A/S
DK-2760 Måløv, Denmark

R. E. Koeppe II
Department of Chemistry and Biochemistry
University of Arkansas
Fayetteville, AR 72701, USA

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:75–82

Introduction

The fluid-mosaic membrane model evolved from thermodynamic considerations about the organization of the main membrane components - phospholipids, cholesterol, and proteins (Singer and Nicolson, 1972). The guiding principle underlying the development of the model, the need to maximize hydrophobic and hydrophilic interactions, has served as a central organizing theme in all subsequent work. The main feature of the model, that the lipids are organized in a liquid-crystalline

bilayer in which integral membrane proteins are imbedded, had a similar immediate appeal. A weakness of the model was that the lipid bilayer component was assumed to be a passive entity only - a permeability barrier that separated the extracellular and intracellular aqueous phases. This point of view was strengthened by numerous studies on the permeability of lipid bilayers to small polar solutes (e.g. Walter and Gutknecht, 1986), which showed that the lipid bilayer could be approximated as being a ~5 nm thin sheet of liquid hydrocarbon. The possible importance of geometric packing criteria (Israelachvili, 1977), and the material properties of the lipid bilayer (Helfrich, 1973; Evans and Hochmuth, 1978), for lipid- protein interactions and the lateral organization of biological membranes largely were ignored.

The failure to appreciate the significance of the liquid-crystalline organization of lipid bilayers, with the associated material properties (thickness and compression modulus, curvature and bending modulus), also has implications for attempts to understand the mechanisms underlying the control of membrane protein function by the membrane lipids. Numerous studies (e.g. Devaux and Seigneuret, 1985; Bienvenüe and Marie, 1994) have shown that membrane protein function is affected by the membrane lipid composition - and by whether the lipids are in the gel or liquid-crystalline state. The view of the lipid bilayer as a sheet of liquid hydrocarbon led to the notion of bilayer fluidity as an important determinant of protein function.

The limitations of this notion were exposed by Lee (1991), who pointed out that a change in bilayer fluidity alone cannot explain a shift in the conformational preference of integral membrane proteins. If not fluidity, what then?

Membrane Protein Conformation Changes and Bilayer Perturbations

Structural studies on membrane proteins show that membrane protein function may involve changes in protein structure that affect the protein/lipid interface (Unwin and Ennis, 1984; Unwin et al., 1988). The hydrophobic coupling between the membrane-spanning domain of integral membrane proteins and the bilayer core will cause such a protein conformational change to perturb the structure of the immediately surrounding bilayer (Fig. 1). The free energy difference (ΔG^0_{tot}) between two protein conformations is the sum of contributions from the protein *per se* (ΔG^0_{prot}) and terms that arise from the protein's interactions with the environment, which include the deformation energy (ΔG^0_{def}) arising from the bilayer perturbation. This separation of the total free energy into intrinsic and extrinsic terms may be problematic, but the distinction helps to differentiate energetic contributions that arise from intramolecular rearrangements in the protein interior from the energetic cost of the intermolecular reorganization that occurs at the protein surface.

ΔG^0_{def} varies as a function of the material properties of the lipid bilayer, which, in principle, provides the means for control of the protein conformational preference and function by the bilayer lipid composition. Bilayer deformations (bilayer compression and bending) can be described using the theory of liquid-crystal deformations (Helfrich, 1973). This notion was further developed by Huang (1986) to describe the energetics of inclusion-induced membrane deformations (monolayer bending and compression). In such models of elastic membrane deformations, ΔG^0_{def} will be a function of the membrane deformation (u). When the energetic penalty associated with exposing hydrophobic groups to water is much larger than the

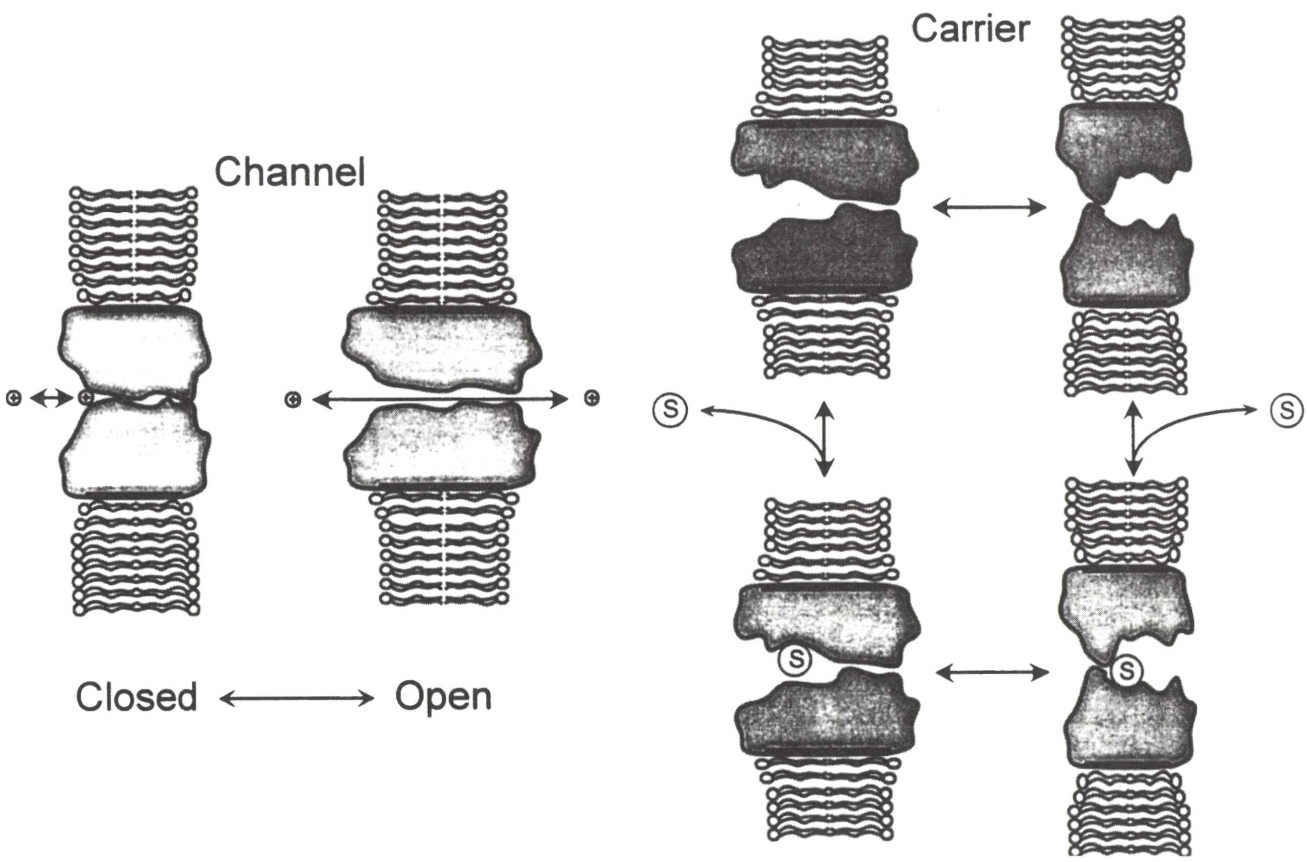


Figure 1. Solute transfer by membrane-spanning channels (left) and conformational carriers (right).

membrane deformation energy, i.e. in the limit of strong hydrophobic coupling , then u will be equal to the difference between the membrane hydrophobic thickness (d) and the protein's hydrophobic exterior length (l). In elastic membrane models, ΔG^0_{def} can in many cases be described as a quadratic function of u (Lundbæk et al., 1996; Nielsen et al., 1998):

$$\Delta G^0_{\text{def}} = A \cdot u^2 \qquad (1)$$

where A is a phenomenological spring constant associated with the membrane deformation. Is the bilayer deformation energy of sufficient magnitude to affect the protein conformational preference and function? To address this question it is necessary to have quantitative measurements that probe how the lipid bilayer affects structurally well-defined conformational transitions in membrane proteins (Gruner, 1991).

The energetic coupling between proteins and bilayers can affect the function of all imbedded proteins. There is, however, a fundamental difference between the way in which membrane-spanning channels and conformational carriers catalyze the transmembrane transfer of selected solutes (Fig. 1), which has implications for how the function of channels and carriers is affected by the lipid bilayer. In channels, the control of function arises from conformational changes between non-conducting (closed) and conducting (open) states. The catalytic event (the transfer of a solute/ion across the membrane) is uncoupled from such large-scale protein conformational changes. One therefore can use the channel-mediated ionic current to monitor directly the distribution between non-conducting (closed) and conducting (open)

channel states (conformations). In carriers, the catalytic events are inextricably coupled to protein conformation changes. A change in the equilibrium constant between the major conformers (the binding site exposed toward the left or the right in Fig. 1) will affect the rate constants for both the forward and the backward transitions (usually in opposite directions). This complicates attempts to understand how a change in bilayer material properties will affect the carrier function. The turnover rate, for example, may be a non-monotonic function of $\Delta G^0_{\rm def}$. Channels, therefore, offer advantages not enjoyed by the carriers for attempts to elucidate the basis for membrane control of protein function. (Similar advantages are offered by membrane-spanning receptors, where one likewise can monitor the equilibrium distribution between different conformations, see e.g. Brown (1994)).

Ion Channels as Tools to Study Protein-Membrane Interactions

Among ion permeable channels, the gramicidin A (gA) monomer↔dimer equilibrium, associated with the formation of membrane-spanning gA channels, constitutes a reasonably well-defined structural transition in a membrane inclusion (Figure 2). Standard gA channels are miniproteins formed by the transmembrane assembly (O'Connell et al., 1990) of two $\beta^{6.3}$-helical monomers (He et al., 1994), that join at their formyl-NH-termini to form the conducting channels (see Andersen and Koeppe, 1992; Killian, 1992, and Koeppe and Andersen, 1996, for reviews). Most, if not all, membrane-spanning gA dimers are conducting channels (Veatch et al., 1975), and there is no evidence for specific interactions between gA channels and their host bilayer (Providence et al., 1995; Girshman et al., 1997). These properties make gA suitable for investigating the bilayer mechanical properties.

The gA dimerization constant is $K_D = [D]/[M]^2$, where $[D]$ and $[M]$ denote the surface densities of gA dimers and monomers. Assuming that $\Delta G^0_{\rm def}$ is the only extrinsic contribution to $\Delta G^0_{\rm tot}$,

$$\begin{aligned} K_D &= \frac{[D]}{[M]^2} = \exp\{-\Delta G^0_{\rm tot}/kT\} \\ &= \exp\{-(\Delta G^0_{\rm prot} + \Delta G^0_{\rm def})/kT\} \\ &= K_D^{\rm prot} \cdot \exp\{-\Delta G^0_{\rm def}/kT\} \end{aligned} \quad (2)$$

where k is Boltzmann's constant, T the temperature in Kelvin, and $K_D^{\rm prot} = \exp\{-\Delta G^0_{\rm prot}/kT\}$, Rewriting Eq. 2 gives

$$\Delta G^0_{\rm def} = kT \cdot \ln\left\{\frac{[D]}{K_D^{\rm prot} \cdot [M]^2}\right\}. \quad (3)$$

The gA channel-associated membrane conductance $G = [D] \cdot g$, where g is the single-channel conductance, and Eq. 3 becomes

$$\Delta G^0_{\rm def} = kT \cdot \ln\left\{\frac{G/g}{K_D^{\rm prot} \cdot [M]^2}\right\} \quad (4)$$

which relates bilayer energetics and electrophysiological measurements. When $K_{\rm D}^{\rm prot}$ is unknown, Eq. 4 can be used to measure changes in $\Delta G^0_{\rm def}$ ($\Delta\Delta G^0_{\rm def}$) in the limit when $[D] << [M]$ (Lundbæk and Andersen, 1994; Lundbæk et al., 1997):

$$\Delta\Delta G^0_{\rm def} = -kT \cdot \ln\left\{\frac{G_{\rm exptl}/g_{\rm exptl}}{G_{\rm cntrl}/g_{\rm cntrl}}\right\}, \quad (5)$$

where the subscripts denote the experimental and control situations, respectively. It is thus possible to show that relatively modest modifications of the bilayer properties can change $\Delta G^0_{\rm def}$ by 10-15 kJ/mole (Lundbæk and Andersen, 1994; Lundbæk et al., 1997) - indicating that the bilayer deformation energy may be of sufficient magnitude to affect protein function.

Rather than $\Delta G^0_{\rm def}$, one can measure the disjoining force the bilayer imposes on the membrane-spanning gA dimers, which affects both the association (k_1) and dissociation (k_{-1}) rate constants. k_{-1} is of primary interest because $k_{-1} = 1/\tau$,

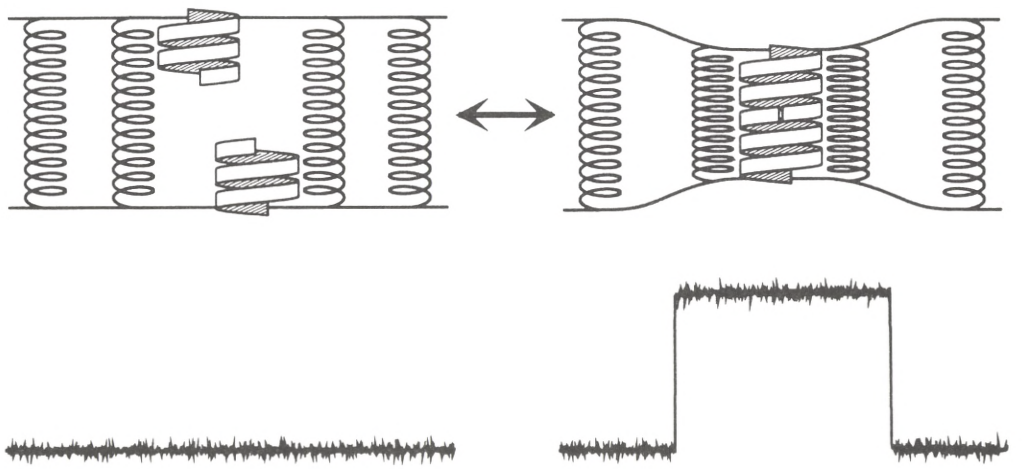


Figure 2. The gA monomer↔dimer reaction can be observed electrophysiologically. Top: schematic representation of the gramicidin monomer↔dimer equilibrium and the membrane perturbation that is associated with channel formation. Bottom: the current signal associated with channel formation/dissociation.

where τ is the average dimer (channel) lifetime, which is directly measurable

$$k_{-1} = \frac{1}{\tau_0} \cdot \exp\{-\Delta G^{\ddagger}/kT\}, \qquad (6)$$

where $\Delta G^{\ddagger}$ is the activation energy for dimer dissociation and $1/\tau_0$ is a frequency factor (in Eyring's Transition State Theory $1/\tau_0 = kT/h$). The transition state for dimer dissociation occurs when the monomers move a distance δ apart, and $\Delta G^{\ddagger}$ is the sum of the intrinsic activation energy ($\Delta G^{\ddagger}_{\rm prot}$) and the difference in bilayer deformation energy ($\Delta G^{\ddagger}_{\rm def}$) for a deformation of u and $u - \delta$. Using Eq. 1,

$$\begin{aligned} \Delta G^{\ddagger} &= \Delta G^{\ddagger}_{\rm prot} + \Delta G^{\ddagger}_{\rm def} \\ &= \Delta G^{\ddagger}_{\rm prot} + A \cdot ([u-\delta]^2 - u^2) \qquad (7) \\ &= \Delta G^{\ddagger}_{\rm prot} - A \cdot (2 \cdot u - \delta) \cdot \delta \end{aligned}$$

cf. (Lundbæk et al., 1996), and

$$\begin{aligned} \tau &= \tau_{\rm prot} \cdot \exp\{\Delta G^{\ddagger}_{\rm def}/kT\} \\ &= \tau_{\rm prot} \cdot \exp\{-A \cdot (2 \cdot u - \delta) \cdot \delta/kT\}, \qquad (8) \end{aligned}$$

where $\tau_{\rm prot} = \tau_0 \cdot \exp\{\Delta G^{\ddagger}_{\rm prot}/kT\}$. Assuming that the hydrophobic coupling is sufficiently strong (that $u = d - l$), A can be determined from the variation of τ as a function of membrane thickness (J. A. Lundbæk and O. S. Andersen, manuscript in preparation). The resulting value of A is large, indicating that the bilayer deformation energy associated with a hydrophobic mismatch can affect protein function - as well as the lateral organization of proteins in the membrane (cf. Mouritsen and Bloom, 1984).

Hydrophobic Coupling - and its Limitations

The hydrophobic coupling between integral membrane proteins and their surrounding bilayer is a central element in models of membrane organization. Limitations arise, however, for at least two reasons.

First, the hydrophobic/hydrophilic boundary is fuzzy - because of the imprecise relation between the positions of C_α and the charged, or

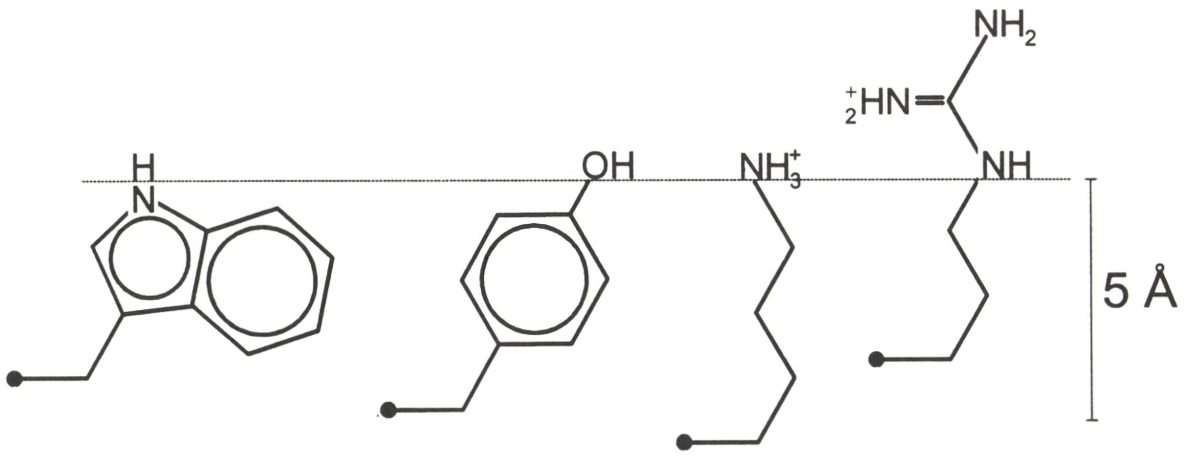


Figure 3. Amphipathic amino acids at the membrane/solution interface. The membrane/solution interface is denoted by ..... ; the C_α by •.

polar, moieties in the anchor residues that delimit membrane-spanning α-helices (Fig. 3). The $(CH_2)_3$ and $(CH_2)_4$ linkers in Arg and Lys, for example, allow the effective length of membrane-spanning α-helix to vary by several Å. Interestingly, the amphipathic aromatic residues may define the hydrophobic/hydrophilic boundary better, because the rigid ring structure provides for a better defined relation between C_α and the polar moiety. This may account for the preponderance of Trp and Tyr residues at the hydrophobic/hydrophilic boundary of integral membrane proteins (cf. Landolt-Marticorena et al., 1993).

Second, as u increases, the notion of strong hydrophobic coupling will fail ($u \neq d - l$) because ΔG^0_{def} eventually will become so large that it becomes advantageous to allow hydrophobic residues to be in direct contact with water. For example, the effective spring constant for membrane deformations adjacent to an integral membrane protein of radius 30 Å is ~4 kJ/(mol · Å^2) (C. Nielsen, M. Goulian and O. S. Andersen, in preparation). For the same protein, the hydrophobic penalty associated with a hydrophobic mismatch is ~20 kJ/(mol · Å), which means that the incremental deformation energy will exceed the incremental hydrophobic energy when $u > 2.5$ Å. Strong hydrophobic coupling therefore will fail for larger membrane deformations - and, even for small deformations, there may be some slippage.

Perspectives

The bilayer and its imbedded proteins exert reciprocal effects upon each other:

Protein Conformational Change
⇳
Bilayer Deformation Energy.

The reciprocity emphasizes the dynamic implications of the hydrophobic coupling between bilayer and proteins. That is, in addition to serving as an organizing principle for the folding of membrane proteins, the need to minimize the exposure of hydrophobic groups to water (Singer and Nicolson, 1972) also serves as an organizing principle for the regulation of protein function by the bilayer. It thus becomes important to understand how membrane lipid heterogeneity affects the dynamics and

energetics of protein conformational changes:

1. Does the local membrane lipid composition reorganize in response to membrane protein conformational changes?
2. How will such reorganization affect the lipid bilayer material properties - and the protein conformational changes?
3. What is the significance of lipid components that form non-bilayer structures; can a bilayer be too stable - e.g. because protein conformational transitions are facilitated close to the lamellar/non-lamellar phase transition for the membrane lipids?

Finally, implicit in the above is that the control of protein function by the membrane lipids *to a first approximation* is a "simple" energetic question, which can be addressed using the continuum theory of liquid-crystal deformations with minimal chemical specificity. That is, one can to a first approximation disregard the existence of numerous different membrane lipid components, and describe the bilayer as an elastic sheet. The situation thus becomes similar to that for electrified interfaces, where the Gouy-Chapman theory of the diffuse double layer serves as a major organizing principle (e.g. McLaughlin, 1989).

References

Andersen, O.S., and R.E. Koeppe, II. 1992. Molecular determinants of channel function. *Physiol. Rev.* 72:S89-S158.

Bienvenüe, A., and J.S. Marie. 1994. Modulation of protein function by lipids. *Curr. Top. Membr.* 40:319-354.

Brown, M.F. 1994. Modulation of rhodopson function by properties of the membrane bilayer. *Chem. Phys. Lipids* 73:159-180.

Devaux, P.F., and M. Seigneuret. 1985. Specificity of lipid-protein interactions as determined by spectroscopic techniques. *Biochim. Biophys. Acta* 822:63-125.

Evans, E.A., and R.M. Hochmuth. 1978. Mechanochemical properties of membranes. *Curr. Top. Membr. Transp.* 10:1-64.

Girshman, J., J.V. Greathouse, R.E. Koeppe, II, and O.S. Andersen. 1997. Gramicidin channels in phospholipid bilayers having unsaturated acyl chains. *Biophys. J.* 73:1310-1319

Gruner, S.M. 1991. Lipid membrane curvature elasticity and protein function *In*: Biologically Inspired Physics (Peliti, L., Ed.) Plenum Press, New York, Pp. 127-135

He, K., S.J. Ludtke, Y. Wu, H.W. Huang, O.S. Andersen, D. Greathouse, and R.E. Koeppe, II. 1994. Closed state of gramicidin channel detected by X-ray in-plane scattering. *Biophys. Chem.* 49:83-89.

Helfrich, W. 1973. Elastic properties of lipid bilayers: theory and possible experiments. *Z. Naturforsch.* 28C:693-703.

Huang, H.W. 1986. Deformation free energy of bilayer membrane and its effect on gramicidin channel lifetime. *Biophys. J.* 50:1061-1070.

Israelachvili, J.N. 1977. Refinement of the fluid-mosaic model of membrane structure. *Biochim. Biophys. Acta* 469:221-225.

Killian, J.A. 1992. Gramicidin and gramicidin-lipid interactions. *Biochim. Biophys. Acta* 1113:391-425.

Koeppe, R.E., II, and O.S. Andersen. 1996. Engineering the gramicidin channel. *Annu. Rev. Biophys. Biomol. Struct.* 25:231-258.

Landolt-Marticorena, C., K.A. Williams, C.M. Deber, and R.A.F. Reitmeier. 1993. Non-random distribution of amino acids in the transmembrane segments of human type I single span membrane proteins. *J. Mol. Biol.* 229:602-608.

Lee, A.G. 1991. Lipids and their effects on membrane proteins: Evidence against a role for fluidity. *Prog. Lipid Res.* 30:323-348.

Lundbæk, J.A., and O.S. Andersen. 1994. Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *J. Gen. Physiol.* 104:645-673.

Lundbæk, J.A., P. Birn, J. Girshman, A.J. Hansen, and O.S. Andersen. 1996. Membrane stiffness and channel function. *Biochemistry* 35:3825-3830.

Lundbæk, J.A., A.M. Maer, and O.S. Andersen. 1997. Lipid bilayer electrostatic energy, curvature stress, and assembly of gramicidin channels. *Biochemistry* 36:5695-5701.

McLaughlin, S. 1989. The electrostatic properties of membranes. *Annu. Rev. Biophys. Biophys. Chem.* 18:113-136.

Mouritsen, O.G., and M. Bloom. 1984. Mattress model of lipid-protein interactions in membranes. *Biophys. J.* 46:141-153.

Nielsen, C., M. Goulian, and O.S. Andersen. 1998. Energetics of inclusion-induced bilayer deformations. *Bio-*

phys. J. 74, in press.

O'Connell, A.M., R.E. Koeppe, II, and O.S. Andersen. 1990. Kinetics of gramicidin channel formation in lipid bilayers: transmembrane monomer association. *Science* 250:1256-1259.

Providence, L.L., O.S. Andersen, D.V. Greathouse, R.E. Koeppe, II, and R. Bittman. 1995. Gramicidin channel function does not depend on phospholipid chirality. *Biochemistry* 34:16404-16411.

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720-731.

Unwin, N., C. Toyoshima, and E. Kubalek. 1988. Arrangement of the acetylcholine receptor subunits in the resting and desentisitized states, determined by cryoelectron microscopy of crystalllized Torpedo postsynaptic membranes. *J. Cell. Biol.* 107:1123-1138.

Unwin, P.N.T., and P.D. Ennis. 1984. Two configurations of a channel-forming membrane protein. *Nature* 307:609-613.

Veatch, W.R., R. Mathies, M. Eisenberg, and L. Stryer. 1975. Simultaneous fluorescence and conductance studies of planar bilayer membranes contaning a highly active and fluorescent analog of gramicidin A. *J. Mol. Biol.* 99:75-92.

Walter, A., and J. Gutknecht. 1986. Permeability of small nonelectrolytes through lipid bilayer membranes. *J. Membrane Biol.* 77:255-264.

Helix Stability and Interactions in Membrane Proteins

Donald M. Engelman, Karen G. Fleming *and* Kevin R. MacKenzie

Abstract

The feasibility of a qualitative separation of interaction energies between those producing stable transbilayer helices and those resulting in the side-to-side association of such helices is supported by a growing body of data. We find that the specific side-to-side interactions in a dimeric association of glycophorin A transmembrane helices can be understood in terms of a detailed packing of their van der Waals surfaces with each other. Thus, a chemical approach to membrane protein folding may succeed.

Donald M. Engelman, Karen G. Fleming *and* Kevin R. MacKenzie
Department of Molecular Biophysics and Biochemistry
Yale University
New Haven, CT 06520, USA
email: jmurphy@paradigm.csb.yale.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:83–86

Introduction

Some years ago, we discussed principles of stability for helices in membranes and the use of those principles in folding and oligomerization (Engelman and Steitz, 1981; Popot and Engelman, 1990). It appears that the notion of a separation of energies into two distinct stages may have some utility in describing the ways in which helical membrane proteins fold and engage in oligomeric associations. The two-stage model for membrane protein folding (Popot and Engelman, 1990) proposes the existence of two energetically distinct events: the formation of independently stable transmembrane alpha-helices and their side-to-side association to form higher order structure (Fig. 1). Early data from bacteriorhodopsin (Popot et al., 1987) and more recent work on glycophorin (Lemmon et al., 1992; Fleming et al., 1997) generally support the ideas and have resulted in deeper insights into the chemical principles involved. However, it may be that some modifications to the model will be required; for example, it may prove to be the case that helical hairpins rather than single helices will act as stable motifs in some instances. The following is a general discussion of a current view of these ideas.

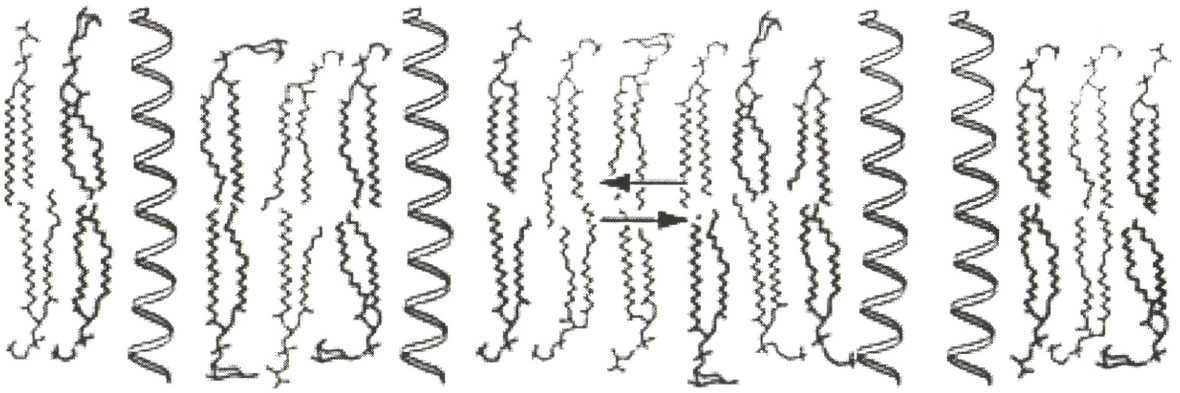


Figure 1. Two-stage model for helix association and membrane protein folding.

Helix Stability in Bilayers (Stage I)

If we consider a polypeptide having largely hydrophobic sidechains and traversing a phospholipid bilayer as a helix, we can ask which energies might contribute to its stability. It seems that two energy terms dominate in the stability of such a structure. The first is the hydrophobic effect, which favors partitioning of the helix into a hydrophobic environment and away from the aqueous environment. Based on the notion that the hydrophobic effect can be treated in terms of buried surface area, tens of kilocalories favor such a partitioning for a helix with aliphatic sidechains. The other strongly favorable energy term stabilizing the helix structure arises from the fact that the mainchain hydrogen bonds are in a low dielectric environment in which alternative donors and acceptors are absent. Thus, breaking mainchain hydrogen bonds in the hydrophobic region of the lipid bilayer carries a strong energy penalty, probably more than 5 kcal/mol per hydrogen bond. This greatly exceeds the energy corresponding to the entropy gained in a helix-coil transition, so one expects that helices will be strongly favored (or, as is well known from the porins, the alternative structure of a beta barrel may form, which also satisfies all of the hydrogen bonds). This balance of energies is supported by the observation that hydrophobicity drives helix formation even when β-branched sidechains are present (Li and Deber, 1994). Against these two stabilizing terms, and in addition to the chain entropy already mentioned, the presence of polar groups will oppose the hydrophobic effect. Some contribution of this kind is to be expected both from the mainchain of the helix and from polar sidechains. On balance, and generalizing from the widespread observation of helical structure in many membrane proteins, it appears that helical structures are strongly favored.

Helix-Helix Interactions (Stage II)

Useful views of helix-helix interactions come from studies of human glycophorin A, which forms dimers through interactions of its transmembrane helices. We have established that the individual helices are stable in the presence of sequence alterations that abolish dimerization while preserving

hydrophobicity. The fact that glycophorin dimers are stable in SDS has allowed an extensive study of sequence alterations that perturb the stability of the dimer, resulting in a large database of phenotypes for different sequences (Lemmon et al., 1992). Molecular dynamics and simulated annealing methods have been used to find side-to-side helix contacts that are favorable for the formation of dimeric structures of the glycophorin transmembrane region. Only a few favorable contacts are found; one of these agrees well with the data from sequence variation, and has led to the choice of a model for the structure (Adams et al., 1996). Using NMR spectroscopy we have now derived a structure that is independent of the previous data, and that agrees well with the model (MacKenzie et al., 1997). We have also learned a great deal more from the NMR structure concerning the details of the interaction. The fact that a largely correct model was derived from a combination of modeling and sequence variation encourages the view that reliable models for membrane proteins may be attainable without detailed crystallographic or NMR study.

Using the NMR structure, we have examined the basis of the effects of different mutations on the stability of the dimer, and we find that the dimerization is largely driven through the precise van der Waals fit of two complementary surfaces. Mutations that introduce steric clashes are strongly disruptive of the interaction, and mutations that remove van der Waals contacts also weaken the dimerization. Thus, the second stage of the two-stage model - lateral association of TM helices - can be modulated by sequence changes that affect the detailed packing between helices

We have also developed a protocol to measure the energy of interaction of the glycophorin dimer and sequence variants using analytical ultracentrifugation (Fleming et al., 1997). By using a detergent with a micelle buoyant density similar to that of buffered solvent, we observe the equilibration of the monomer and dimer of a construct of the glycophorin transmembrane domain coupled to staphylococcal nuclease. From the equilibrium distribution, we obtain equilibrium constants, and, therefore, free energies. We observe a larger proportion of dimer than was reported by the SDS assay; however, the energy changes for residue substitutions are in the same rank order of importance. Thus, it appears that the micelle environment, which is different in the two cases since different detergents were used, produces a change in the overall association constant but does not alter the hierarchy of interactions of different glycophorin molecules. If we combine this with the observation that the modeling that was successful in identifying choices for the structure is conducted *in vacuo*, we arrive at the view that the interaction of helix surfaces produces a key energy term that is not sensitive to the lipid environment. The separability of helix-helix interaction energies from the energy contributed by helix-lipid interaction would be a great simplification in understanding membrane protein folding.

Further Development of Ideas Concerning Membrane Protein Folding

While the two-stage model appears to describe much of what we have observed, it appears that some modifications may emerge. Studies of the individual helices of bacteriorhodopsin (BR) have revealed that, while the first five helices are capable of independently forming transbilayer helical structures, the last two may not be (Li and Deber, 1994), yet they are capable of reforming BR when combined with retinal and a peptide containing the first five helices (Ozawa, 1997). It may be that the link between them is necessary to stabilize a "hairpin" structure that promotes their stability as a subsection of the structure, or, possibly, that interactions with the other helices in the molecule are required to stabilize their transbilayer helical organization.

Summary

The notions of independent stability of helices and the role of helix-helix interfaces in forming membrane protein structures appear to be useful. Lipid-protein interactions, while they probably contribute to the stability of membrane proteins, may not strongly influence the details of helix interactions. If the separability of energies that we have observed is supported by further tests, it may be that good models for the transmembrane structures of helical membrane proteins can be developed on the basis of a combination of relatively limited data with molecular modeling approaches.

References

Adams, P.D., D.M. Engelman, and A.T. Brünger. 1996. An improved prediction for the structure of the dimeric transmembrane domain of glycophorin A obtained through global searching, *Proteins* 26:257-261.

Engelman, D.M., and T.A. Steitz. 1981. The spontaneous insertion of proteins into and across membranes: The helical hairpin hypothesis, *Cell* 23: 411-422.

Fleming, K.G., A.L. Ackerman, and D.M. Engelman. 1997. The effect of point mutations on the free energy of transmembrane -helix dimerization, *J. Mol. Biol.* 272:266-275.

Lemmon, M.A., J.M. Flanagan, H.R. Treutlein, J. Zhang, and D.M. Engelman. 1992. Sequence specificity in the dimerization of transmembrane α-helices, *Biochemistry* 31: 12719-12725.

Li, S.C., and C.M. Deber. 1994. A measure of helical propensity for amino acids in membrane environments, *Nature Struc. Biol.* 1:368-373.

MacKenzie, K.R., J.H. Prestegard, and D.M. Engelman. 1997. A transmembrane helix dimer: structure and implications, *Science* 276:131-133.

Ozawa, S., R. Hayashi, A. Masuda, T. Ito, and S. Takahashi. 1997. Reconstitution of bacteriorhodopsin from a mixture of a proteinase V8 fragment and two synthetic peptides, *Biochem. Biophys. Acta* 1323:145-153.

Popot, J.-L., and D.M. Engelman. 1990. Membrane protein folding and oligomerization: The two-stage model, *Biochemistry* 29: 4031-4037.

Popot, J.-L., S.E. Gerchman, and D.M. Engelman. 1987. Refolding of bacteriorhodopsin in lipid bilayers: A thermodynamically controlled two-stage process, *J. Mol. Biol.* 198:655-676.

Membrane Specificities of Antimicrobial Peptides

Huey W. Huang

Abstract

Antimicrobial peptides target the lipid matrix of plasma membranes. Their biological functions can be understood in terms of the free energy of peptide-membrane interactions.

Huey W. Huang
Physics Department
Rice University
Houston, TX 77005, USA
e-mail: huang@ion.rice.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:87–92

During the past decade, endogenous antimicrobial peptides have become recognized as important, ubiquitous, and ancient contributors to the innate mechanisms which permit animals (including humans) and plants to resist infection (Boman et al., 1994). Most of these host defense peptides are small (18-35 amino acids), amphipathic and possess either an α-helical or cystine-stabilized β-sheet structure. They are most likely too small for enzymatic function and, so far, no specific receptors have been found. All evidence indicates that their target of action is the lipid matrix of the plasma membranes. What are the molecular mechanisms of these peptides? How do they kill bacteria without harming the host cells? Indeed the most interesting question is: how do they accomplish the membrane specificities? These questions provide a good test to our current understanding of membrane properties.

In this review article, we will concentrate on helical peptides. In particular, we will discuss the experimental data of alamethicin (a fungal peptide) and magainin (a peptide secreted in frog skin). Magainin exhibits a broad-spectrum of antibacterial, antifungal, and tumoricidal activities. However, at the bactericidal concentrations, the peptide does not harm eukaryotic cells–it does so only if the concentrations are increased 100- to 1000-fold. On the other hand, alamethicin is hemolytic at the concentrations it is bactericidal. However, this does not mean that alamethicin lyses all cells equally. Even among various bacteria, there is a 1000-fold difference in their sensitivity to alamethicin (Jen et al., 1987).

In their natural environment, antimicrobial peptides interact with cell membranes in two steps.

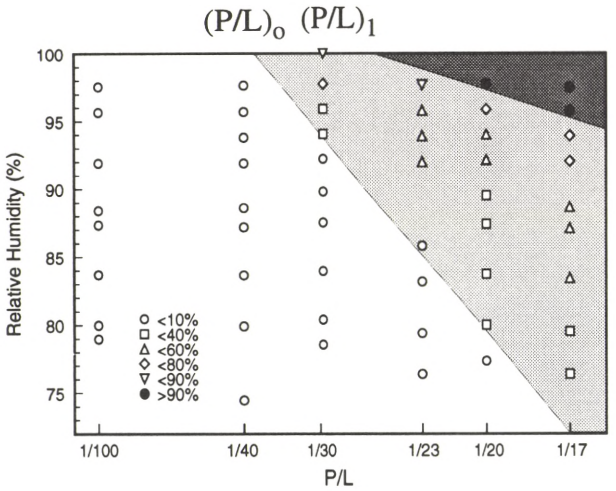


Figure 1. Orientation of alamethicin helices in DPhPC bilayers in the phase diagram P/L vs relative humidity. The symbols represent the percentage of alamethicin oriented perpendicularly.

First they bind to the lipid matrix of the membranes. This step has been studied by vesicle binding experiments. As expected, positively charged peptides preferentially bind to acidic lipids (which are present on the outer surface of bacterial plasma membranes). However, if the binding of peptides were enough to kill cells, this electrostatic effect alone does not provide a good selectivity. Why? Because these peptides are amphipathic and they do bind to neutral lipid membranes. Besides, there are examples countering the electrostatic argument: for instance, melittin is more effective in lysing neutral liposomes than acidic liposomes. We have to examine the second step, i.e., what happens after the peptides bind to the membranes.

Matsuzaki et al. (1995) showed that once a peptide binds to a bilayer surface, it quickly redistributes itself to both sides of the bilayer by translocation. Therefore it is appropriate to consider peptide-membrane interactions with the peptides bound to both sides of the membrane. Our experiments started with a peptide-lipid mixture at a molar ratio (P/L). The mixture was aligned into parallel lamellae. The water content of the sample was determined by the relative humidity which the sample was equilibrated with. Since the sample tends to flow off the substrate if the relative humidity is $\geq$ 98%, the experimental range of the relative humidity was ~70% to ~98% RH (most experiments were performed in the L_α phase). The hydration dependence of our experiment is extremely helpful for interpreting the results. In particular we are able to extrapolate the results to the full hydration from the humidity dependent data.

First we measured the orientation of the helical axes of the peptides relative to the plane of the lipid bilayers. This was conveniently accomplished by the method of oriented circular dichroism (Wu et al., 1990). Fig. 1 shows the orientation of alamethicin helices in diphytanoylphosphatidylcholine (DPhPC) bilayers at various P/L and RH. Most surprisingly, the result resembles a phase diagram. For P/L below a critical value, $(P/L)_0$ ~1/40, the helical peptides adsorb parallel to the membrane surface. Above the critical concentration, however, a fraction of the peptides are oriented perpendicular to the bilayers while the rest remain on the membrane surface. Above an even higher concentration, $(P/L)_1$, all of the peptides are oriented perpendicular to the plane of the membranes. It appears that this is the general behavior of alamethicin in all lipid bilayers (Huang and Wu, 1991). However, in most lipids, the critical concentration $(P/L)_0$ as well as $(P/L)_1$ are very low, so that within the experimental range of P/L where the peptide orientation is detectable,

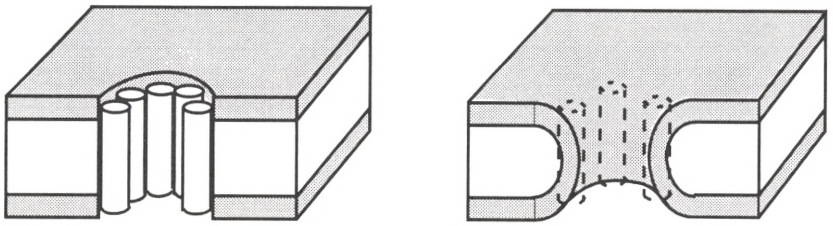


Figure 2. Alamethicin inserts into a lipid bilayer by forming a cylindrical pore with the peptide monomers lining the periphery. (The small cylinders represent the helical peptide monomers. The shaded area represents the headgroup region of the lipid bilayer. The dotted cylinders represent the peptide monomers embedded in the headgroup region.) Magainin always associates with the headgroups of the lipid. When magainins insert, they carry the headgroups with them. So the top monolayer bends and merges with the bottom monolayer like the inside of a torus.

one sees only the phase of perpendicular orientation (e.g., in DMPC, POPC, etc.). Magainin also exhibits a similar phase diagram, but whether it has an all-perpendicular concentration $(P/L)_1$ is not clear (Ludtke et al., 1994).

The samples with $P/L > (P/L)_0$ were examined by neutron scattering (He et al., 1995; 1996a; Ludtke et al., 1996). We found pores in the membranes whenever there are peptides perpendicularly oriented. Interestingly alamethicin and magainin form two different types of pore (Fig. 2). Both pores are large (water pathways $\geq$ 20 Å in diameter). A high density of pores in the cell membrane is apparently lethal to the cell. On the other hand, below the critical concentration, $P/L < (P/L)_0$, the great majority of the peptide are adsorbed on the surface. Through thermal fluctuations, a small number of transient pores (lifetimes being ms) may appear as detected by patch-clamp measurements. Because cells have repair mechanisms, these transient pores are presumably nonlethal (Boman et al., 1994). Therefore, what determines the action of a peptide is its membrane-bound concentration relative to the critical concentration $(P/L)_0$. To see the significance of the phase transition, let us imagine that the pore state and the surface state are two states of the peptide with different energies. Without cooperativity, the fraction of the peptide forming pores is determined by a Boltzmann factor, independent of the peptide concentration. With a phase transition, the system gains a control parameter, i.e., the concentration. And there can be a range of concentration in which the peptide is a very effective antimicrobial (Fig. 3).

To see what causes the phase transition, we have to examine the energetics of the peptide-membrane interactions beyond the principle of hydrophobic matching. The clue comes from x-ray diffraction experiments. We found that the membrane thickness decreases linearly with the peptide concentration on its surface (Fig. 4). This is the evidence that the peptide is adsorbed within the headgroup region of the bilayer. The adsorption expands the bilayer laterally and hence reduces its thickness (Fig. 5). From the (fractional) decrease in the thickness, one obtains the (fractional) increase in the lipid area $\Delta A/A_0$ as a function of P/L. We found $\Delta A(L/P)$ equal to the cross section of alamethicin lying parallel to the bilayer (Wu et al., 1995). Magainin also causes membrane thinning in the same manner (Ludtke et al., 1995). I proposed to describe the bilayer deformation energy F (per unit area) by (Huang, 1986; 1995)

$$F = aB\left[\frac{D(x,y)}{2a}\right]^2 + \frac{K_c}{8}[\Delta D(x,y)]^2 + \frac{K_c}{2}[\Delta M(x,y) - C_0(x,y)]^2. \quad (1)$$

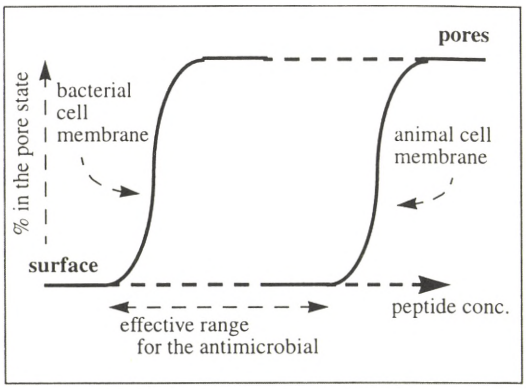


Fig. 3

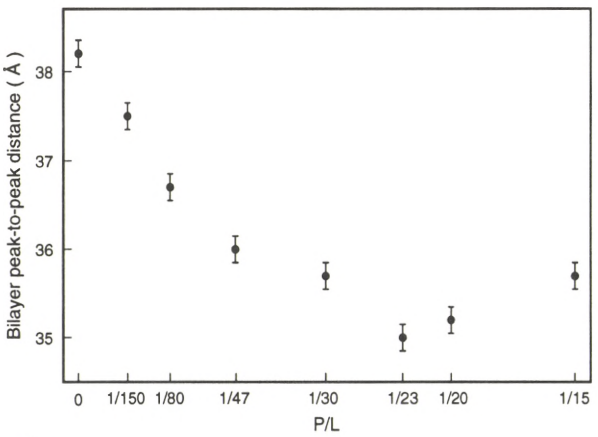


Fig. 4

Figure 3. Significance of phase transitions.

Figure 4. The bilayer thickness is defined as the peak-to-peak (approximately phosphate-to-phosphate) distance in the electron density profiles measured by x-ray diffraction. For $P/L<(P/L)_0$, the thickness decreases linearly with alamethicin concentration.

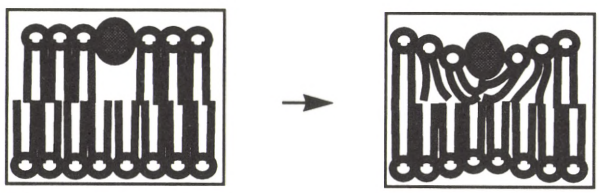


Figure 5. Membrane thinning effect. Imagine a peptide in the headgroup region creating a gap in the chain region. For this gap to be filled, the membrane must become locally thinner.

The unperturbed bilayer is assumed to lie in the xy plane. $D(x,y)$ is the deviation of the bilayer thickness from the equilibrium thickness $2a$ at the coordinate (x,y). $M(x,y)$ is the displacement of the mid-plane of the bilayer from its equilibrium position. Δ is the Laplacian. B is the compressibility modulus of the bilayer. K_c is Helfrich's bending rigidity for a bilayer (Helfrich, 1973). $C_0(x,y)$ is the local spontaneous curvature induced by peptide adsorption. Only the change of the bilayer thickness (the D-mode) will concern us here. The free energy of thickness deformation consists of only the first two terms, the compressibility term and the splay term. The effect of peptides adsorbed on the membrane surface is as follows: The peptide-induced membrane deformation has a characteristic length $\lambda = (aK_c/2B)^{1/4} \sim 13$ Å. There is a membrane mediated interaction between two adsorbed peptide monomers described by the potential $V(x)$, where $x = r/\sqrt{2\lambda}$ and r is the distance between the two monomers (Fig. 6). The potential is repulsive up to about $r \sim 37$ Å. Therefore the peptide is dispersed on the bilayer surface as monomers. At low concentrations (the average inter-peptide distance >37 Å), the total membrane deformation energy is proportional to the peptide concentration. However, if the concentration is high such that the average inter-peptide

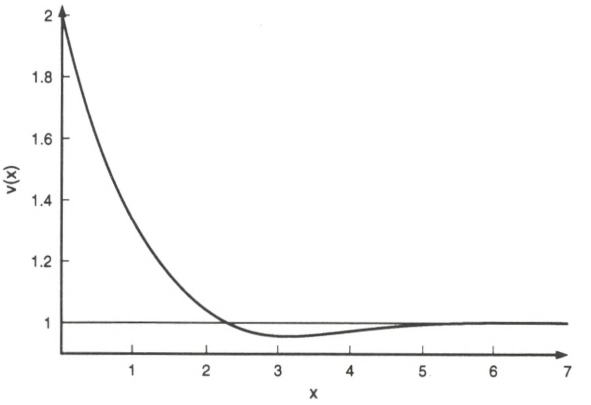


Figure 6. The membrane mediated interacting potential between two peptide monomers adsorbed on the surface, normalized to one at large distance. $x = r/\sqrt{2\lambda}$.

distance is <37 Å, the total deformation energy increases quadratically with the peptide concentration (Huang, 1995).

Thus the chemical potential μ_s of peptide adsorption consists of two parts: the binding energy (primarily due to hydrophobic matching) $-\varepsilon$ and the energy of membrane deformation per peptide f. As mentioned above, at high peptide concentrations f is proportional to P/L. Imagine that we gradually increase the peptide concentration from zero. At first μ_s is a constant. Then as the surface concentration exceeds $\sim (1/\sqrt{2\lambda})^2$, μ_s begins to increase linearly with P/L, until it becomes equal to the chemical potential for insertion μ_I. Until $\mu_s = \mu_I$, the great majority of the peptide molecules are adsorbed on the membrane surface. Only after μ_s reaches μ_I, a macroscopic insertion is possible. (In other words, as long as $\mu_s < \mu_I$, only a small number of transient pores appear in the membrane.) Thus the concentration that satisfies $\mu_s = \mu_I$ defines the critical concentration $(\mathrm{P/L})_0$. A mean-field calculation based on the free energy (1) showed that indeed a phase transition as described by Figs. 1 and 4 takes place (He et al., 1996b). In this model we showed that $(\mathrm{P/L})_0$ is decided by many factors including: the elastic constants of the bilayers, the binding energy difference between the surface state and the pore state, the area expansion of the bilayer per peptide adsorbed and the thickness-matching condition of the bilayer to the inserted peptide.

Thus the specificity (or selectivity) of a peptide toward different cell membranes is determined by its binding coefficient and its critical concentration (of the bound peptide) for pore formation, $(\mathrm{P/L})_0$. Both are sensitive to the lipid composition of the membrane. The selectivities of these peptides are sharp (kill or harmless at a given concentration) because their actions are cooperative phenomena.

As a simple test to the above model, we predicted that if the size of the lipid headgroup is reduced, the bilayer will accommodate a higher concentration of peptide on its surface – in other words, the critical concentration $(\mathrm{P/L})_0$ will increase. By mixing a small amount of DPhPE into the pure DPhPC bilayer (Heller et al., 1997) the theory predicts:

$$\left(\frac{P}{L}\right)_0(\theta) = \left(\frac{P}{L}\right)_0(0) + \theta\left(\frac{\Sigma_{PC} - \Sigma_{PE}}{\Gamma}\right) \quad (2)$$

where θ is the fraction of PE in the PE-PC mixture. $(\mathrm{P/L})_0(\theta)$ is the critical concentration as a function of θ. Σ_{PC} and Σ_{PE} are the cross sections of PC and PE headgroup, respectively. Γ is the cross section of alamethicin. For example, the equation predicts that $(\mathrm{P/L})_0(0.1) \sim 1/25$ at

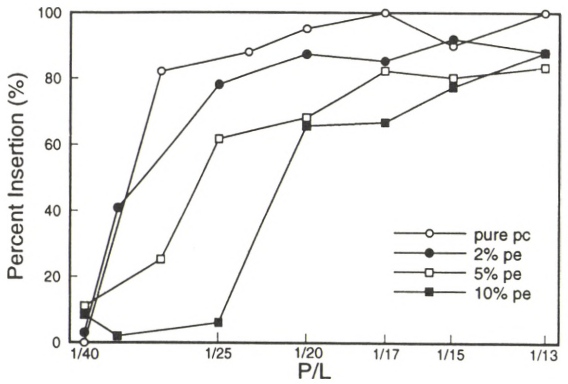


Figure 7. Percentage of alamethicin insertion as a function of P/L, measured by oriented circular dichroism, in pure PC and three PE-PC mixtures.

10%PE and $(P/L)_0(0.05) \sim 1/31$ at 5%PE. Both agree with the experiment quite well (see Fig. 7).

***

This work was supported in part by the NIH grant GM55203 and Biophysics Training grant GM08280; by the DOE grant DE-FG03-93ER61565; NSF grant INT-9312637; and by the Robert A. Welch Foundation.

References

Boman, H.G., J. Marsh, and J.A. Goode. Eds. 1994. Antimicrobial Peptides, Ciba Foundation Symposium 186, pp 1-272, John Wiley & Sons, Chichester.

He, K., S.J. Ludtke, W.T. Heller, and H.W. Huang. 1996b. Mechanism of alamethicin insertion into lipid bilayers, *Biophys. J.* 71: 2669-2679.

He, K., S.J. Ludtke, D.L Worcester, and H.W. Huang. 1995. Antimicrobial peptide pores in membranes detected by neutron in-plane scattering. *Biochemistry* 34: 15614-15618.

He, K., S.J. Ludtke, D.L. Worcester, and H.W. Huang. 1996a. Neutron scattering in the plane of membrane: structure of alamethicin pores. *Biophys. J.* 70: 2659-2666.

Helfrich, W. 1973. Elastic properties of lipid bilayers: theory and possible experiment. *Z. Naturforsch* 28C: 693-703.

Heller, W.T., K. He, S.J. Ludtke, T.A. Harroun, and H.W. Huang. 1997. Effect of changing the size of lipid headgroup on peptide insertion into membranes. *Biophys. J.* 73: 239-244.

Huang, H.W. 1986. Deformation free energy of bilayer membrane and its effect on gramicidin channel lifetime. *Biophys. J.* 50: 1061-1070.

Huang, H.W. 1995. Elasticity of lipid bilayer interaction with amphiphilic helical peptides. *J. Phys. II France* 5: 1427-1431.

Huang, H.W., and Y. Wu. 1991. Lipid-alamethicin interactions influence alamethicin orientation. *Biophys. J.* 60: 1079-1087.

Jen, W.-C., G.A. Jones, D. Brewer, V.O. Parkinson, and A. Taylor. 1987. The antibacterial activity of alamethicin and zervamicins. *J. Applied Bacteriology* 63: 293-298.

Ludtke, S.J., K. He, W.T. Heller, T.A. Harroun, L. Yang, and H.W. Huang. 1996. Membrane pores induced by magainin. *Biophys. J.* 35: 13723-13728.

Ludtke, S.J., K. He, and H.W. Huang. 1995. Membrane thinning caused by magainin 2. *Biochemistry* 34: 16764-16769.

Ludtke, S.J., K. He, Y. Wu, and H.W. Huang. 1994. Cooperative membrane insertion of magainin correlated woth its cytolytic activity. *Biochim. Biophys. Acta* 1190: 181-184.

Matsuzaki, K., O. Murase, N. Fujii, and K. Miyajima. 1995. Translocation of a channel-forming antimicrobial peptide, megainin 2, across lipid bilayers by forming a pore. *Biochemistry* 34: 6521-6526.

Wu, Y., K. He, S.J. Ludtke, and H.W. Huang. 1995. X-ray diffraction study of lipid bilayer membrane interacting with amphiphilic helical peptides: diphytanoyl phosphatidylcholine with alamethicin at low concentrations. *Biophys. J.* 68: 2361-2369.

Wu, Y., H.W. Huang, and G.A. Olah. 1990. Method of oriented circular dichroism. *Biophys. J.* 57: 797-806.

Tryptophan Anchors in Transmembrane Peptides

Roger E. Koeppe II, J. Antoinette Killian,
Denise V. Greathouse, *and* Olaf S. Andersen

Abstract

Geometric considerations prescribe that embedded proteins may be regarded as "defects" in lipid bilayers (unless there is "perfect" structural matching). A critical accumulation of such defects could mandate a lipid phase transition, and such macroscopic effects may lend insight into individual molecular lipid-protein interactions. To investigate these issues, we are using two types of model transmembrane peptides: gramicidins and designed membrane-spanning alpha-helices. Both models are anchored at each membrane/water interface by multiple tryptophans, which appear to be important for modulating the lipid phase behavior.

Roger E. Koeppe II and Denise V. Greathouse
Department of Chemistry and Biochemistry
University of Arkansas
Fayetteville, AR 72701, USA
e-mail: rk27487@uafsysb.uark.edu

J. Antoinette Killian
Center for Biomembranes and Lipid Enzymology
University of Utrecht
3584 CH Utrecht, The Netherlands

Olaf S. Andersen
Department of Physiology and Biophysics
Cornell University Medical College
New York, NY 10021, USA

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:93–98

Introduction

Lipid bilayers vary in hydrophobicity from the polar exterior to the non-polar membrane interior, with the hydrophobicity difference being bridged by the derivatized glycero-phosphate groups. The fluid-mosaic model of the cell membrane provided a way to incorporate proteins into this picture, based on the principle of maximizing hydrophobic and hydrophilic interactions (Singer and Nicolson, 1972). That embedded proteins might influence the host lipids was not considered. Molecular packing considerations (Israelachvili, 1977), nevertheless, reveal that– even in the absence of specific intermolecular interactions between lipids and proteins–the structure or mobility of lipids near a protein must differ from those in the rest of the bilayer. Moreover, geometric constraints impose a structural coupling between proteins and neighboring lipids such that the proteins do not "float

freely" in a sea of lipids (Israelachvili, 1977).

Embedded proteins therefore may be regarded as "defects" within otherwise pure bilayers, which raises questions about how different amino acid sequences will insert, orient and interact with lipids. Depending on their hydrophobicities, different side chains will seek the membrane interior, the aqueous phase or the membrane/water interface. Mismatches in the hydrophobic lengths of lipids and membrane-spanning peptide segments will introduce strain. A low density of defects will increase the bilayer energy marginally and cause local perturbations that could be important for large-scale dynamic processes such as endocytosis, membrane division or fusion. In the extreme, a critical accumulation of packing defects may mandate a phase transition. To address some of these issues, we have made use of membrane-spanning peptides of defined sequence.

The Questions We Wished to Elucidate Were:

A. How do protein sequences fit into the hydrophobicity gradient of the membrane?

B. What determines the "hydrophobic length" of a particular peptide?

C. What are effective anchoring residues for transmembrane segments of proteins? How many such residues are needed per segment?

D. What determines the transmembrane orientation or tilt of embedded protein segments?

Peptide Models

In order to be useful for understanding these questions, peptide models will need to:

(A) adopt defined, non-random, folded structures; and

(B) assume defined orientations within lipid bilayer membranes.

Single-span transmembrane peptides, such as gramicidin channels and non-aggregating α-helical peptides, may satisfy these demands (Killian, 1992; Koeppe and Andersen, 1996; Killian et al., 1996).

The structure and organization of the peptides and lipids will be influenced by:

(1) the relative lengths of the peptides and lipids,

(2) the hydrophobicity of the internal part of the peptide sequence, and

(3) the identity and placement of anchoring residues.

Anchoring residues will seek the aqueous phase or the membrane/water interface (head group region). Charged residues are good anchors, and several transmembrane peptide models have been developed using charged residues as anchors (e.g., Davis et al., 1983; Zhang et al., 1995). Charged anchors are not the only possible anchor residues, however. Peptides that have no side-chain or end-group charges and that fit entirely within the span of the bilayer can use tryptophan indole rings as anchors (Figures 1, 2).

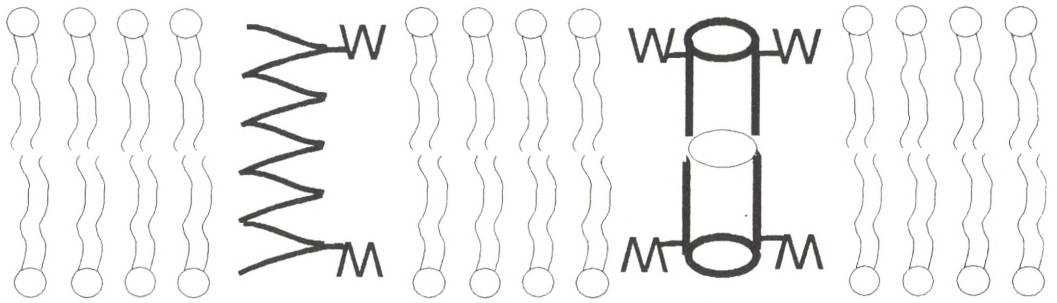


Figure 1. Schematic drawings of a 30-residue gramicidin dimer (channel) and a 17-residue transmembrane α helix, each with tryptophan anchors depicted as "W". Each model peptide has a defined folded conformation and transmembrane orientation. (For clarity, only 50% of the tryptophans are shown.)

gA HCO-VGALAVVVWLWLWLW-$NHCH_2CH_2OH$

WALP17 HCO-AWWLALALALALALWWA-$NHCH_2CH_2OH$

Figure 2. Sequences of the peptide models in Fig. 1: gramicidin A (gA, with D-residues underlined), which spans a bilayer as a $\beta^{6.3}$- helical dimer with W's at both ends; and WALP17 (Killian et al., 1996), which spans a bilayer as an α-helical monomer with all L- residues, and W's at both ends.

Lipid Influence on Peptide Conformation

The linear gramicidins require a lipid bilayer (or bilayer-like environment) to fold properly. If the phospholipid acyl chains are less than eight carbons long, the channel fold is not observed (Figure 3); if the acyl chains are too long, the membrane-spanning channels are destablilized. When the phospholipid acyl chains are lengthened from 16 to 20 carbons, the average channel duration decreases from ~5,000 to ~50 ms (N. Mobashery, C. Nielsen and O. S. Andersen, unpublished observations). If the acyl chain is increased to 22 carbons, the standard gramicidin channels become so destabilized that new phenotypes predominate (Nielsen et al., 1997). These results highlight the importance of hydrophobic matching between peptides and lipids (Owicki et al., 1978; Engelman and Zaccai, 1980; Mouritsen and Bloom, 1984; Mouritsen and Bloom, 1993). Taken together with the results of O'Connell et al. (1990), these results show that the tryptophan indole rings act as anchors that prefer the lipid head groups (over both the aqueous phase and the membrane interior).

In the case of alpha-helical WALP peptides and when the phospholipid and peptide lengths are matched (so that the peptides can span the acyl chain region), hydrophobic WALP peptides such as WALP17 (Figure 2) fold into α-helices with the helix axis in a transmembrane orientation (Figure 4).

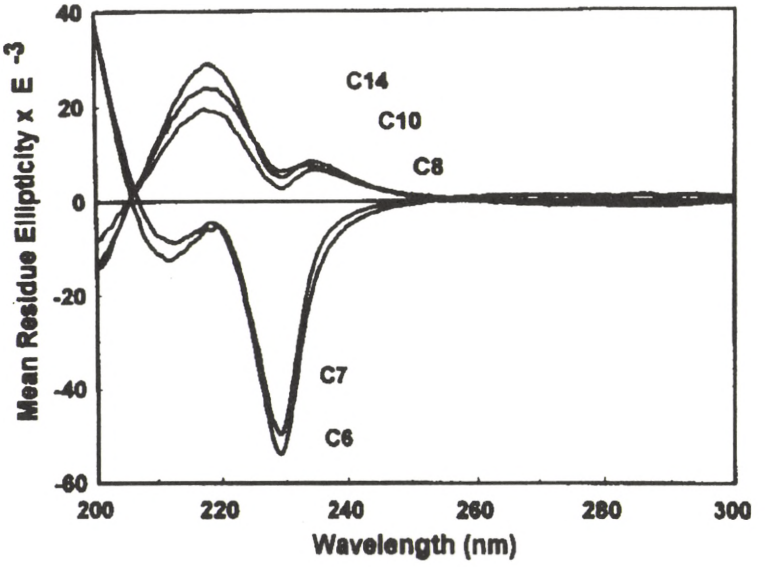


Figure 3. Circular dichroism spectra of aqueous gA/di-Cn-PC dispersions, showing characteristic RH SS $\beta^{6.3}$ channel spectra for $n = 8$, 10 or 14, and LH DS spectra for $n = 6$ or 7 (1 mM gA, 28 mM lipid, 55°C). Reproduced from Greathouse et al. (1994).

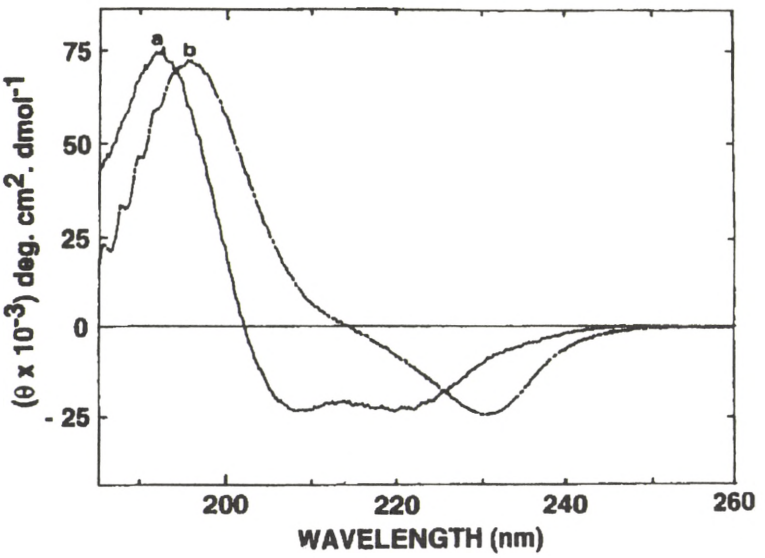


Figure 4. CD spectra of WALP17 in di-C14:0-PC at 1/25 peptide/lipid in (a) sonicated vesicles in excess water, and (b) oriented bilayers. From Killian et al. (1996).

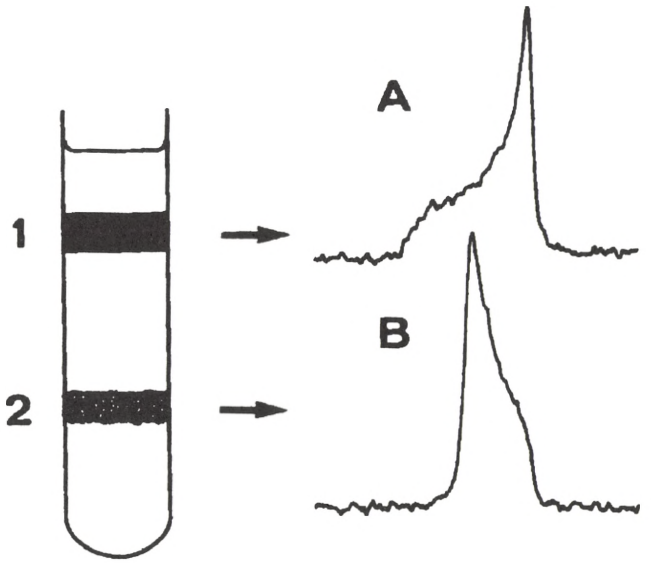


Figure 5. Illustration of the separation by sucrose density gradient centrifugation of a mixture of di-C18:1*c* PC and WALP16 into bilayer (A) and H_{II} components (B). From Killian et al. (1996).

Peptide Influence on Lipid Organization

Peptide-lipid interactions are reciprocal. When gramicidin or WALP17 is incorporated in phosphatidylcholine (PC) bilayers at high peptide/lipid molar ratios, the bilayer responds to a hydrophobic mismatch (in which the peptides are shorter than the lipid acyl chains) by forming non-bilayer structures (Van Echteld et al., 1982; Killian et al., 1989; Killian 1992; Killian et al., 1996). The separation of a peptide/lipid mixture into bilayer and inverted H_{II} phase components is illustrated for a WALP peptide in Figure 5.

Similar phase transitions have not been observed for other hydrophobic peptides in PC systems. The effect is therefore attributed to the presence of multiple tryptophans near the lipid/water interfaces (Killian et al., 1996; c.f., Figure 1). But we cannot at this time exclude that the peptide-induced phase transition is due to a combination of the anchoring effect of hydrogen bonding between the indole NH group and water, and the bulk of the indole ring *per se*, which may perturb the lipid packing.

Significance and Future Questions

The local lipid phase behavior around embedded proteins will affect biological processes that involve membrane rearrangement, fusion or separation. The mechanisms that regulate such processes remain enigmatic.

The tryptophan-anchored peptides, both the gramicidins and the WALP peptides, provide evidence that a build-up of bilayer energy, due to hydrophobic mismatch, can affect the structural organization of both lipids and the imbedded peptides. Remaining questions include:

A. What boundaries or sequence elements determine the effective peptide hydrophobic length? Is the length related to the distance between the two innermost amphipathic or polar side chains?

B. Will embedded helices tilt relative to the membrane normal– particularly, when the effective hydrophobic length of a transmembrane segment is longer than that of the lipids in the bilayer? How will that affect lipid-peptide interactions?

C. What is the minimum number of tryptophans required to induce the lipid phase transitions?

D. What is the relation between the density of membrane- spanning inclusions (proteins or peptides), the hydrophobic mismatch, and the propensity for the lipids to adopt nonbilayer structures?

E. To what extent, if any, is membrane protein function affected by the energetic cost of a hydrophobic mismatch? Again, experiments with model systems that adopt defined conformation will be important. The gramicidin derivative gLW, for example, assumes three different functional membrane-spanning conformations (Koeppe and Andersen, 1996). More examples will be needed.

References

Davis, J.H., D.M. Clare, R.S. Hodges, and M. Bloom. 1983. Interaction of a synthetic amphiphilic polypeptide and lipids in a bilayer structure. *Biochemistry* 22:5298-5305.

Engelman, D.M., and G. Zaccai. 1980. Bacteriorhodopsin is an inside- out protein. *Proc. Natl. Acad. Sci. U.S.A.* 77:5894-5898.

Greathouse, D.V., J.F. Hinton, K.S. Kim, and R.E. Koeppe II. 1994. Gramicidin A/short-chain phospholipid dispersions: chain length dependence of gramicidin conformation and lipid organization. *Biochemistry* 33:4291-4299.

Israelachvili, J.N. 1977. Refinement of the fluid-mosaic model of membrane structure. *Biochim. Biophys. Acta* 469:221-225.

Killian, J.A. 1992. Gramicidin and gramicidin/lipid interactions. *Biochim. Biophys. Acta* 1113:391-425.

Killian, J.A., K.U. Prasad, D.W. Urry, and B. de Kruijff. 1989. A mismatch between the length of gramicidin and the lipid acyl chains is a prerequisite for HII phase formation in phosphatidylcholine model membranes. *Biochim. Biophys. Acta* 978:341-345.

Killian, J.A., I. Salemink, M.R.R. de Planque, G. Lindblom, R.E. Koeppe II, and D. V. Greathouse. 1996. Induction of non-bilayer structures in diacylphosphatidylcholine model membranes by transmembrane α-helical peptides. Importance of hydrophobic mismatch and proposed role of tryptophans. *Biochemistry* 35:1037-1045.

Koeppe, R.E., II, and O.S. Andersen. 1996. Engineering the Gramicidin Channel. *Annu. Rev. Biophys. Biomol. Struct.* 25:231-258.

Mouritsen, O.G., and M. Bloom. 1984. Mattress model of lipid- protein interactions in membranes. *Biophys. J.* 46:141-153.

Mouritsen, O.G., and M. Bloom. 1993. Models of lipid-protein interactions in membranes. *Annu. Rev. Biophys. Biomol. Struct.* 22:145-171.

Nielsen, C., N. Mobashery, and O.S. Andersen. 1997. Membrane dependent changes in conformational preference of gramicidin A channels. *Biophys. J.* 72:A191.

O'Connell, A.M., R.E. Koeppe II, and O.S. Andersen. 1990. Kinetics of gramicidin channel formation in lipid bilayers: transmembrane monomer association. *Science* 250:1256- 1259.

Owicki, J.C., M.W. Springgate, and H.M. McConnell. 1978. Theoretical study of protein-lipid interactions in bilayer membranes. *Proc. Natl. Acad. Sci. U.S.A.* 75:1616-1619.

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720-731.

Van Echteld, C.J.A., B. de Kruijff, A.J. Verkleij, J. Leunissen- Bijvelt, and J. de Gier. 1982. Gramicidin induces the formation of non-bilayer structures in phosphatidylcholine dispersions in a fatty acid chain length dependent way. *Biochim. Biophys. Acta* 692:126-38.

Zhang, Y.P., R.N.A.H. Lewis, and R.N. McElhaney. 1995. Peptide models of helical hydrophobic transmembrane segments of membrane proteins. 1. Studies of the conformation, intrabilayer orientation, and amide hydrogen exchangeability of Ac-K_2-$(LA)_{12}$-K_2-amide. *Biochemistry* 34:2348-2361.

Protein Folding in Membranes: Pondering the Nature of the Bilayer Milieu

Stephen H. White, William C. Wimley,
Alexey Ladokhin *and* Kalina Hristova

Abstract

The fundamental principles of the stability of proteins in membranes are beginning to emerge as a result of structural and thermodynamic studies of the interactions of peptides with lipid bilayers. Some of the results confirm preexisting ideas; others are puzzling and reveal the complexities and subtleties of the bilayer that must be included in the "New Biomembrane Model."

Stephen H. White, William C. Wimley,
Alexey Ladokhin *and* Kalina Hristova
Department of Physiology and Biophysics
University of California
Irvine, CA 92697-4560, USA
email: shwhite@uci.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:99–106

"Curiouser and curiouser!", cried Alice during her "Adventure in Wonderland". Our laboratory can say the same, as a result of our studies of the fundamental principles of membrane protein folding and stability. While there is certainly nothing curious about the well known broad principles (Engelman and Steitz, 1981; Engelman et al., 1986), significant new features of lipid bilayers and their interactions with peptides are beginning to emerge. These features, summarized here, must be included in any "New Biomembrane Model." Some of the features are puzzling; "curious", Alice would say. They reveal, however, the complexities and subtleties of the bilayer milieu that determine the stability of membrane proteins.

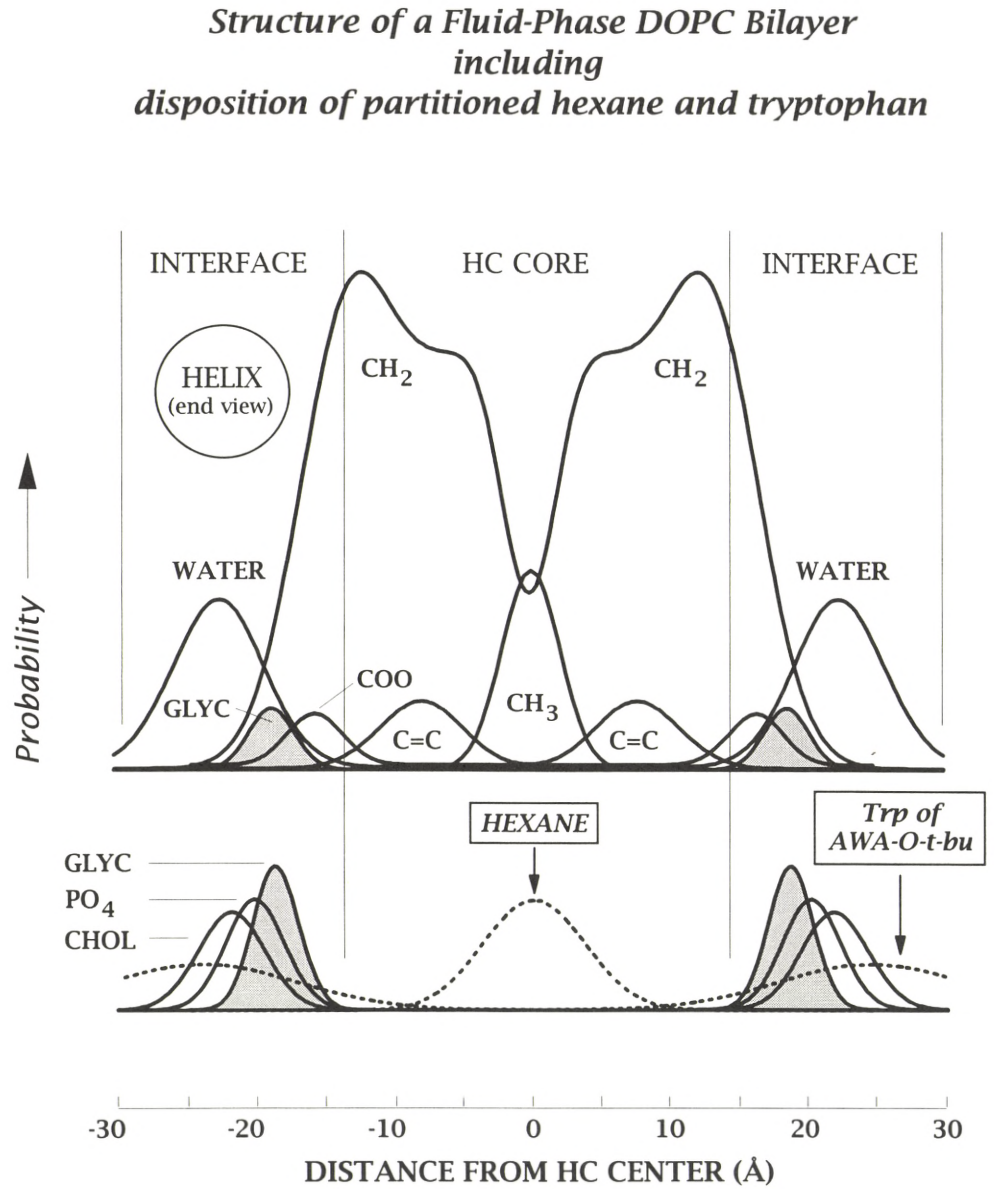


Figure 1. The transbilayer probability distribution functions for the principle structural groups of dioleoylphosphatidylcholine (DOPC) at 66% RH (5.4 waters/lipid) determined by x-ray and neutron diffraction (Wiener and White, 1992; White and Wimley, 1994; White and Wiener, 1996). The areas under the distributions equal the number of components, *e.g.* the area of the distribution in one monolayer for the double-bond is 2, the phosphate distribution 1, etc. The figure is based upon one published by White (1994).

Key Structural Features of Fluid Bilayers

Combined x-ray and neutron diffraction studies (Wiener and White, 1992; White and Wimley, 1994; White and Wiener, 1996) provide a revealing image of the complexity of the bilayer milieu (Figure 1). Several features of the image are especially important. First, there is a great amount of thermal disorder. This is indicated by the widths the transbilayer Gaussian probability densities of the principal structural groups such as the phosphates, carbonyls, and especially the double-bonds. Second, the combined thickness of the interfacial regions (defined by the water distribution), is equal to the 30 Å thickness of the hydrocarbon (HC) core. The thermal thickness of a single interface (15 Å) can easily accommodate unfolded and folded polypeptide chains (the typical α-helix has a diameter of 10 Å, Figure 1). Third, the interfaces are chemically highly heterogeneous. This means that they are rich in possibilities for non-covalent interactions with peptides. Because they are the sites of first contact, they are especially important in the folding and insertion of non-constitutive membrane proteins such as toxins. Structural images such as those of Figure 1 obtained in the presence of peptides allow one to determine where the peptides reside and to understand the structural response of bilayers to them (Jacobs and White, 1989).

Critical Thermodynamic Issues

Constitutive membrane proteins are assembled and inserted by means of a complex translocation process (Simon, 1995) whereas non-constitutive proteins and peptides generally fold and insert spontaneously after binding to the bilayer interface. In either case, the folded protein sits in a free energy minimum determined by its net interactions with water, bilayer interface, and HC core. Thermodynamically, one can thus describe the assembly by the model shown in Figure 2 (Jacobs and White, 1989). Experimental exploration of this model is, unfortunately, not straight forward because membrane proteins, folded or unfolded, are insoluble in the aqueous phase because of their high content of non-polar amino acids. Furthermore, they are also insoluble as unfolded chains in the HC because of the very high cost of partitioning peptide bonds that do not participate in H-bonds (Engelman et al., 1986; Wimley and White, 1996). Our current approach to working around these problems can be summarized as follows.

The Unfolded (Virtual) Reference State. The reference state is taken as the unfolded protein in the interface. However, as far as we know, one cannot actually achieve this state with constitutive membrane proteins because of the solubility problems nor with small non-constitutive membrane-active peptides because binding usually induces secondary structure (partitioning-folding coupling). Thus, as is often the case in solution thermodynamics, the reference state must be a virtual one. We define it by means of an experimental interfacial hydrophobicity scale (Wimley and White, 1996) derived from the partitioning of tri- and pentapeptides (Jacobs and White, 1989; Wimley and White, 1996) that have no secondary structure in the aqueous or interfacial phases. This scale, that includes the peptide bonds as well as the sidechains, can be used to calculate the virtual free energy of transfer of an unfolded chain into the interface. The most important feature of whole-residue partitioning is that the energetics are dominated by the peptide bonds (Wimley and White, 1996).

Partitioning-Folding Coupling and the Energetics of Interfacial Folding. A number of small peptides, such as melittin (Vogel, 1981), are unfolded in the aqueous phase, but are fully structured upon partitioning into the interface. Even though the unfolded state is inaccessible, the en-

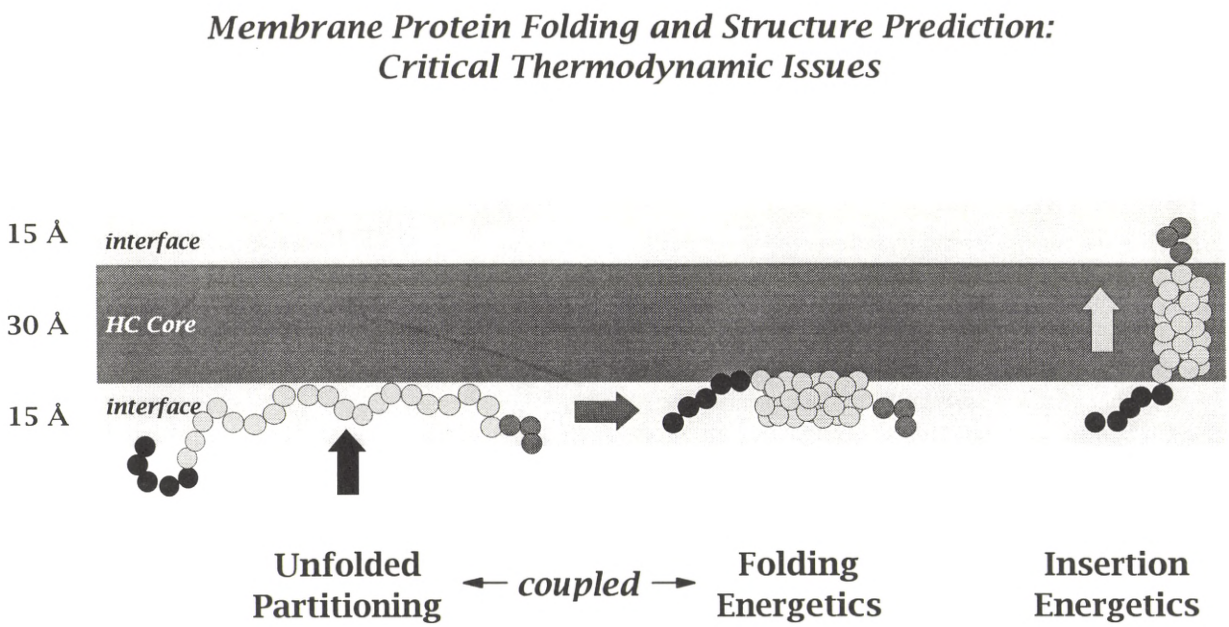


Figure 2. Summary of the critical thermodynamic issues of membrane protein folding and structure prediction. The figure emphasizes the three major classes of thermodynamic measurements that must be made in order to describe the stability of membrane proteins.

ergetics of the folding can be estimated from the difference between the virtual free energy of transfer of the unfolded state (calculated using the interfacial hydrophobicity scale) and the measured free energy of transfer of the folded peptide. Secondary structure formation appears to be driven by the reduction in the free energy of partitioning of peptide bonds that accompanies hydrogen bond formation. We estimate the reduction to be 0.2 to 0.4 kcal/mol per peptide bond. The accumulative effect of this modest reduction can be very large (~10 kcal/mol for melittin).

Energetics of Bilayer Insertion. This last step in folding is the crucial one, but the least adequately studied because of the insolubility and aggregation of hydrophobic peptides. Direct measurement of the partitioning of a hydrophobic α-helix or β-barrel across a membrane is absolutely essential because we must know the true cost of partitioning a hydrogen-bonded peptide bond into the bilayer HC. Estimates for this cost vary from 0 to +1.6 kcal/mol (Engelman et al., 1986; Roseman, 1988; Ben-Tal et al., 1996). This means that calculations of insertion free energy based on sidechain free energies could be over-estimated by as much as +30 kcal/mol for a 20-residue helix!

Our experience has been that hydrophobic peptides that partition into and across bilayers as α-helices and that seem to be soluble in the aqueous phase, are actually multimers. This greatly complicates the thermodynamics. Insertion energetics can be reliably determined only if the following conditions are satisfied: (1) The helix (or unfolded peptide) must be monomeric in the aqueous phase, (2) the transmembrane geometry must be verified experimentally, (3) helical conformation in the transmembrane geometry must be verified, (4) all bound helices must be transmembrane or the ratio of trans and non-trans must be known, and (5) the helix must be monomeric in the membrane or the equilibrium constant between monomer and multimer must be known.

Subtleties of Bilayer Partitioning

Included in the structural image of DOPC (Figure 1) are two additional distributions, determined directly by neutron diffraction: partitioned hexane (White et al., 1981) and the Trp of partitioned Ala-Trp-Ala-*O-tert*-butyl (Jacobs and White, 1989). These are included to note that (1) the HC is not thermodynamically equivalent to a bulk hydrocarbon (White, 1976) and (2) tryptophan, and probably other aromatics, play a crucial role in peptide-bilayer interactions (Wimley and White, 1996).

Alkanes in the Hydrocarbon Core. The transbilayer distribution of *n*-hexane in DOPC shown in Figure 1 shows that the hexane is confined to the central part of the bilayer HC. Because it is not uniformly distributed, the activity coefficient is position dependent. Extensive studies of the thermodynamics of the solubility of a variety of alkane isomers in black lipid films showed (White, 1977), now long ago, that the enthalpies and entropies of alkane solubility depended dramatically on the structure of the isomer, including branching and chain length. For example (White, 1976), the enthalpy of transfer of *n*-hexadecane from bulk to the interior of glycerol monoöleate bilayers is +3.8 kcal/mol! To the extent that non-polar amino acid sidechains behave like alkane isomers, one must assume that the free energy of sidechain partitioning will depend upon position within the thickness of the HC.

Indoles in the Interface. The location of the Trp of the Ala-Trp-Ala-*O-tert*-butyl peptide in Figure 1 came as a surprise. Even though the hydrophobicity of Trp suggested that at least its non-imidated ring should be well buried in the HC, its location corresponds largely to the distribution of the water of hydration of the headgroup. One explanation for this location is that it is a result of complex interactions of the peptide with the bilayer interface. However, partitioning studies of several indole compounds (Wimley and White, 1992; Wimley and White, 1993) suggested that this is the preferred location of the indole ring itself. NMR studies in progress in the laboratory of Klaus Gawrisch of the NIH confirm this conclusion. We do not understand the nature of the indole-headgroup interaction that causes the indole to prefer the interface. It is not determined solely by the imide group because indene compounds also largely prefer this location. Whatever the nature of the interaction may be, it must be important for membrane protein stability because all membrane proteins whose 3D structures are known have aromatic residues preferentially located at the membrane interface.

The Bilayer Effect. The partitioning of the indole compounds reveals another important aspect of the bilayer as a solvent for hydrophobic molecules that is illustrated in Figure 3. Specifically, the bilayer responds thermodynamically to the partitioning of solutes. This 'bilayer effect', sometimes referred to as the 'non-classical' hydrophobic effect, often causes the partitioning of hydrophobic compounds to be driven by enthalpy rather than entropy. We have shown that the relative contributions of the bilayer and hydrophobic effects can be established through measurements of the heat capacity associated with partitioning (Wimley and White, 1993). Their relative contributions for *N*-methylindole and 3-methylindole are summarized in Figure 3. Note the great difference in the relative magnitudes of the effects associated with the two compounds. We do not understand the exact origin of the bilayer effect or why it can change so dramatically with relatively modest changes in solute structure.

The Interfacial Solvation Parameter. Figure 4 compares the partitioning of the peptides Acetyl-Trp-Leu_m ($m = 1 - 6$) into the bilayer interface with the partitioning into *n*-octanol (Wimley et al., 1996; Wimley and White, 1996). We have shown (Wimley et al., 1996) that the partitioning of the peptide sidechains into octanol is fully accounted for by the hydrophobic effect described by a solvation parameter of 22.8 cal/mol/Å^2, which is the same value obtained for the partitioning of non-polar compounds between water and bulk non-polar phases (Reynolds et al., 1974). The slope of the line in Figure 4 is 0.49, meaning that

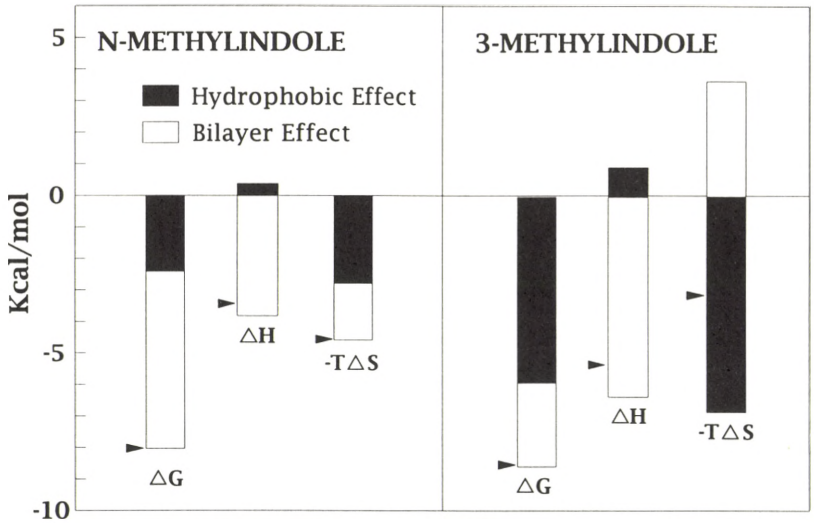


Figure 3. Relative contributions of the hydrophobic effect and so-called bilayer effects to the thermodynamics of the partitioning of *N*-methylindole and 3-methylindole into palmitoyloleoylphosphatidylcholine (POPC) bilayers. The net free energies (ΔG), enthalpies (ΔH), and the entropic (ΔS) contributions to free energies ($T\Delta S$) are indicated by the small arrows. Despite their structural similarity, the two tryptophan analogs have quite different thermodynamic properties. The figure is based upon work published by Wimley and White (1993).

the solvation parameter for interfacial partitioning is reduced to 11.2 cal/mol/Å^2, very close to the value of 12.4 obtained from the partitioning of hydrophobic tripeptides (Jacobs and White, 1989). This 50% reduction is thus a characteristic of the phosphatidylcholine bilayer interface. We do not understand its origin. Furthermore, we are intrigued by the fact that it apparently applies to polar interactions as well because the free energy cost of partitioning the peptide bond into the interface (1 kcal/mol) is one-half the value observed for octanol partitioning (2 kcal/mol).

Through the Looking-Glass

The bilayer milieu has not turned out to be the simple one of the fluid mosaic model (Singer and Nicolson, 1972) that pretty much considered the bilayer to be a thin slab of bulk hydrocarbon. The realm we have entered through closer inspection of the bilayer and its interactions with peptides is far more complex and interesting. We hope that as the subtleties of the bilayer milieu are understood, a coherent structural and thermodynamic framework will emerge. But, as Alice said, "You can just see a little *peep* of the passage in the *Looking-Glass* House. ... you know it may be quite different on beyond."

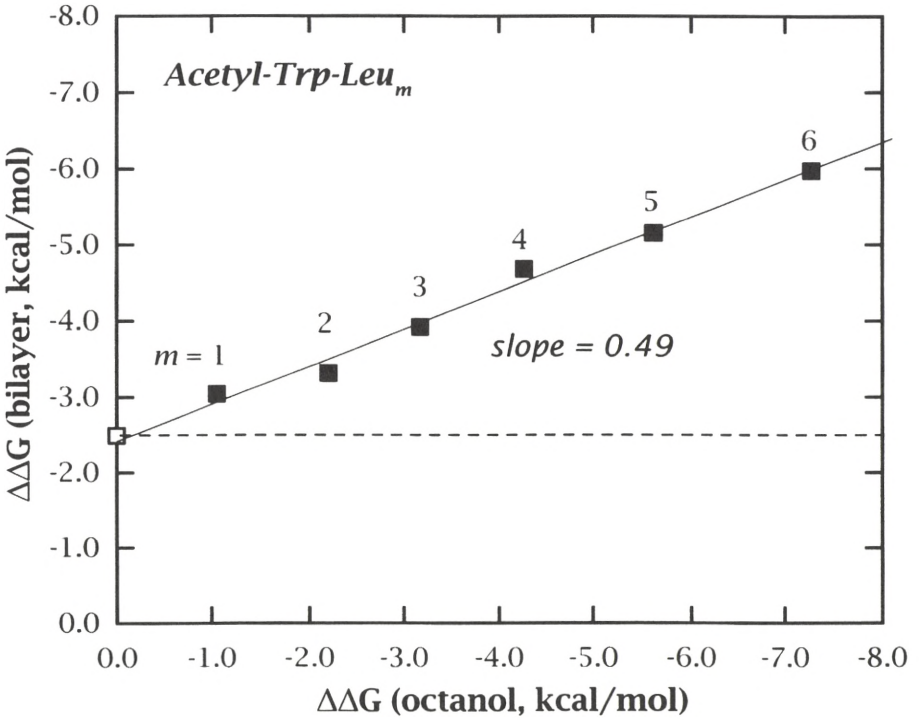


Figure 4. Comparison of the free energies of transfer of the peptides Ac-Trp-Leu$_m$ (m = 1 to 6) from water to POPC bilayer interfaces and from water to n-octanol. The slope of 0.49 indicates that the hydrophobic-contribution to partitioning into bilayers is apparently only half that expected for partitioning into bulk non-polar phases. The figure is based upon one published by Wimley and White (1996).

References

Ben-Tal, N., A. Ben-Shaul, A. Nicholls, and B. Honig. 1996. Free-energy determinants of a-helix insertion into lipid bilayers. *Biophys.J.* 70:1803-1812.

Engelman, D.M. and T.A. Steitz. 1981. The spontaneous insertion of proteins into and across membranes: The helical hairpin hypothesis. *Cell* 23:411-422.

Engelman, D.M., T.A. Steitz, and A. Goldman. 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15:321-353.

Jacobs, R.E. and S.H. White. 1989. The nature of the hydrophobic binding of small peptides at the bilayer interface: Implications for the insertion of transbilayer helices. *Biochemistry* 28:3421-3437.

Reynolds, J.A., D.B. Gilbert, and C. Tanford. 1974. Empirical correlation between hydrophobic free energy and aqueous cavity surface area. *Proc. Natl. Acad. Sci. USA* 71:2925-2927.

Roseman, M.A. 1988. Hydrophobicity of the peptide C=O...H-N hydrogen-bonded group. *J. Mol. Biol.* 201:621-625.

Simon, S.M. 1995. Protein-conducting channels for the translocation of proteins into and across membranes. *Cold Spring Harbor Symp.Quant.Biol.* 60:57-69.

Singer, S.J. and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720-731.

Vogel, H. 1981. Incorporation of melittin into phosphatidylcholine bilayers: Study of binding and conformational changes. *FEBS Lett.* 134:37-42.

White, S.H. 1976. The lipid bilayer as a "solvent" for small hydrophobic molecules. *Nature (London)* 262:421-422.

White, S.H. 1977. Studies of the physical chemistry of planar bilayer membranes using high precision measurements of specific capacitance. *Ann. N.Y. Acad. Sci.* 303:243-265.

White, S.H. 1994. Hydropathy plots and the prediction of membrane protein topology. *In* Membrane Protein Structure: Experimental Approaches, S. H. White, editor. Oxford University Press, New York. 97-124.

White, S.H., G.I. King, and J.E. Cain. 1981. Location of hexane in lipid bilayers determined by neutron diffrac-

tion. *Nature (London)* 290:161-163.

White, S.H. and M.C. Wiener. 1996. The liquid-crystallographic structure of fluid lipid bilayer membranes. *In* Membrane Structure and Dynamics, K. M. Merz and B. Roux, editors. Birkhäuser, Boston. 127-144.

White, S.H. and W.C. Wimley. 1994. Peptides in lipid bilayers: Structural and thermodynamic basis for partitioning and folding. *Cur. Opinion Struc. Biol.* 4:79-86.

Wiener, M.C. and S.H. White. 1992. Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. III. Complete structure. *Biophys. J.* 61:434-447.

Wimley, W.C., T.P. Creamer, and S.H. White. 1996. Solvation energies of amino acid sidechains and backbone in a family of host-guest pentapeptides. *Biochemistry* 35:5109-5124.

Wimley, W.C. and S.H. White. 1992. Partitioning of tryptophan side-chain analogs between water and cyclohexane. *Biochemistry* 31:12813-12818.

Wimley, W.C. and S.H. White. 1993. Membrane partitioning: Distinguishing bilayer effects from the hydrophobic effect. *Biochemistry* 32:6307-6312.

Wimley, W.C. and S.H. White. 1996. Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nature Struct. Biol.* 3:842-848.

The Courtship and Marriage of K^+ Channel Subunits

Carol Deutsch

Abstract

Assembly of oligomeric membrane proteins is complex. It is even more complicated in the case of a polytopic protein such as a voltage-gated K^+ channel. However, one can engineer a particular biophysical function of such a channel to reveal the prior history of its subunits during assembly. These functional tagging experiments entail either heterologous expression of a wild-type subunit with a mutant subunit, or heterologous expression of a mutant subunit in a cell expressing endogenous wild-type channels. The method of analysis of the appropriately modified function assumes a binomial distribution for the random formation of homo- and heteromultimeric channels. Application of this general strategy to the T lymphocyte K^+ channel, Kv1.3, has revealed that subunits are recruited randomly into tetramers from mixed pools of wild-type and mutant monomers, that tetramers in the plasma membrane of the T cell do not dissociate, and that temporal, but not spatial, segregation of wild-type and mutant subunits occurs within this cell.

Carol Deutsch
Department of Physiology
University of Pennsylvania
Philadelphia, PA 19104, USA
e-mail: cjd@mail.med.upenn.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:107–113

Voltage-gated K^+ channels are homotetrameric membrane proteins, each subunit containing six putative transmembrane segments, S1-S6. The four channel subunits are not linked covalently, so what holds them together? And where, when, and how do channels form? We often modify structure to understand function; our approach, however, is to engineer the function of a channel to reveal its prior history, namely, to learn something about the assembly of individual subunits. In this regard the strategy and methods described below can be used to answer the following questions: Does synthesis and assembly of different channel subunits occur in the same shared compartment? Are subunits recruited randomly or preferentially? Does multimer formation occur in the plasma membrane? Are channel monomers and multimers in equilibrium in the plasma membrane? Is channel diversity

temporally or spatially regulated? To the extent that many of these events occur in the membrane, any new model of biomembranes should provide for these features of oligomer assembly.

Kv1.3, a voltage-gated *Shaker*-like K^+ channel in human T lymphocytes, opens in response to depolarization (Matteson and Deutsch, 1984; DeCoursey et al., 1984). Upon prolonged depolarization, it inactivates by a mechanism known as C-type inactivation. The wild-type homotetramer inactivates with a time constant of 200 ms and a point mutation in the S6 segment of this channel produces a mutant homotetramer that inactivates with a time constant of 4 ms, 50× faster than the wild-type channel (Panyi et al., 1995). Using this functionally-tagged subunit along with the wild-type subunit, we have shown that C-type inactivation is cooperative, in that each of the four subunits in a Kv1.3 channel contributes equal free energy to the transition from the open state to an inactivated conformation (Panyi et al., 1995). Moreover, by studying the inactivation kinetics, we have shown that simultaneous heterologous expression of wild-type and mutant subunits in a mammalian cell results in a randomly mixed pool of subunits, and therefore that the resulting population of expressed channel types can be described by a binomial distribution of tetramers containing from zero to four mutant subunits. This has allowed us to predict the inactivation kinetics for a population of such channels, which we verified by constructing tandem dimers containing a wild-type and mutant subunit covalently linked, so that the resulting tetramer had a defined 2:2 stoichiometry.

How can we use such analyses of inactivation kinetics to learn about *in vivo* assembly of native Kv1.3? We transfected Jurkat cells, which express endogenous Kv1.3, with a mutant Kv1.3 subunit. At the time of transfection there could be any number of preformed wild-type channels already in the membrane. We asked "Can heterologous and endogenous subunits mix? Do they use the same compartments for assembly, and if so what can a kinetic analysis of inactivation tell us about assembly? What distribution of channel types might we predict occurs in such an experiment?" Among the possible cases, a channel distribution that is binomial (to a constant) will occur only if tetramers are formed irreversibly from a pool of mixed monomers in the presence of preformed wild-type channels in the plasma membrane. Our results showed that endogenous subunits can mix with heterologous subunits to form channels, i.e., that synthesis and assembly of different subunits occurs in the same shared compartment, that Kv1.3 subunits are recruited randomly from integrated monomer pools, that tetramer formation occurs prior to residence of the channel in the plasma membrane, that once tetramers are inserted into the plasma membrane, thereafter they do not dissociate, and finally that regulation of endogenous K^+ channel diversity in mammalian cells is temporally, and not spatially, regulated (Panyi and Deutsch, 1996).

These experiments suggest a general strategy for exploring the history of channel subunits during assembly. This strategy proceeds in two steps. As outlined in Figure 1, the first test determines the relative affinities of mixed subunits. Wild-type (WT) and mutant (MUT) subunits are heterologously co-expressed in a cell devoid of the channel in question, and the resulting channel population either conforms to or fails to conform to a binomial distribution. If it conforms, this means that WT and MUT subunits are recruited randomly and independently with the same probability. Failure means that subunits are not selected randomly, but since they are present at the same time in the same heterologous cell compartment, failure means there is some cooperativity, positive ("like prefers like"), or negative ("like avoids like"). This will be manifest as an excess of homomultimers or an excess of heteromultimers, respectively, compared to a binomial distribution.

The second test determines whether subunits are recruited from integrated or segregated monomer pools and what must be the nature of the segregation. It should be used after verifying that the first test shows no cooperativity. MUT subunits are heterologously expressed in a cell which already has endogenous WT subunits. A binomial distribution can be interpreted as evidence that random recruitment of subunits occurred from an

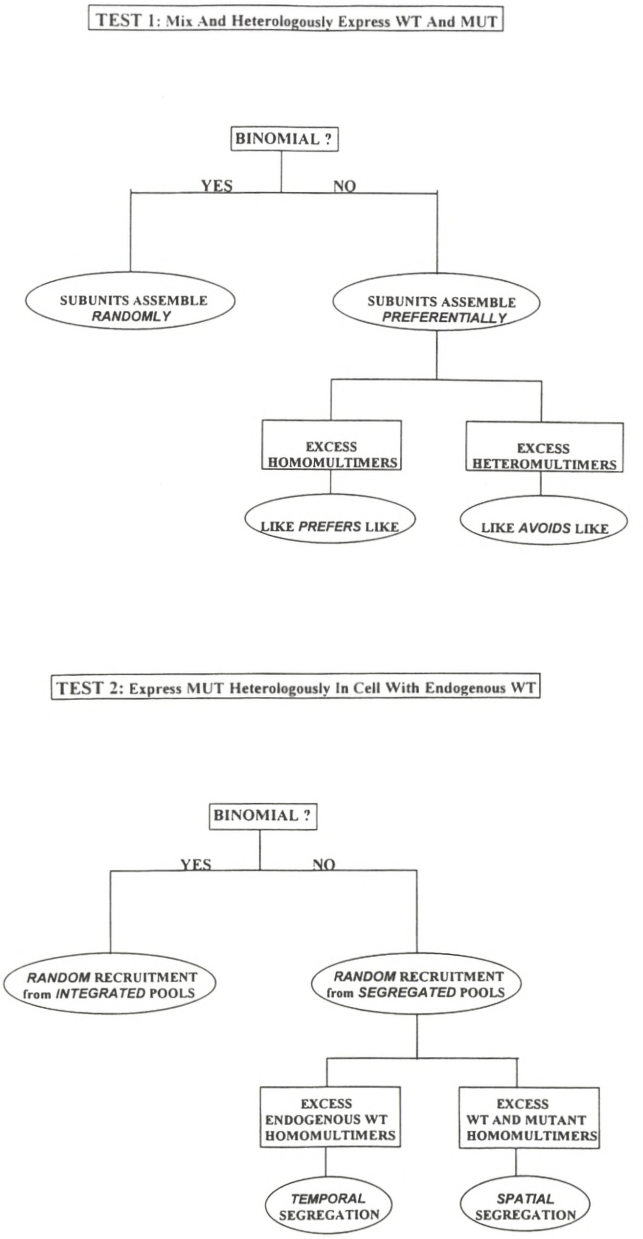


Figure 1. Prior history of channel subunits. Flow diagram illustrating tests for subunit preferences in association (Test 1) and for segregation of subunits (Test 2). The results (rectangles) and interpretations (ovals) of heterologous co-expression of wild-type (WT) and mutant (MUT) subunits in a cell are shown in Test 1. The results and interpretations of heterologous expression of MUT in a cell already expressing endogenous WT are shown in Test 2, where it is known that WT and MUT subunits show no preferential association.

integrated pool of subunits. This test can only fail the binomial distribution if there is some segregation, assuming that these subunits did not fail the first test, i.e. did not show cooperativity. Segregation will be manifest either as an excess of endogenous WT channels or as an excess of both WT and MUT channels compared to the binomial distribution. The first case indicates temporal segregation of subunits (i.e., multimers were formed irreversibly at different times), while the second indicates spatial segregation (i.e., WT and MUT homomultimers were formed in spatially separate compartments).

This method of analysis assumes a binomial distribution for the random formation of heteromultimeric channels. For a multimer of N subunits, the fraction of channels with exactly m mutant subunits will be

$$B(N,p,m) = \frac{N!}{m!(N-m)!}p^m(1-p)^{N-m} \quad (1)$$

where p is the fraction of mutant subunits in the membrane. The wild-type homomultimer is represented by $m = 0$, whereas $m = N$ represents the homomultimeric mutant channel. If the biophysical properties of each member of this population are known, it is possible to estimate both p and the validity of the underlying assumption, namely that WT and mutant subunits assemble randomly. Biophysical properties that can be quantified in this way include the kinetics of inactivation, the affinity of an open-channel blocker, and the single-channel conductance. Any functional property of the channel can be used for this purpose. The major criterion that must be met, however, is that an order of magnitude difference in the chosen functional parameters for the wild-type and mutant subunits must exist, regardless of which parameter is being studied, whether it be time constants of gating, binding constants of some ligand, or single-channel conductances.

Three similar equations (Figure 2) may be used to fit the data obtained from a cell expressing both endogenous and heterologous subunits, depending on the biophysical parameter to be measured. In the case of gating kinetics, $I(t)$ is the current at time t and $Y_m(t)$ is a function describing the gating kinetics for a channel with m mutant subunits (see example below). In the case of blocker affinity, $I([bk])$ is the current in the presence of blocking agent, $I(O)$ is the current in the absence of blocking agent, bk is the blocker molecule, $[bk]$ is the blocker concentration, and $F_{unbk,m}([bk])$ is the fraction of unblocked current for a channel with m mutant subunits. In the case of single-channel conductance, i_m is the single-channel current for a channel with m mutant subunits. The conclusions from our T-cell studies have led us to ask a whole new set of questions. If subunit recruitment is random and subunits diffuse to find their correct partners, then what are the recognition signals for assembly? Do recognition and assembly occur in a membrane compartment? If the tetramer never dissociates, then what are the stabilization interactions holding the tetramer together? And finally, if the cell must regulate the time of subunit expression, and possibly the kinetics of synthesis and assembly of channel proteins in order to produce separate channel isoforms in the same spatial compartment, then what are the mechanisms of kinetic control of K^+ channel diversity?

Neither these mechanisms nor the interacting surfaces across subunit boundaries are known. Pfaffinger and Li, as well as my laboratory, have found recognition domains in the N-terminal cytoplasmic tail of K^+ channels, referred to as T1 ("first tetramerization") domains, that are known to tetramerize *in vitro* and to confer subfamily specificity (Li et al., 1992; Shen et al., 1993; Shen and Pfaffinger, 1995; Tu et al., 1995; Xu et al., 1995). But this finding does not preclude other recognition signals nor inform us about the stabilization interactions. In fact, we made a deletion mutant of Kv1.3 that lacks the first 141 amino acids (Kv1.3($T1^-$)), and in oocytes it produces currents whose biophysical properties are identical to those produced by full-length Kv1.3 (Tu et al., 1995; 1996). We have interpreted this finding to mean that there are association sites in the central core of Kv1.3 that provide sufficient stabilization interactions for channel assembly.

To probe for these putative interaction sites across subunit boundaries, our approach has been to test the ability of a series of hydrophobic Kv1.3 peptide fragments to suppress Kv1.3 cur-

Gating Kinetics: $$I(t) = \sum_{m=0}^{N} B(N,p,m) Y_m(t)$$

Affinity of Channel Blocker: $$\frac{I([bk])}{I(0)} = \sum_{m=0}^{N} B(N,p,m) F_{unbk,m}([bk])$$

Single Channel Conductance: $$\frac{number\ of\ openings\ to\ level\ i_m}{total\ number\ of\ openings} = B(N,p,m)$$

$$B(N,p,m) = \frac{N!}{m!(N-m)!} p^m (1-p)^{N-m}$$

Figure 2. Read-outs of functionally-tagged subunits. Equations that describe gating kinetics, open-channel block, and single-channel conductance. In each case, $B(N,p,m)$ represents the binomial distribution, as described in the text, along with the functions and symbols used in these equations.

rent when they are heterologously co-expressed with Kv1.3($T1^-$) subunits in Xenopus oocytes, a so-called dominant negative suppression strategy. We have experimentally demonstrated several prerequisites in order to use this strategy to infer putative interaction sites (Tu et al., 1996; Sheng and Deutsch, 1997; Sheng et al., 1997). Thus, we can interpret suppression by a specific peptide as evidence that it competes with full-length subunits for self-association sites involved in assembly. Our results from both electrophysiological experiments and from immunoprecipitation experiments identify specific intramembrane association (IMA) sites in Kv1.3 and support a model in which association between subunits occurs between transmembrane segments in the plane of the lipid bilayer (Tu et al., 1996; Sheng et al., 1997). Furthermore, our studies show that synthesis and integration of interacting proteins into microsomal membranes occur rapidly and that the protein-protein interaction itself is the rate-determining membrane-delimited step in association (Sheng et al., 1997).

These results are consistent with the endoplasmic reticulum membrane itself facilitating K^+ channel tetramerization even when subunits are expressed at low levels, thus permitting efficient and rapid oligomerization relative to non-membrane assembly compartments (Helenius et al., 1992). We propose that Kv1.3 contains intersubunit IMA sites, located between S1 and S5, which may serve a specific role in tetramer assembly. Moreover, the S1-S2-S3 segments may be important for tetramer stabilization. The voltage-gated K^+ channel protein has multiple functions (Figure 3) that can be assigned to discrete structural domains, which may be created by the primary, secondary, tertiary, and/or quaternary conformations of the protein. The subunit stoichiometry of each of these functions has yet to be deter-

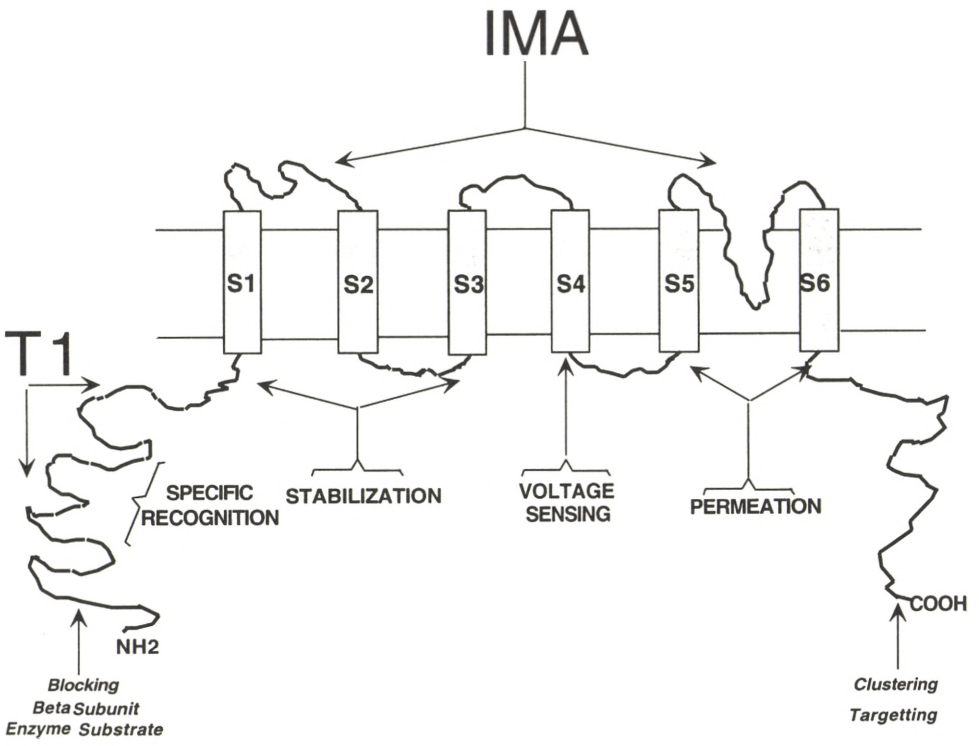


Figure 3. Modular functions of a voltage-gated K^+ channel.

mined. For instance, we know that all four subunits participate in voltage sensing, in pore formation, and in the binding sites for a variety of channel blockers, but what is the subunit participation in recognition? Do all four T1 domains participate? Do IMA sites from all four subunits participate equally in tetramer stabilization? And finally, do tetramers form by stepwise addition of monomers or, as we propose, do tetramers arise from association of two dimers, using different interaction mechanisms and IMA sites from those used in the initial monomer-monomer interaction? Where and at which stage in tetramer formation are T1 and IMA domains critical? The major question that these studies pose for our new model of biomembranes is how does the bilayer accommodate the random aspects of channel assembly and the modes of spatial and temporal segregation involved in oligomerization of polytopic membrane proteins.

References

DeCoursey, T.E., K.G. Chandy, S. Gupta, and M.D. Cahalan. 1984. Voltage-Gated K^+ Channels in Human T Lymphocytes: A Role in Mitogenesis? *Nature (London)* 307: 465-468.

Li, M., Y.-N. Jan, and L.-Y. Jan. 1992. Specification of Subunit Assembly by the Hydrophilic Amino-Terminal Domain of the *Shaker* Potassium Channel. *Science* 257: 1225-1230.

Helenius, A., Marquardt, T., and Braakman, I. 1992. The Endoplasmic Reticulum as a Protein-Folding Compartment. *Trends in Cell Biology* 2: 227-231.

Matteson, D.R., and C. Deutsch. 1984. K^+ Channels in T Lymphocytes: A Patch Clamp Study Using Monoclonal Antibody Adhesion. *Nature* 307: 468-471.

Panyi, G., and C. Deutsch. 1996. Assembly and Suppression of Endogenous Kv1.3 Channels in Human T Cells. *J. Gen. Physiol.* 107: 409-420.

Panyi, G. Z. Sheng, L.W. Tu, and C. Deutsch. 1995. C-Type Inactivation of a Voltage-Gated K^+ Channel Occurs by a Cooperative Mechanism. *Biophys. J.* 69: 896-904.

Shen, N.V., X. Chen, M.M. Boyer, and P.J. Pfaffinger. 1993. Deletion Analysis of K^+ Channel Assembly. *Neuron* 11:67-76.

Shen, N.V., and P.J. Pfaffinger. 1995. Molecular Recognition and Assembly Sequences Involved in the Subfamily-Specific Assembly of Voltage-Gated K^+ Channel Subunit Proteins. *Neuron* 14: 625-633.

Sheng, Z.F., and C. Deutsch. 1997. Assembly of Ion Channels, Methods in Enzymology, (in press).

Sheng, Z.F., W. Skach, V. Santarelli, and C. Deutsch. 1997. Evidence for Interaction Between Transmembrane Segments in Assembly of Kv1.3. *Biochemistry*, (in press).

Tu, L., V. Santarelli, and C. Deutsch. 1995. Truncated K^+ Channel DNA Sequences Specifically Suppress Lymphocyte K^+ Channel Gene Expression. *Biophys. J.* 68:147-156.

Tu, L., V. Santarelli, Z.-F. Sheng, W. Skach, D. Pain, and C. Deutsch. 1996. Voltage- gated K^+ Channels Contain Multiple Intersubunit AssociationSites. *J. Biol. Chem.* 271: 18904-18911.

Xu, J., W. Yu, Y.-N. Jan, L.-Y. Jan, and M. Li. 1995. Assembly of Voltage-gated Potassium Channels. Conserved Hydrophilic Motifs Determine Subfamily-Specific Interactions between the α-Subunits. *J. Biol. Chem.* 270: 24761-24768.

How Lipids Interact with an Intrinsic Membrane Protein: the Case of the Calcium Pump

A.G. Lee

Abstract

The Ca-ATPase has been purified from skeletal muscle sarcoplasmic reticulum and reconstituted into phospholipid bilayers of defined chemical composition. These studies show that the effects of phospholipid structure on the activity of the Ca-ATPase are complex and these and related studies on simple model peptides show that both phospholipids and proteins distort to optimize lipid-protein interactions.

A.G. Lee
Department of Biochemistry
University of Southampton
Southampton, SO16 7PX, United Kingdom
e-mail: A.G.Lee@soton.ac.uk

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:115–120

Effects of Lipid Structure on the Function of a Membrane Protein are Complex

We have been studying the interactions between phospholipids and the Ca^{2+}-ATPase purified from skeletal muscle sarcoplasmic reticulum (SR). The Ca^{2+}-ATPase is an enzyme, coupling the hydrolysis of one molecule of ATP to the transport of two Ca^{2+} ions across the membrane. Mixing the purified ATPase with lipid in detergent followed by removal of the detergent allows the reconstitution of the ATPase into lipid bilayers of defined composition. If the ATPase is reconstituted into membrane fragments, ATPase activity (hydrolysis of ATP) can be measured in the absence of net accumulation of Ca^{2+}, removing any problems associated with leak of Ca^{2+} across the membrane.

We have shown that the function of the Ca^{2+}-ATPase depends on the chemical structure and physical phase of the lipids surrounding it in the membrane. ATPase activity is highest in bilayers of dioleoylphosphatidylcholine (di(C18:1)PC) and activities are low in bilayers with shorter (C14) or longer (C22) fatty acyl chains so that fatty acyl chain length, and thus bilayer thickness, is important for the proper functioning of the

ATPase (Starling et al., 1993). Activities in bilayers of phosphatidylethanolamine are the same as in phosphatidylcholine so that the exact structure of a zwitterionic phospholipid headgroup appears not to be important (Starling et al., 1996a). However, activities are low in bilayers of phosphatidylserine or phosphatidic acid so that a negatively charged headgroup supports low activity (Dalton et al., 1997). Activities in gel phase lipid are very low, and activities in phosphatidylethanolamines under conditions where the phosphatidylethanolamine is in the hexagonal H_{II} phase are also low (Starling et al., 1995a, 1996a). Thus a liquid crystalline bilayer of the appropriate thickness is required for high activity; these experiments provide no evidence that the exact phospholipid composition of the native SR membrane is in any way special, at least as far as ATPase activity is concerned.

We have also shown that there is no one common mechanism that explains the effects of all phospholipids on the function of the ATPase; the activity observed in a bilayer of any one particular phospholipid depends on the unique conformational state of the ATPase in that particular bilayer. This can be illustrated by studies of the effects of bilayer thickness on the ATPase. In short (C14) or long (C24) chain phosphatidylcholines, the stoichiometry of Ca^{2+} binding changes from the usual 2 Ca^{2+} ions bound per ATPase molecule to 1 Ca^{2+} ion bound; the rate of dephosphorylation of E2P decreases; the E1/E2 equilibrium shifts towards E1 in di(C14:1)PC but not in di(C24:1)PC; and the rate of phosphorylation decreases in di(C14:1)PC but not di(C24:1)PC (Michelangeli et al., 1991; Starling et al., 1993, 1994, 1995b). The observed effects of chain length on Ca^{2+} binding and on the rate of dephosphorylation are distinct; in di(C22:1)PC the stoichiometry of Ca^{2+} binding is normal, but the rate of dephosphorylation is decreased (Starling et al., 1996b).

An effect of bilayer thickness on Ca^{2+} binding is, perhaps, not surprising since the Ca^{2+} binding sites on the ATPase are located between trans-membrane α-helices embedded in the lipid bilayer (Figure 1). However, what is then surprising is that Ca^{2+} binding is unaffected by lipid head group structure (Dalton et al., 1997) or by a lipid phase transition from the liquid crystalline into the gel or hexagonal H_{II} phases (Starling et al., 1995a, 1996a). Indeed, phosphorylation and dephosphorylation of the ATPase are more sensitive to lipid structure than is Ca^{2+} binding in that changes are seen in phosphorylation and dephosphorylation not only with changes in phospholipid chain length but also with changes in lipid phase and lipid headgroup structure. Thus in gel phase lipid the rate of phosphorylation becomes very slow (Starling et al., 1995a) and in hexagonal H_{II} phase lipid, although the rate of phosphorylation is normal, the rate of dephosphorylation becomes slow (Starling et al., 1996a). Phosphorylation and dephosphorylation of the ATPase occur on the cytoplasmic domain of the ATPase, located a considerable distance above the bilayer surface (Baker et al., 1994). Changes in bilayer thickness must be sensed by the trans-membrane α-helices, since these are the parts of the ATPase in contact with the bilayer. Changes in helix packing in response to changes in bilayer thickness must then be linked to changes in the nucleotide binding and phosphorylation domains of the ATPase.

These effects follow from distinct effects on the conformational states of the ATPase. Lipid fluidity is not involved; as described, changes in a number of equilibrium properties of the ATPase have been observed on changing lipid structure, and changes in fluidity (a dynamic property of the system) cannot result in a change in an equilibrium property (Lee, 1991). Experimentally, no correlation is observed between lipid order parameter and ATPase activity (East et al., 1984). Changes in the aggregation state of the ATPase are not involved in effects of chain length or lipid phase; low ATPase activities are observed in short or long chain phospholipids or in gel phase lipids when the ATPase is reconstituted into sealed vesicles containing isolated, single ATPase molecules, where aggregation is not possible (Starling et al., 1995c). However, low activities observed in anionic lipids follow in large part from a decrease in the proportion of the ATPase able to bind ATP, and could result from formation of dimers (in phosphatidylserine) or trimers (in phosphatidic acid) with only one ATPase molecule per oligomer being able to bind ATP (Dalton et al., 1997).

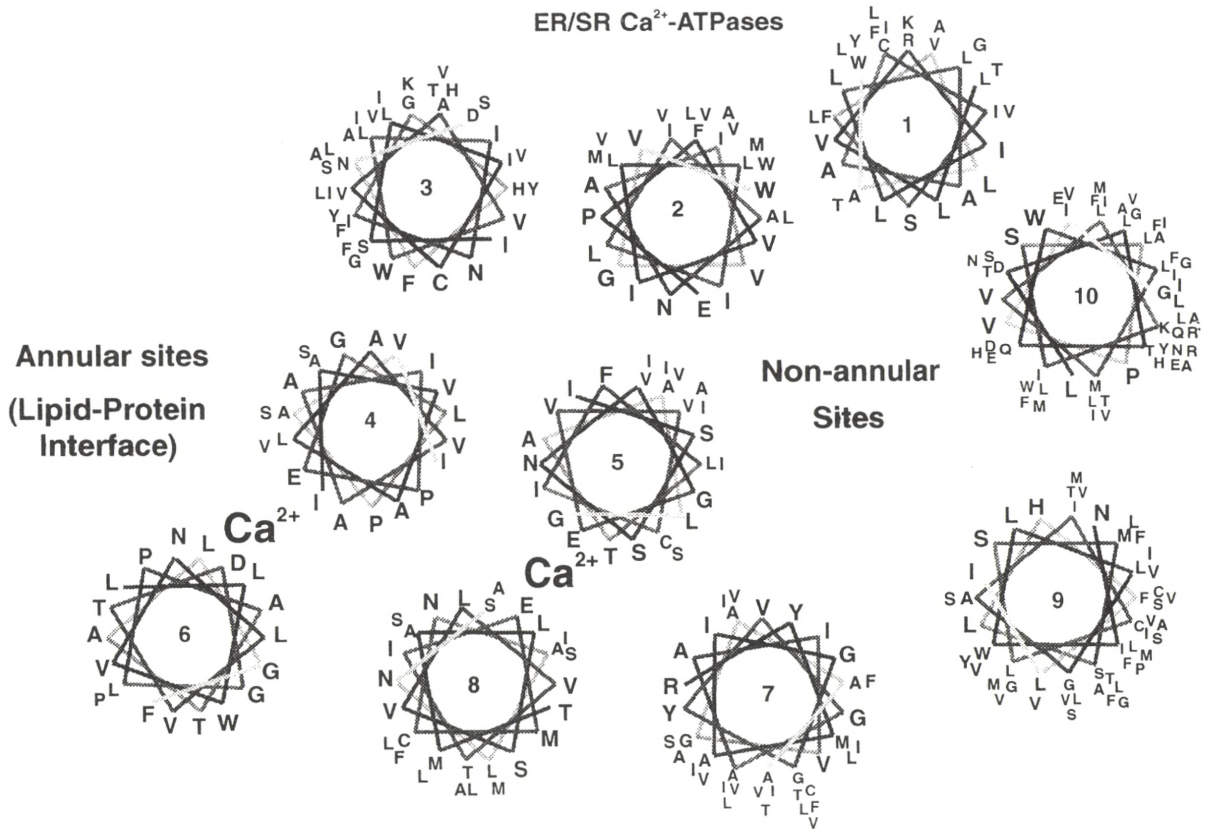


Figure 1. Possible packing of the 10 transmembrane helices of the Ca^{2+}-ATPase. The model is based on that of Stokes et al. (1994). Residues conserved amongst the ER/SR Ca^{2+}-ATPases are shown in large capitals. Residues not conserved are shown by small capitals. Ca^{2+} binding sites are located in a channel between helices 4, 5, 6 and 8. Non- specific binding sites for the bulk phospholipids in the membrane are proposed to exist around the outer surface of the structure (annular sites). Specific binding sites for a small number of "special" phospholipids could exist in the regions between helices, marked as non-annular sites.

Both Lipids and Proteins Distort to Optimise Lipid-protein Interactions

These marked effects of phospholipid structure on ATPase activity presumably follow from changes in the conformational state of the ATPase. There must be an energetic cost to these conformational changes, which would be expected to be reflected in different binding constants for phospholipids of different structure. In fact, phosphatidylcholines with chain lengths between C14 and C24 bind to the ATPase with equal strengths (East and Lee, 1982). However, although the energetics of the interaction of one phospholipid with the ATPase may change little with chain length, the ATPase is surrounded by about 30 phospholipid molecules in the membrane (East et al., 1985) and the total effect of all these phospholipids could be sufficient to result in significant conformational effects; consistent with this interpretation, the effects of chain length on the function of the Ca-ATPase have been

Table 1. Relative phospholipid binding constants for peptides P_{16} and P_{22}

Lipid	Bilayer Thickness (Å)[a]	Emission Maximum (nm)		Relative Binding Constant[b]	
		P_{16}^{c}	P_{22}^{d}	P_{16}^{c}	P_{22}^{d}
di(C14:1)PC	22.8	328	330	0.4	0.9
di(C16:1)PC	26.3	325	328	0.8	0.7
di(C18:1)PC	29.8	323	325	1.0	1.0
di(C20:1)PC	33.3	322	323	0.7	1.8
di(C22:1)PC	36.8	-	323	-	2.0
di(C24:1)PC	40.3	-	323	-	1.5

[a] Bilayer hydrophobic thickness d calculated from $d = 1.75(n - 1)$ where n is the number of carbon atoms in the fatty acyl chain (Sperotto and Mouritsen, 1988).
[b] Binding constant relative to that for di(C18:1)PC calculated from equations 1 and 2 with $n = 2.7$ for P_{16} and $n = 2.3$ for P_{22}.
[c] Estimated hydrophobic length 27 Å.
[d] Estimated hydrophobic length 36 Å.

shown to be highly cooperative (Starling et al., 1993).

Figure 1 shows a possible arrangement for the transmembrane helices of the Ca^{2+}- ATPase, based on the EM studies of David Stokes (Stokes et al., 1994). The bulk, solvent, or annular lipids interact with the outside surfaces of this bundle of helices (Lee et al., 1995). The interaction is short lived, as shown by the rapid rate of lipid exchange at these sites (East et al., 1985). It is relatively non-specific, since it is independent of chain length, as already described, and is unaffected by methyl branching of the chains (Froud et al., 1986). Binding of phosphatidylethanolamines is a factor of 2 weaker than binding of phosphatidylcholines (East and Lee, 1982), but anionic lipids bind as strongly as phosphatidylcholines (Dalton et al., 1997). Binding of gel phase lipid is a factor of 20 weaker than binding of lipids in the liquid crystalline phase (East and Lee, 1982), presumably as a result of poor van der Waals contact between rigid chains and the rough protein surface. We conclude that phospholipids are not bound at distinct 'sites' around the ATPase but rather should be pictured as interacting with the surface of the ATPase.

The conformational changes induced in the ATPase by changes in phospholipid structure presumably follow from changes in the packing of the trans-membrane α-helices embedded in the bilayer; changes in the structures of the α-helices themselves are unlikely. One obvious change enabling the ATPase to respond to changes in the thickness of the bilayer is a tilting of the helices. We have investigated this process using simple peptides of the type Ac-K_2-G-L_m-W-L_n-K_2-A-amide (P_{m+n}) containing a central Trp residue to act as a fluorescence reporter group and a pair of Lys residues at each end to anchor the peptide across the lipid bilayer (Webb et al., 1997). The peptides have been incorporated into bilayers of phosphatidylcholines with chain lengths between C14 and C24 by mixing peptide and excess phospholipid in organic solvent, removing the

solvent, and hydrating the mixture to give a bilayer containing the peptide. The peptide P_{22} ($m = 10, n = 12$) incorporates into all bilayers but P_{16} ($m = 7, n = 9$) does not incorporate into bilayers when the fatty acyl chain length is C24, and only partly incorporates into bilayers where the chain length is C22. This asymmetry follows because a too-long peptide can be matched to a too-thin bilayer both by stretching the lipid and by tilting the peptide. However, a too-thin peptide can only be matched to a too-thick bilayer by compression of the lipid, which becomes energetically unfavourable when the difference between the bilayer thickness and the peptide length exceeds about 10 Å. In the region of hydrophobic mismatch where the peptide still incorporates into the bilayer, there will be an energetic cost associated with stretching or compressing the lipid fatty acyl chains, which will be reflected in values of relative lipid binding constants. As shown in Table 1, strongest binding of lipid to P_{16} is observed for di(C18:1)PC and for P_{22} strongest binding is observed with di(C22:1)PC, as expected for optimal matching. However, it is clear that effects of a too-thin bilayer are relatively small, and level out at a factor of 2- 2.5 reduction in binding constant at a mismatch of about 6 Å.

These experiments with simple peptides therefore show that peptides can reorient in a lipid bilayer to help achieve an optimal match between the hydrophobic thickness of the lipid bilayer and the hydrophobic length of the peptide. It is not yet clear how these ideas should be extended to effects of bilayer thickness on a membrane protein such as the Ca^{2+}- ATPase containing a large number of trans-membrane α-helices. In a too-thin bilayer helices in the Ca^{2+}-ATPase could tilt to match the bilayer thickness, this resulting in changes in the conformation of the ATPase and changes in activity. The response to a too-thick bilayer is much less clear. The problem here is that, for the Ca^{2+}-ATPase, a too-thick bilayer is defined in a functional sense as one in which activity is less than in a bilayer of optimal thickness (provided by C18 chains). In terms of hydrophobic mismatch, it could be that the lengths of the helices in the Ca^{2+}-ATPase match the thickness of a C24 bilayer, explaining the observation that the Ca^{2+}-ATPase incorporates normally into bilayers of di(C24:1)PC. Some tilting of the helices would then be observed in di(C18:1)PC, and it would then have to follow that this degree of tilting corresponded to a conformational state for the ATPase which showed maximal ATPase activity. Finally, in di(C14:1)PC the degree of tilt would have become too extreme, giving a conformational state of low ATPase activity.

***

I thank Kate A. Dalton, J. Malcolm East, Sanjay Mall, Susan Oliver, Ram P. Sharma, Anthony P. Starling and Richard J. Webb, who carried out the unpublished work included here, and the BBSRC for financial support.

References

Baker, K.J., J.M. East, and A.G. Lee. 1994. Localization of the hinge region of the Ca-ATPase of sarcoplasmic reticulum using resonance energy transfer. *Biochim. Biophys. Acta* 1192: 53-60.

Dalton, K.A., J.M. East, S. Mall, S. Oliver, A.P. Starling, and A.G. Lee. 1997. Interaction of phosphatidic acid and phosphatidylserine with the Ca-ATPase of sarcoplasmic reticulum. and the mechanism of inhibition. *Biochem. J.*, in press.

East, J.M., and A.G. Lee. 1982. Lipid selectivity of the calcium and magnesium ion dependent adenosinetriphosphatase, studied with fluorescence quenching by a brominated phospholipid. *Biochemistry* 21: 4144-4151.

East, J.M., O.T. Jones, A.C. Simmonds, and A.G. Lee. 1984. Membrane fluidity is not an important physiological regulator of the (Ca^{2+}-Mg^{2+})-dependent ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 259: 8070-8071.

East, J.M., D. Melville, and A.G. Lee. 1985. Exchange rates and numbers of annular lipids for the calcium and magnesium ion dependent adenosinetriphosphatase. *Biochemistry* 24: 2615-2623.

Froud, R.J., C.R.A. Earl, J.M. East, and A.G. Lee. 1986. Effects of lipid fatty acyl chain structure on the activ-

ity of the (Ca^{2+} + Mg^{2+})-ATPase. *Biochim. Biophys. Acta* 860: 354-360.

Lee, A.G. 1991. Lipids and their effects on membrane proteins: evidence against a role for fluidity. *Prog. Lipid Res.* 30: 323-348.

Lee, A.G., K.A. Dalton, R.C. Duggleby, J.M. East, and A.P. Starling. 1995. Lipid structure and Ca-ATPase function. *Biosci. Rep.* 15: 289-298.

Michelangeli, F., E.A. Grimes, J.M. East, and A.G. Lee. 1991. Effects of phospholipids on the function of the (Ca-Mg)-ATPase. *Biochemistry* 30: 342-351.

Sperotto, M.M. and O.G. Mouritsen. 1988. Dependence of lipid membrane phase transition temperature on the mismatch of protein and lipid hydrophobic thickness. *Eur. Biophys.* J. 16: 1-10.

Starling, A.P., J.M. East, and A.G. Lee. 1993. Effects of phosphatidylcholine fatty acyl chain length on calcium binding and other functions of the (Ca-Mg)-ATPase. *Biochemistry* 32: 1593-1600.

Starling, A.P., Y.M. Khan, J.M. East, and A.G. Lee. 1994. Characterization of the single Ca binding site on the Ca-ATPase reconstituted with short and long chain phosphatidylcholines. *Biochem. J.* 304: 569-575.

Starling, A.P., J.M. East, and A.G. Lee. 1995a. The effects of gel phase phospholipid on the Ca-ATPase. *Biochemistry* 34: 3084-3091.

Starling, A.P., J.M. East, and A.G. Lee. 1995b. Effects of phospholipid fatty acyl chain length on phosphorylation and dephosphorylation of the Ca-ATPase. *Biochem. J.* 310: 875-879.

Starling, A.P., J.M. East, and A.G. Lee. 1995c. Evidence that the effects of phospholipids on the activity of the Ca-ATPase do not involve aggregation. *Biochem. J.* 308: 343-346.

Starling, A.P., K.A. Dalton, J.M. East, S. Oliver, and A.G. Lee. 1996a. Effects of phosphatidylethanolamines on the activity of the Ca-ATPase of sarcoplasmic reticulum. *Biochem. J.* 3A20: 309-314.

Starling, A.P., J.M. East, and A.G. Lee. 1996b. Separate effects of long-chain phosphatidylcholines on dephosphorylation of the Ca^{2+}-ATPase and on Ca^{2+} binding. *Biochem. J.* 318: 785-788.

Stokes, D.L., W.R. Taylor, and N.M. Green. 1994. Structure, transmembrane topology and helix packing of P-type ion pumps. *FEBS Lett.* 346: 32-38.

Webb, R.J., J.M. East, R.P. Sharma, and A.G. Lee. 1997. Hydrophobic mismatch and the incorporation of peptides into lipid bilayers: a possible mechanism of Golgi retention. *Biochemistry*, in press.

Electrostatic Interaction of Myristoylated Proteins with Membranes

Stuart McLaughlin, Diana Murray *and* Anna Arbuzova

Abstract

Membrane binding of a number of important peripheral proteins (e.g. Src, MARCKS, HIV-1 Gag, and K-Ras) requires nonspecific electrostatic interactions between a cluster of basic residues on the protein and acidic phospholipids in the membrane. Simple theoretical models based on the Poisson-Boltzmann equation can describe the experimentally measured electrostatic interactions and illustrate how electrostatics contributes to the formation of lateral domains.

Stuart McLaughlin, Diana Murray *and* Anna Arbuzova
Department of Physiology & Biophysics
Health Science Center at SUNY at Stony Brook
Stony Brook, NY 11794, USA
e-mail: smcl@epo.som.sunysb.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:121–125

Hydrophobic and electrostatic interactions act in concert to anchor several important myristoylated (e.g. Src, HIV-1 Gag, MARCKS) and farnesylated (e.g. K-Ras) proteins to membranes. Two recent reviews focus on how the attachment of acyl and isoprenyl groups to proteins influences membrane binding (Resh, 1996; Bhatnagar and Gordon, 1997). We stress the role electrostatics plays in binding myristoylated proteins to membranes and in forming lateral domains.

Recent structural studies of c-Src have revealed how the binding of a phosphorylated tyrosine (Tyr 527) in the C-terminal tail to the SH2 domain juxtaposes the SH3 domain with the polyproline type-II helix that links the SH2 and kinase domains (Xu et al., 1997). The intramolecular interaction between the SH3 and kinase domains holds the protein in an inactivate conformation. Membrane association of Src requires both the N-terminal myristate and cluster of basic residues. Membrane binding increases the effective concentration of Src in a thin ($d \sim 1$ nm) surface layer adjacent to the membrane. For a spherical cell with a radius equal to a few micrometers, the volume of the surface phase ($V = 4\pi R^2 d$) is about 1/1000 the volume of the cell ($V = 4\pi R^3/3$); thus anchoring Src to the membrane increases its effective concentration 1000-fold and enhances its ability to phosphorylate its membrane-bound substrates.

Myristate (14-carbon fatty acid) is attached co-

translationally to the N-terminal glycine of Src by the enzyme N-myristoyl transferase (Resh, 1996; Bhatnagar and Gordon, 1997). Myristate is required for Src membrane binding and binding is required for function: nonmyristoylated v-Src mutants are found in the cytoplasm and do not transform cells, even though the kinase activity of the protein is unaffected. Simple myristoylated peptides bind to electrically neutral phospholipid vesicles with a unitary binding energy of 8 kcal/mol or a molar partition coefficient of $10^4 M^{-1}$ (Peitzsch and McLaughlin, 1993; Buser et al., 1994). Measurements of the membrane partitioning of acylated peptides show that the binding energy increases 0.8 kcal/mol for each CH_2 group added to the acyl chain, in agreement with Tanford's observations on the hydrophobic partitioning of fatty acids into oil from water (Peitzsch and McLaughlin, 1993; Tanford, 1991). Thus (8 kcal/mol)/(0.8 kcal/mol per CH_2) = 10 CH_2 groups penetrate the hydrocarbon core of the membrane, 4 traverse the polar head group region, while the N-terminal glycine is located just outside the envelope of the polar head group region. Recent spin label (Cafiso, 1997), monolayer, and circular dichroism (Buser et al., 1994, Murray et al., 1997) measurements show that the N-terminal residues of Src do not penetrate the membrane and indicate that a myristoylated peptide corresponding to this region has an extended conformation when it binds to a membrane.

Although myristate is required for Src membrane binding, it is not sufficient (McLaughlin and Aderem, 1995). The Src protein binds to electrically neutral membranes with a molar partition coefficient of $10^3 M^{-1}$ (Sigal et al., 1994); the concentration of lipid in the plasma membrane of a 10 μm radius cell is about 10^{-3}M, so myristate alone would bind only half the protein. Src's N-terminal cluster of basic residues augments the binding due to myristate. Studies with peptides corresponding to the N-terminus of Src show that adding 33% acidic lipid to electrically neutral membranes increases the binding 1000-fold (Buser et al., 1994). The same 1000-fold enhancement is seen with the intact Src protein (Sigal et al., 1994). Mutating away the N-terminal basic residues weakens the binding *in vitro* and produces non-transforming phenotypes *in vivo* (Sigal et al., 1994). These observations provide strong evidence that the N-terminal basic residues contribute to the membrane binding of Src by interacting electrostatically with acidic lipids.

To a first approximation, the hydrophobic and electrostatic binding energies add (or the molar partition coefficients multiply). This observation follows from simple "ball and string" models that consider the acyl chain and basic cluster as small balls connected by a flexible string of length r (Buser et al., 1994; Ghomashchi et al., 1995). Binding of myristate confines the basic cluster to a hemisphere of radius r above the membrane surface and facilitates its binding. Although the simple models account for the synergism between electrostatic and hydrophobic interactions, they are descriptive rather than predictive. Using atomic models of Src's N-terminus and phospholipid bilayers, a continuum representation of the solvent, and the nonlinear Poisson-Boltzmann equation we can describe theoretically the electrostatic binding (Ben-Tal et al., 1996; Ben-Tal et al., 1997; Murray et al., 1997).

For these calculations, each atom is assigned a radius and a partial charge, and the peptide/membrane model is mapped onto a three-dimensional lattice of points. The nonlinear Poisson-Boltzmann equation is solved numerically for the electrostatic potential adjacent to the peptide and the membrane when they are far apart and when they are close together. These potentials are used to calculate the decrease in the electrostatic free energy as the peptide approaches the membrane. The free energy curve exhibits a long-range Coulombic attraction and short-range Born repulsion that result in the free energy minimum at distance $h \sim 3$ Å. The peptide concentration at each distance h is a product of the peptide concentration in the bulk solution and the exponent of the interaction energy. (In practice, the peptide concentration at h is calculated by averaging over many orientations of the peptide with respect to the membrane in order to approximate a complete ensemble of different configurations.) Integrating the excess peptide concentration over h gives the

Gibbs surface excess, which represents the number of moles of peptide bound per unit area of membrane surface (Ben-Tal et al., 1996). The Gibbs surface excess is simply related to the molar partition coefficient that is measured experimentally.

The theoretical methodology has been used to describe the membrane binding of basic peptides (Ben-Tal et al., 1996; Murray et al., 1997) and toxins (Ben-Tal et al., 1997): the model predicts how the binding is affected by changes in the ionic strength of the solution, the net positive charge of the peptide, or the mole % acidic lipid in the membrane. For example, the model correctly predicts that the binding of charybdotoxin decreases by five orders of magnitude when the salt concentration is increased from 10 mM to 150 mM (Ben-Tal et al., 1997). These results indicate the model describes well the long-range ($h \geq 3$Å) electrostatic attraction that gives rise to the membrane binding. Nevertheless, the calculated binding energies based on electrostatics alone consistently underestimate the observed values by 1-2 kcal/mol. This implies the model ignores some attractive interactions (Ben-Tal et al., 1997), overestimates the repulsive interactions, or both. Further theoretical and experimental work is required to obtain a more accurate description of the short-range ($h < 3$ Å) interactions, particularly when hydrophobic residues penetrate the polar head group region, a phenomenon investigated experimentally by several groups (Wimley and White, 1996).

Other proteins that use either myristate or farnesyl and a cluster of basic residues to bind to membranes include HIV-1 Gag (Zhou et al., 1994), K-Ras 4B (Hancock et al., 1990; Hancock et al., 1991), and MARCKS (myristoylated alanine-rich C kinase substrate) (Swierczynski and Blackshear, 1996; Seykora et al., 1996). The N-terminal cleavage product of HIV-1 Gag, the matrix protein, contains the two membrane binding motifs (Zhou et al., 1994). Structural studies show the basic residues are clustered in a patch that forms a membrane-binding surface (Massiah et al., 1994; Hill et al., 1996). K-Ras, a small GTPase, uses farnesyl (15 carbon isoprenoid) rather than myristate to bind to membranes. The farnesyl chain, like myristate, does not provide sufficient hydrophobic energy to anchor the protein to membranes (Silvius and l'Heureux, 1994). Adding 20% acidic lipid to electrically neutral membranes enhances the binding of farnesylated peptides corresponding to the C-terminus of K-Ras 300-fold (Ghomashchi et al., 1991). Spin-label EPR experiments show the MARCKS basic effector region, MARCKS (151-175), lies at the membrane interface in an extended conformation; although most of the binding energy is due to electrostatics, its five phenylalanines penetrate the polar head group region (Qin and Cafiso, 1996). MARCKS is interesting because protein kinase C (PKC) phosphorylation of three serines within the basic effector region weakens the electrostatic interaction and causes the MARCKS protein to translocate from the plasma membrane to the cytoplasm in many cell types (Kim et al., 1994); this mechanism has been termed the "myristoyl-electrostatic" switch (McLaughlin and Aderem, 1995). Recent results show that the binding of peptides corresponding to the MARCKS effector region is a diffusion limited process and that calmodulin can produce a rapid desorption of these peptides from the membrane (Arbuzova et al., 1997).

Lateral organization at the membrane surface may provide a mechanism for facilitating or regulating the interaction of membrane-bound molecules. For example, many important signaling molecules (e.g. Src family members Lck and Fyn, G-protein alpha subunits, H-Ras, eNOS, and phosphatidylinositol 4,5-bisphosphate, PIP_2) are concentrated in plasma membrane organelles called caveolae (Simons and Ikonen, 1997). MARCKS has a punctate distribution in the plasma membrane of macrophages and is concentrated, with PKCα, in nascent phagosomes (Allen and Aderem, 1996). In phospholipid vesicles, the basic effector region of MARCKS (Glaser et al., 1996) and simple basic peptides like pentalysine (Denisov et al., 1998), which corresponds to the first five residues of the MARCKS effector region, form discrete lateral domains enriched in monovalent acidic phospholipids and PIP_2. The results with pentalysine indicate that electrostatics can play a major role in domain formation and that PIP_2 can be sequestered in domains by nonspecific electrostatic

interactions, a phenomenon with interesting physiological implications.

***

This work was supported by grants MCB-9419175 from the National Science Foundation and GM-24971 from the National Institutes of Health to SM and a Helen Hay Whitney Foundation postdoctoral fellowship to DM.

References

Allen, L.A., and A. Aderem. 1996. Mechanisms of phagocytosis. *Current Opinion in Immunology* 8: 36-40.

Arbuzova, A., J. Wang, D. Murray, J. Jacob, D.S. Cafiso, and S. McLaughlin. 1997. MARCKS, membranes, and calmodulin: Kinetics of interaction. *Journal of Biological Chemistry* . 272: 27167-27177.

Ben-Tal, N., B. Honig, C. Miller, and S. McLaughlin. 1997. Electrostatic binding of proteins to membranes: Theoretical prediction and experimental results with charybdotoxin and phospholipid vesicles. *Biophys. J.* 73: 1717-1727.

Ben-Tal, N., B. Honig, R.M. Peitzsch, G. Denisov, and S. McLaughlin. 1996. Binding of small basic peptides to membranes containing acidic lipids: Theoretical models and experimental results. *Biophys. J.* 71: 561-575.

Bhatnagar, R.S., and J.I. Gordon. 1997. Understanding covalent modifications of proteins by lipids: where cell biology and biophysics mingle. *Trends in Cell Biology* 7: 14-21.

Buser, C.A., C.T. Sigal, M.D. Resh, and S. McLaughlin. 1994. Membrane binding of myristylated peptides corresponding to the NH_2-terminus of Src. *Biochemistry* 33: 13093-13101.

Cafiso, D. 1997. Personal Communication.

Denisov, G., S. Wanaski, P. Luan, M. Glaser, and S. McLaughlin. 1997. Binding of basic peptides to membranes produces lateral domains enriched in the acidic lipids phosphatidylserine and phosphatidylinositol 4,5-bisphosphate: An electrostatic model and experimental results. *Biophysical J.* In press.

Ghomashchi, F., X. Zhang, L. Liu, and M.H. Gelb. 1995. Binding of prenylated and polybasic peptides to membranes: affinities and intervesicle exchange. *Biochemistry* 34: 11910-11918.

Glaser, M., S. Wanaski, C.A. Buser, V. Boguslavsky, W. Rashidzada, A. Morris, M. Rebecchi, S.F. Scarlata, L.W. Runnels, G.D. Prestwich, J. Chen, A. Aderem, J. Ahn, and S. McLaughlin. 1996. MARCKS produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. *J. Biol. Chem.* 271: 26187-26193.

Hancock, J.F., K. Cadwallader, H. Paterson, and C.J. Marshall. 1991. A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *EMBO J.* 10: 4033-4039.

Hancock, J.F., H. Paterson, and C.J. Marshall. 1990. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* 63: 133-139.

Hill, C.P., D.P. Bancroft, A.M. Christensen, and W.T. Sundquist. 1996. Crystal structures of the trimeric human immunodeficiency virus type I matrix protein: Implications for membrane binding and assembly. *Proc. Natl. Acad. Sci. U.S.A.* 93: 3099.

Kim, J., T. Shishido, X. Jiang, A. Aderem, and S. McLaughlin. 1994. Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles. *J. Biol. Chem.* 269: 28214-28219.

Massiah, M.A., M.R. Starich, C. Paschall, M.F. Summers, A.M. Christensen, and W. Sundquist. I. 1994. Three-dimensional structure of the human immunodeficiency virus type 1 matrix protein. *J. Molec. Biol.* 244: 198-223.

McLaughlin, S., and A. Aderem. 1995. The myristoyl-electrostatic switch: A modulator of reversible protein-membrane interactions. *Trends Biochem. Sci.* 20: 272-276.

Murray, D., L. Hermida-Matsumoto, C.A. Buser, J. Tsang, C. Sigal, N. Ben-Tal, B. Honig, M.D. Resh, and S. McLaughlin. 1997. Electrostatics mediates the membrane association of Src: Theory and experiment. *Biochemistry.* In press.

Peitzsch, R.M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry* 32: 10436-10443.

Qin, Z., and D.S. Cafiso. 1996. Membrane structure of the protein kinase C and calmodulin binding domain of Myristoylated Alanine Rich C Kinase Substrate determined by site-directed spin labeling. *Biochemistry* 35: 2917-2925.

Resh, M.D. 1996. Regulation of cellular signalling by fatty acid acylation and prenylation of signal transduction proteins. *Cellular Signalling* 8: 403-412.

Seykora, J.T., M.M. Myat, L.A. Allen, J.V. Ravetch, and A. Aderem. 1996. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J.Biol.Chem.* 271: 18797-18802.

Sigal, C.T., W. Zhou, C.A. Buser, S. McLaughlin, and M.D. Resh. 1994. The amino terminal basic residues of Src mediate membrane binding through electrostatic interaction with acidic phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* 91: 12253-12257.

Silvius, J.R., and F. l'Heureux. 1994. Fluorimetric evaluation of the affinities of isoprenylated peptides for lipid bilayers. *Biochemistry* 33: 3014-3022.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature* 387: 569-572.

Swierczynski, S.L., and P.J. Blackshear. 1996. Myristoylation-dependent and electrostatic interactions exert independent effects on the membrane association of the Myristoylated Alanine-rich C-kinase Substrate. *J. Biol. Chem.* 271: 23424-23430.

Tanford, C. 1991. The Hydrophobic Effect: Formation of Micelles and Biological Membranes 2nd ed. Malabar, FL: Krieger Publishing Co.

Wimley, W.C., and S.H. White. 1996. Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nature Structural Biology* 3: 842-848.

Xu, W., S.C. Harrison, and M.J. Eck. 1997. Three dimensional structure of the tyrosine kinase c-Src. *Nature* 385: 595-602.

Zhou, W., L.J. Parent, J.W. Wills, and M.D. Resh. 1994. Identification of a membrane-binding domain within the amino- terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J. Virol.* 68: 2556-2569

Lipid Sorting by Membrane Proteins and Membrane Organization

J.-F. Tocanne

Abstract

In this contribution it is shown that a mismatch between the hydrophobic lengths of transmembrane proteins and the hydrophobic thickness of the surrounding lipid bilayer may generate physical forces which can promote molecular mechanisms of lipid sorting by the proteins and then contribute to the supramolecular organization of biological membranes.

J.-F. Tocanne
Institut de Pharmacologie et Biologie Structurale du CNRS
F-31062 Toulouse Cedex, France
e-mail: tocanne@ipbs.fr

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:127–131

Biological membranes are complex assemblies composed of a wide variety of lipid and protein molecular species. In current models of membranes, the lipid bilayer is considered as a two-dimensional fluid in which lipids and intrinsic proteins are free to diffuse. As a direct consequence, one would expect both types of molecules to be randomly distributed within the membrane. In fact, membrane organization is certainly more complex and evidence is accumulating to indicate the occurrence of both a transverse (Devaux, 1993) and lateral (Tocanne, 1992; Glaser, 1993) compartmentation of membranes which can be described in terms of lipid and protein macro- and microdomains. Macrodomains, a few micrometers in size, have been recognized in the plasma membrane of specialized cells such as spermatozoa, eggs and epithelia (Tocanne et al., 1994a). The two leaflets of the lipid bilayer can also be considered as two large and nearly independent membrane domains but with the possibility of transbilayer lipid redistribution mediated by a specific and energy-dependent aminophospholipid transporter (Devaux, 1993). Microdomains extending from the submicron to the molecular scale have been recognized in the plasma membrane of many cell types and by means of a quite wide variety of approaches (Tocanne, 1992) among which Fluorescence Recovery After Photobleaching (FRAP) (Tocanne et al., 1994) and Single Particle Track-

ing (SPT) (Saxton and Jacobson, 1997) techniques have proved very useful.

At this stage, one can have the feeling that membranes are structurally well known and that the concept of membrane domain is firmly established. In fact, and as emphasized by other contributors, one has to realize that the word 'domain' is generic and applies to a wide variety of structures and that the view one can have of these domains still strongly depends on the technique and the lipid or the protein probes used for their detection. There is certainly not a unique definition of membrane domains and as a matter of fact, the size of these domains, their composition, the time- and space-scale over which they exist may vary from one cellular system to another. The nature of the interactions and forces responsible for their formation have also to be clearly identified and remains, in addition to the previous points, one of the many challenging problems in membranology.

With respect to proteins, one current opinion is to consider their confinement in domains is due principally to interactions of their cytosolic part with membrane skeleton and/or cytoskeleton elements (Sheets et al., 1995; Kusumi and Yako, 1996). In this model, the barriers to protein diffusion are located outside the membrane, not within the membrane.

Like proteins, lipids exhibit a wide diversity in structure, at the level of both their polar headgroups and acyl chains. The causal structure-function relationship which may easily be put forward for membrane proteins is not so obvious in the case of lipids. From a physical point of view, the structural diversity they display is not required to maintain bilayer assembly and fluidity. Gel-fluid phase lateral separations have been suggested as being responsible for lateral heterogeneities in membranes (Vaz, 1992). Such a possibility, which can occur in some specific cases, like in ram sperm plasma membrane (Wolf et al., 1990), does not seem to be of general relevance to natural membranes. In many cases, the lateral heterogeneities in lipid distribution detected in natural membranes are no longer observed in the corresponding protein-free lipid bilayers (Tocanne et al., 1994b) and there is increasing evidence that proteins are responsible for membrane organization (Edidin, 1997). From another point of view, we recently arrived at the conclusion that FRAP experiments conducted at variable beam radius provide a way to detect the presence of domains in membranes and to evaluate their size. When applied to published data obtained for lipids and proteins on various cellular membranes, this approach indicates the presence of closed protein and lipid domains in these membranes, with nearly the same size (Salomé et al., 1997). This implies that in addition to interactions of membrane proteins with the membrane skeleton, protein-protein interactions occur within the membrane. They lead to the formation of intramembrane barriers which prevent lipids and proteins from long-range lateral diffusion. However, if protein-protein interactions seem to play an important role, we still have no direct information on the molecular organization of lipids in contact with or far from the proteins, nor on the potential specificity of lipid distribution around membrane proteins, nor on the contribution of protein-lipid interactions to protein agregation.

The possibility of specific interactions between lipids and transmembrane proteins is not to be discarded (Tocanne et al., 1994b; see also D. Marsh's contribution in the present issue). However, this kind of interaction remains limited to specific cases and is not expected to contribute in any great extent to membrane organization. In contrast, and because of the long-range dispersion of elastic forces in membranes (principle of cooperativity), the consequences of non specific protein-lipid interactions on lipid dynamics may extend over a few lipid layers around each protein and therefore may contribute on a large scale to membrane organization. One particularly attractive case is the existence of a mismatch between the hydrophobic thicknesses of a transmembrane protein and the supporting lipid bilayer. As is now well evidenced by theoretical and experimental data, a hydrophobic mismatch between lipids and proteins can generate physical forces capable of promoting an aggregation of proteins within the membrane, changes in the organization and dynamics of those lipid molecules which are in contact with proteins,

and a sorting of lipids by the proteins.

First developed from a theoretical point of view (Mouritsen and Bloom, 1984), the concept of hydrophobic mismatch has been checked experimentally with the reaction center and the light-harvesting antenna from *Rhodobacter sphaeroides* (Riegler and Möhwald, 1986; Peschke et al., 1987) and bacteriorhodopsin from *Halobacterium halobium* (Piknova et al., 1993) reconstituted in phosphatidylcholines with different acyl chain lengths. Depending on the relative hydrophobic thickness of the proteins and the supporting lipid bilayers, upward or downward shifts in the phase transition temperature of the lipids were detected with the three proteins which could be accounted for in the light of the current theories, thus providing strong experimental support to the concept that hydrophobic mismatch can be at the origin of great changes in the physical state of lipids. Coherence lengths of 12 - 15 Åwere determined, which means that at least three to four lipid layers around the proteins are significantly affected by the hydrophobic mismatch.

The Ca-ATPase from muscle sarcoplasmic reticulum reconstituted in phosphatidylcholines substituted by various monounsaturated fatty acids has long been shown to exhibit a bell-shape activity versus acyl chain length curve, with maximum activity for oleic acid (Starling et al., 1993). As recently shown, these changes in activity are closely related to the aggregation state of the protein, the lower the activity of the protein in the short or long chain PC species, the higher its agregation state in the proteoliposomes (Cornea and Thomas, 1994).

In this contribution, it is pointed out that a mismatch between the hydrophobic lengths of transmembrane proteins and the hydrophobic thickness of the surrounding lipid bilayer may generate physical forces which can promote molecular mechanisms of lipid sorting by the proteins and then contribute to the supramolecular organization of biological membranes.

In mixtures of lipids with different acyl chain lengths, a transmembrane protein is expected to be solvated by the lipid species capable of best matching its hydrophobic surface (Sperotto and Mouritsen, 1993). This theoretical prediction is now supported experimentally by data obtained with the pulmonary surfactant protein SP-C and bacteriorhodopsin (BR), through fluorescence energy transfer experiments. SP-C, reconstituted in surfactant lipids, is excluded from gel phase palmitoyl lipids and prefers shorter chain and unsaturated lipids below the bulk lipid phase transition (Horowitz, 1995). BR was reconstituted in DLPC/DSPC mixtures and its behaviour was analyzed quantitatively by comparing simulated and experimental data. In a quite consistent way, the theoretical and experimental approaches show that at low temperature, when all the lipids are in the gel state, BR is embedded in the nearly pure DLPC phase. At high temperature, when all the lipids are in the fluid state, the protein is preferentially surrounded by DSPC at the expense of DLPC. Quite interestingly, at moderate temperature, when DLPC is in the fluid phase while DSPC is still in the gel phase, BR is found to be located at the gel-fluid phase boundary (Dumas et al., 1997). This last conclusion is also supported by indirect arguments developed by Schram and Thompson who studied the phase behaviour and dynamics of BR/DMPC/DSPC mixtures (Schram and Thompson, 1997).

Even in the absence of hydrophobic mismatch, the dynamics of lipids may also be affected by interactions with membrane proteins. Thus, in the course of our studies on the influence of proteins on the lateral diffusion of lipids, comparison of simulated and experimental FRAP data led us to conclude that in egg yolk phosphatidylcholine, bacteriorhodopsin is not to be viewed as naked but rather as surrounded by about two layers of lipids with restricted lateral mobility (Schram et al., 1994).

From a biophysical point of view, the hydrophobic matching condition can be considered as a very useful and operational concept. From a biological point of view, one can argue that this concept has nothing to do with membrane organization because in a given membrane, proteins and lipids show, on the average, similar hydrophobic thicknesses. The influence of cholesterol, which is known to have a buffering effect on the dynam-

ics of the lipid acyl chains, should also be considered. However, this concept has been recently introduced in cellular biology to explain some aspects of the sorting of membrane proteins from the reticulum to the plasmalemma, through the Golgi apparatus (Bretscher and Munro, 1993). Moreover, careful inspection of protein sequences and structures show that the various transmembrane segments of membrane proteins may differ in hydrophobic length (by at least a few angstrom) and orientation with respect to the bilayer normal. In other words, the concept of hydrophobic mismatch and lipid sorting by proteins can be extended to a submolecular level. A membrane protein may be seen now surrounded by various lipid species (including cholesterol), each being selected to match best its various hydrophobic segments (Dumas et al., 1997).

Protein-lipid interactions are believed to contribute important molecular forces which, on account of the relatively high protein surface density in membranes, can propagate over long distances. In the presence of too strong protein-lipid hydrophobic mismatch, protein aggregation provides a way for the system to decrease the importance of protein-lipid interactions and therefore to relieve an excess of energy. Another and not contradictory possibility is to generalize the concept of lipid sorting by proteins to the whole system and to consider now the membrane as a supramolecular assembly in which microdomains are the consequence of a good structural matching of the various protein and lipid components. However, the final organization of the membrane and in particular the structure of the protein fences are also expected to depend critically on protein-protein interactions. These various interaction parameters, their relative extent and interplay need to be firmly established before any clear picture of membrane organization can be drawn. Models of membranes should not be thought of as being static and in equilibrium. They should include the dynamics of the protein and lipid components and of the membrane itself.

References

Bretscher, M.S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science* 26: 1280-1281.

Cornea, R.L., and D.D. Thomas. 1994. Effects of membrane thickness on the molecular dynamics and enzymatic activity of reconstituted Ca-ATPase. *Biochemistry* 33: 2912-2920.

Devaux, P.F. 1993. Lipid transmembrane asymmetry and flip-flop in biological membranes and lipid bilayers. *Curr. Opin. Struct. Biol.* 3: 489-494.

Dumas, F., M.M. Sperotto, C. Lebrun, J.-F. Tocanne, and O.G. Mouritsen. 1997. Molecular sorting of lipids by bacteriorhodopsin in DLPC/DSPC lipid bilayers. *Biophys. J.* 73: 1940-1953.

Edidin, M. 1997. Lipid microdomains in cell surface membranes. *Curr. Opin. Struct. Biol.* 7: 528-532

Glaser, M. 1993. Lipid domains in biological membranes. *Curr. Opin. Struct. Biol.* 3: 475-481.

Horowitz, A.D. 1995. Exclusion of SP-C, but not SP-B, by gel phase palmitoyl lipids. *Chem. Phys. Lipids* 76: 27-39.

Kusumi, A., and Y. Sako. 1996. Cell surface organization by the membrane skeleton. *Curr. Opin. Cell. Biol.* 8: 566-574

Mouritsen, O.G., and M. Bloom. 1984. Mattress model of lipid-protein interactions in membranes. *Biophys. J.* 46: 141-153.

Peschke. J., J. Riegler, and H. Möhwald. 1987. Quantitative analysis of membrane distorsions induced by mismatch of protein and lipid hydrophobic thickness. *Eur. Biophys. J.* 14: 385-391.

Piknova, B., E. Perochon, and J.-F. Tocanne. 1993. Hydrophobic mismatch and long-range protein-lipid interactions in bacteriorhodopsin/phosphatidylcholine vesicles. *Eur. J. Biochem.* 218: 385-396.

Riegler, J., and H. Möhwald. 1986. Elastic interactions of photosynthetic reaction center proteins affecting phase transitions and protein distributions. *Biophys. J.* 49: 1111-1118.

Salomé, L., J.L. Cazeils, A. Lopez, and J.-F. Tocanne. 1997. Characterization of membrane domains by FRAP experiments at variable observation areas. Submitted.

Saxton, M.J., and K. Jacobson. 1997. Single-particle tracking: application to membrane dynamics. *Annu. Rev. Biophys. Biomol. Struct.* 26: 371-397.

Schram, V., J.-F. Tocanne, and A. Lopez. 1994. Long range lateral diffusion of lipids in obstructed bilayers. Computer simulation of FRAP experiments and application to bacteriorhodopsine/phosphatidylcholine recombinants. *Eur. Biophys. J.* 23: 337-348.

Schram, V., and T.E. Thompson. 1997. Influence of the intrinsic membrane protein bacteriorhodopsin on gel-phase domain topology in two-component phase-separated bilayers. *Biophys. J.* 72: 2217-2225.

Sheets, E.D., R. Simson, and K. Jacobson. 1995. New insights into membrane dynamics from the anlysis of cell surface interactions by physical methods. *Curr. Opin. Cell. Biol.* 7: 707-714.

Sperotto, M., and O.G. Mouritsen. 1993. Lipid enrichment and selectivity of integral membrane proteins in two component lipid bilayers. *Eur. Biophys. J.* 22: 323-328.

Starling, A.P., J.M. East, and A.G. Lee. 1993. Effects of phosphatidylcholine fatty acyl chain length on calcium binding and other functions of the (Ca^{++} - Mg^{++})-ATPase. *Biochemistry* 32: 1593-1600.

Tocanne, J.-F. 1992. Detection of lipid domains in biological membranes. *Comments Mol. Cell. Biophys.* 8: 53-72.

Tocanne, J.-F., L. Dupou-Cézanne, and A. Lopez. 1994a. Lateral diffusion of lipids in model and natural membranes. *Progr. Lipid Res.* 33: 203-237.

Tocanne, J.-F., L. Dupou-Czanne, A. Lopez, E. Perochon, B. Piknova, V. Schram, J.F. Tournier, and M. Welby. 1994b. Lipid domains and lipid/protein interactions in biological membranes. *Chem. Phys. Lipids* 73: 139-158

Vaz, W.L.C. 1992. Translational diffusion in phase-separated lipid bilayer membranes. *Comments Mol. Cell. Biophys.* 8: 17-36

Wolf, D.E., V.M. Maynard, C.A. McKinnon, and D.L. Melchior. 1990. Lipid domains in the ram sperm plasma membrane demonstrated by differential scanning calorimetry. *Proc. Natl. Acad. Sci. USA* 87: 6893-6896

Plasma Membrane Organization by the Membrane Skeleton

Akihiro Kusumi

Abstract

The membrane skeleton provides both corralling and binding effects on the movement of membrane proteins. I propose that these effects can play pivotal roles in the molecular organization of the plasma membrane, especially in the formation of special membrane domains.

Akihiro Kusumi
Department of Biological Science
Graduate School of Science
Nagoya University
Nagoya 464-01, Japan
e-mail: akusumi@bio.nagoya-u.ac.jp

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:137–142

Introduction

Various structures and arrays of proteins and lipids exist within and around the plasma membrane, which are essential for the proper functioning of these molecules in the plasma membrane. These supramolecular complexes include (1) multimers of receptor molecules or receptor and effector molecules, which are thought to be the first trigger for the subsequent reactions in cells after ligand binding (Grasberger et al., 1986, Metzger, 1992), (2) specialized membrane domains, such as synapses, clathrin-coated lattices and pits, caveolae, and cell-cell and cell-substrate adhesion structures, in which specific proteins and lipids are assembled to carry out specific functions (Klymkowski and Parr, 1995, Miyamoto et al., 1995), and (3) the polarized distribution of various proteins in epithelial and neuronal cells (Nelson, 1992). The constituent molecules of such supramolecular structures are recruited and assembled from the plasma membrane and the cytoplasm, and by intracellular vesicular transport. In this contribution, I am mostly concerned with the recruitment of membrane proteins within the plasma membrane.

In the recruitment, multimerization, and assembly of specific membrane proteins and lipids, one of the critical processes is the regulation of the movement of these molecules. Proteins are not free-floating in a sea of excess lipids, i.e., the cells have various means to control the mobility and specific assembly of membrane proteins into specialized domains. Of particular interest is the

involvement of membrane-skeletal elements in mediating or inhibiting movements of cell surface receptors, and their participation in the formation of specialized domains and in signal transduction in the plasma membrane (Kusumi and Sako, 1996).

Barriers to Lateral Diffusion of Membrane Receptors as Studied by Single Particle Tracking and Laser Tweezers: Fence and Tether

Movements of transferrin and alpha2-macroglobulin receptor molecules in the plasma membrane of cultured normal rat kidney (NRK) fibroblastic cells were investigated by video-enhanced optical microscopy with a nanometer-level spatial precision and a temporal resolution up to 0.2 ms by labeling the receptors with the ligand-coated nanometer-sized colloidal gold particles (Kusumi et al., 1993; Sako and Kusumi, 1994). For both receptor species, approximately 90% of the movement trajectories are of the confined diffusion type, within domains of 0.25 μm^2 (500-700 nm in diagonal length). Movement within the domains is random with a microscopic diffusion coefficient $D_{micro} \simeq 10^{-9} cm^2/s$, which is consistent with a value expected for freely diffusing proteins in the plasma membrane. The receptor molecules move from one domain to one of the adjacent domains every 25 s on average, indicating that the plasma membrane is compartmentalized for diffusion of membrane receptors and that long-range diffusion occurs as a result of successive intercompartmental hops. The macroscopic diffusion coefficients for these two receptor molecules are $\simeq 3 \times 10^{-11} cm^2/s$, which is smaller than D_{micro} by a factor of 30. The above results indicate that the macroscopic diffusion is slowed due to confinement by the boundaries, and not due to the intrinsically slow rate of diffusion. Partial destruction of the cytoskeleton and partial deletion of the cytoplasmic domains of many membrane receptors strongly influenced their diffusion properties, indicating that the boundaries between compartments are made of the membrane-associated part of the cytoskeleton or the membrane skeleton (membrane-skeleton fence model).

The mechanical properties of intercompartmental boundaries were then studied by tagging transferrin receptor (TR) with either 210 nm latex or 40 nm colloidal gold particles, and by dragging the particle-TR complexes laterally along the plasma membrane using laser tweezers (Sako and Kusumi, 1995). Approximately 90% of the TR-particle complexes, which showed confined-type diffusion with D_{micro} of $\simeq 10^{-9} cm^2/s$, could be dragged past the intercompartmental boundaries in their path by laser tweezers at a trapping force of 0.35 - 0.8 pN. At the dragging forces between 0.05 and 0.1 pN, particle-TR complexes tended to escape from the laser trap at the boundaries, and such escape occurred in both the forward and backward directions of dragging. The boundaries are elastic with an effective elastic constant of 1-10 $pN/\mu m$. These results are consistent with the proposal that the compartment boundaries consist of membrane skeleton. Approximately 10% of TR exhibited slower diffusion $D_{micro} \simeq 10^{-10} - 10^{-11} cm^2/s$ and binding to elastic structures.

Regulation of the Movements of Erythrocyte Band 3 As Studied by Single Particle Tracking and Laser Tweezers

We have proposed a "membrane-skeleton fence model", in which close apposition of the membrane skeleton meshwork to the membrane gives effective barriers for free diffusion of membrane proteins due to steric hindrance. This model was examined by single particle tracking with a high speed camera and by laser tweezers.

(1) Erythrocyte band 3 was labelled by paucivalent colloidal gold. The mobile fraction of band 3 was 65% at 37°C. These molecules undergo free diffusion $D_{\text{micro}} \simeq 5.3 \times 10^{-9} \text{cm}^2/\text{s}$ within domains of $\approx$ 110-nm in diameter, and hop to adjacent domains every 350 ms on average. D_{macro} was one-sixtieth of D_{micro}.

(2) The cytoplasmic domain of band 3 was removed by brief trypsin treatment, which did not cleave spectrin and actin. The domain size and D_{micro} was the same after cleavage, but only the hop rate increased by a factor of 6 to once every 60 ms on average.

(3) When the membrane skeleton was dragged laterally by optical tweezers via attached latex bead (1 micron-diameter), mobile band 3 was also moved along with the membrane skeleton. This result indicates collision of band 3 with the membrane skeleton.

These results support the membrane skeleton fence model.

Regulation of Band 3 Diffusion by Dissociation-Association Equilibrium of the Erythrocyte Membrane Skeleton

The mechanism of intercompartmental hop of band 3 was investigated using optical tweezers.

(1) Band 3 was dragged along the membrane by optical tweezers at several different scan rates. At velocities lower than 1.6 μm/s, band 3 could be moved freely. But at velocities higher than 1.6 μm/s, band 3 often escaped from the trap. These results suggest that, when dragged at a velocity lower than 1.6 μm/s, band 3 tends to pass a fence before band 3 collides with the next fence, and that the fence undergoes conformational change every 70 ms on average (= 110 nm / 1.6 μm/s) that allow the passage of band 3.

(2) The conformational change may be either dissociation of spectrin from tetramers to dimers or conformational fluctuation of the tetramers. When the membrane skeletal network was dragged and elongated by optical tweezers, the hop rate increased for the elongated fence. Since the fluctuation of spectrin should be smaller when spectrin is elongated, the intercompartmental hop of band 3 is likely to be caused by dissociation of spectrin tetramers to dimers.

We propose that the passage of band 3 over the spectrin fence is facilitated by dissociation of spectrin tetramers to dimers, which takes place on the average of once every 70 ms.

Membrane-Skeleton Fence Model

Since variations in the particle size (40 and 210 nm; the particles are on the extracellular surface of the plasma membrane) hardly affect the diffusion rate and behavior in the dragging experiments, and since treatment with either cytochalasin *D* or vinblastin affects the movements of TR, the boundaries are likely to be present in the cytoplasmic domain. The rebound motion of the particle-TR complexes when they escape from the laser tweezers at the compartment boundaries suggests that the boundaries are elastic structures. These results are consistent with the proposal that the compartment boundaries consist of a membrane-associated portion of the cytoskeleton (membrane-skeleton fence model).

In this model, the membrane skeleton provides a barrier to free diffusion of membrane proteins due to steric hindrance (the space between the membrane and the cytoskeleton is too small to allow the cytoplasmic portion of the membrane protein to pass), thus compartmentalizing the membrane into many small domains of 0.1 - 1 μm^2. The membrane proteins can escape from one domain and move to adjacent compartments due to the dynamic properties of the membrane skeleton: the distance between the membrane and the skeleton may fluctuate over time (or the membrane skeleton may dissociate from the membrane), or the membrane-skeleton network may form and break continuously due to dissociation-association equilibrium, thus giving the membrane proteins an opportunity to pass through the mesh barrier. Furthermore, the membrane protein molecules that have sufficient kinetic energy will be able to cross the boundaries.

Confined lateral diffusion and intercompartmental hop diffusion of membrane proteins have been observed in a variety of membrane proteins in all cells studied thus far. We propose that compartmentalization of the plasma membrane by a membrane-skeleton/cytoskeleton meshwork (membrane-skeleton fence structure) is a basic feature of the plasma membrane. For individual protein species, more specific mechanisms such as direct binding to the cytoskeleton may be at work. However, what should be emphasized here is that the fence effect of the membrane skeleton is superimposed on the specific effect for individual protein species. In the case of E-cadherin, some molecules that are bound to the flexible cytoskeleton (possibly thin actin filaments) "feel" the presence of the membrane skeleton fence as they move about with the attached cytoskeleton (Sako and Kusumi, unpublished observation).

Binding and Transport of Membrane Proteins by the Membrane Skeleton

Binding of membrane proteins to the membrane skeleton has been found for almost all proteins investigated so far, including the receptors for transferrin, EGF, and alpha2-macroglobulin, E- and T-cadherins, and band 3 anion channel in erythrocytes. (The strength of the interaction between the cytoskeleton and the membrane bilayer has been estimated to be 2 - 8 pN.) These bound proteins undergo various types of motion. Some show no motion, while some show oscillatory movements without real translation. Some proteins show long-range translational diffusion while they are apparently bound to the membrane skeleton (which is known because these particles cannot be dragged more than 100 nm by laser tweezers). Some show directed transport-type movements probably due to the active movement of the cytoskeleton to which they are bound.

The mechanism by which the cells control these processes and exert the fence effect of the membrane skeleton has yet to be elucidated. The amount of E-cadherin bound to the membrane-

skeleton decreases after a calcium switch. The size of the compartment as "felt" by the Na, K-ATPase in the dorsal/apical membrane decreases by a factor of 2 after the calcium switch in MDCK cells. Specific binding to a particular membrane skeleton/cytoskeleton may be controlled by phosphorylation. It is likely that cells are using the fence effect and active transport by the membrane-skeleton/cytoskeleton to assemble specific membrane proteins into specialized domains. However, exactly how cells do this is not known and is one of the most important issues in membrane biology.

Control Mechanisms for the Formation of Supramolecular Arrays and Assemblies in and around the Plasma Membrane

We envisage three basic processes for the assembly of membrane proteins through movements in the plasma membrane. These are basic concepts and are not mutually exclusive. (1) Diffusion of the membrane protein and entrapment at specific sites in the membrane can occur, possibly due to preassembly of cytoplasmic proteins on the cytoplasmic surface of the membrane. Cooperative assembly of intramembrane proteins and peripheral proteins is a possibility. (2) Cells take advantage of thermal diffusion to drive the movements of membrane proteins, but regulate the direction of the movements by varying the structure of the membrane skeleton using free energy released by decomposition of ATP. In this working hypothesis, we postulate that the free energy generated by ATP decomposition is not used to drive the movements but to regulate the movements. The basic idea for this hypothesis is that rather than simple self-assembly of molecules, cells actively regulate thermal movements to construct supramolecular complexes. (3) Gross, active movements of the membrane-skeleton network may occur to move the membrane proteins trapped in the compartments (like many fish in a fishnet) or those bound to the skeletal network. For example, we envisage that oligomers and aggregates are bound to the membrane skeleton and are carried by the skeleton as a single cargo. The key idea is that cells would not move membrane proteins one by one because it is energetically and temporally too inefficient.

We believe that the cytoskeleton/membrane-skeleton works as an organizer of molecules in the plasma membrane. In addition to regulating the movements of membrane proteins, the membrane-skeleton/cytoskeleton may regulate other undercoat structures of the plasma membrane such as caveolae and clathrin-coated structures.

References

Gadella, T.W.J., and T.M. Jovin. 1995. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J. Cell. Biol.* 129:1543-1558.

Grasberger, B., A.P. Minton, C. DeLisi, and H. Metzger. 1986. Interaction between proteins localized in membranes. *Proc. Natl. Acad. Sci. USA* 83:6258-6262.

Klymkowski, M.W., and B. Parr. 1995. The body language of cells: the intimate connection between cell adhesion and behavior. *Cell* 83:5-8.

Kusumi, A., Y. Sako, and M. Yamamoto. 1993. Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. *Biophys. J.* 65:2021-2040.

Kusumi, A., and Y. Sako. 1996. Cell surface organization by the membrane skeleton. *Curr. Opinion Cell Biol.* 8:566-574.

Metzger, H. 1992. Transmembrane signaling: the joy of aggregation. *J. Immunol.* 149:1477-1487.

Miyamoto, S., S.K. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation

in integrin transmembrane function. *Science* 267:883-885.

Nelson, W.J. 1992. Regulation of surface polarity from bacteria to mammals. *Science* 258:948-955.

Sako, Y., and A. Kusumi. 1994. Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. *J. Cell Biol.* 125:1251-1264.

Sako, Y., and A. Kusumi. 1995. Barriers for lateral diffusion of transferrin receptor in the plasma membrane as characterized by receptor dragging by laser tweezers: fence versus tether. *J. Cell Biol.* 129:1559-1574.

Molecular Basis for Red Cell Membrane Properties

Narla Mohandas *and* J. Aura Gimm

Abstract

Encouraging recent developments have begun to bridge the gap between what we know about membrane biochemistry and our knowledge of *in situ* physical properties of the red cell membrane, a prototypical biological membrane. This review provides a contemporary view of the relationship between red cell membrane molecular architecture and membrane material properties, emphasizing new developments that underscore the subtleties of soft cell interfaces.

Narla Mohandas *and* J. Aura Gimm
Lawrence Berkeley National Laboratory
University of California
Berkeley, 94720 USA
e-mail: mnarla@lbl.gov

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:143–146

The red cell membrane exhibits complex material behavior. It is highly elastic (100-fold softer than latex rubber of comparable thickness), responds rapidly to applied stresses (time constants in the range of 100 milliseconds) and is capable of undergoing large membrane extensions without fragmentation. These unusual material properties are the consequence of evolution-driven engineering which evolved a composite structure in which a plasma membrane envelope composed of amphiphilic surfactant molecules is anchored to a network of skeletal proteins through tethering sites (transmembrane proteins) in the bilayer. Whereas the lipid bilayer is a condensed fluid surface, the skeletal network can exhibit varying degrees of extensional rigidity. The roles for these two structural components appear to be as follows: the lipid bilayer (plus transmembrane proteins) chemically isolates and regulates the cell interior and the skeletal network provides rigid support and stability to the bilayer interface. Though much is known regarding the red cell membrane, the essential question is how to relate material properties of the red cell to the molecular composition and architecture of the membrane. To address this question, we begin the discussion with a brief overview of the biochemical composition and the structural organization of the red cell membrane and note the insights gained from studies of red cell membrane pathologies. Then in the context of these molecular features, we can discuss the current working model for the structural basis of red cell membrane material properties.

Biochemical Composition and Structural Organization of the Red Cell Membrane

The red cell membrane has been very well characterized biochemically and the structural organization of the various lipid and protein components is continuing to evolve. About 52% of the membrane mass is protein, 40% is lipid and 8% is carbohydrate. The major lipid components of the membrane bilayer are unesterified cholesterol and phospholipids which are present in nearly equimolar quantities. The principal components of the skeletal network, spectrin, actin, protein 4.1, adducin, tropomyosin and tropomodulin, form an hexagonal lattice of junctional complexes through specific protein-protein interactions (Mohandas and Evans, 1994). The physical linkage of membrane skeleton to the lipid bilayer is mediated by a number of transmembrane proteins including band 3 and glycophorin C (Mohandas and Evans, 1994).

Much of our understanding of the structural organization of the red cell membrane has been derived from carefully designed biochemical studies using purified components. However, recently developed biophysical approaches have enabled the documentation of the various membrane component interactions in intact membranes (Discher et al., 1994; Discher and Mohandas, 1996). For example, studies on normal red cells and pathologic red cells with deficiencies in specific membrane components using the newly developed technique of Fluorescence-Imaged Microdeformation has enabled the unequivocal documentation of *in situ* linkages between band 3 and the membrane skeleton mediated by ankyrin and between glycophorin C and the membrane skeleton mediated by protein 4.1 (Discher et al., 1994; Gimm et al., 1997). Additional linkages that have not been previously recognized have also been identified and these include the association of the Rh complex with the membrane skeleton.

Effect of Bilayer-Skeletal Protein Network Linkages on Membrane Cohesion

The importance of bilayer-skeletal protein network linkages on membrane cohesion is documented by studies using red cells that are totally deficient in either band 3 or ankyrin (Gimm et al., 1997; Peters et al., 1996). Reductions in the number of linkages between the bilayer and the membrane skeleton due to absence of either band 3 or ankyrin results in decreased cohesion between the bilayer and membrane skeleton resulting in membrane vesiculation and loss of membrane surface area (Gimm et al., 1997; Peters et al., 1996). Decreased membrane spectrin content, which also leads to reductions in the number of linkages between the bilayer and membrane skeleton, results in loss of membrane surface area (Chasis et al., 1988). Thus a critical number of linkages between the bilayer and the membrane skeleton are required for maintaining membrane cohesion.

Effect of Bilayer-Skeletal Protein Network Linkages on Membrane Rigidity

Physical association of the network to the bilayer is also an important determinant of the elastic resilience of the red cell membrane. While a minimum number of linkages between the bilayer and the membrane skeleton are needed for membrane cohesion, a large increase in the number of linkages will hinder the ability of the spectrin molecules to undergo the conformational rearrangements needed for deformation. The marked decrease in the ability of the hereditary ovalocytic red cells to undergo membrane deformation is an excellent example of the importance of this mechanism in regulating membrane deformation. Increased interaction of the mutant band 3 with the membrane skeleton in the ovalocytic red cells has been shown to account for marked increases in membrane rigidity of these membranes (Mohandas et al., 1992). Changes in the extents of association of the cytoplasmic domains of bilayer spanning proteins with the membrane skeleton can regulate membrane rigidity.

Effect of Lateral Skeletal Protein Linkages on Membrane Mechanical Stability

A critical role for lateral protein-protein linkages (spectrin-spectrin interaction and spectrin-actin-protein 4.1 interaction) in regulating membrane mechanical stability has been documented by studies using red cells with defects in spectrin and protein 4.1 (Chasis and Mohandas, 1986; Mohandas and Chasis, 1993). Weakening or disruption of either spectrin-spectrin interaction or spectrin-actin-protein 4.1 interaction in the spectrin-based membrane skeleton results in loss of membrane mechanical integrity and leads to cell fragmentation (Chasis and Mohandas, 1986; Mohandas and Chasis, 1993). Qualitative defects in protein 4.1 and alpha- and beta-spectrin that result in the weakening of lateral skeletal protein linkages have been shown to result in decreased mechanical stability (Chasis and Mohandas, 1986; Mohandas and Chasis, 1993; Mohandas and Evans, 1994). Thus lateral protein associations in the membrane skeleton play a key role in regulating membrane mechanical integrity and cohesion.

This concise summary of red cell membrane physiology illustrates some new insights into the origins of material behavior of red cell membrane and also illustrates how studies of red cell membrane pathologies and genetic structural mutations provide exceptional opportunities to test critically hypothesis for the origin of material behavior at the molecular level. It is hoped that our improved understanding of a simple biological membrane at the molecular level will further our abilities to unravel mysteries of biomolecular design.

References

Chasis, J.A., and N. Mohandas. 1986. Erythrocyte membrane deformability and stability : two distinct membrane properties that are independently regulated by skeletal protein associations. *J. Cell Biol.* 103: 343-350.

Chasis, J.A., P. Agre, and N. Mohandas. 1988. Decreased membrane mechanical stability and in vivo loss of surface area reflect spectrin deficiencies in hereditary spherocytosis. *J. Clin. Invest.* 82: 617-623.

Discher, D., N. Mohandas, and E.A. Evans. 1994. Molecular maps of red cell deformation : hidden elasticity and in situ connectivity. *Science* 266: 1032-1035.

Discher, D.E., and N. Mohandas. 1996. Kinematics of red cell aspiration by fluorescence-imaged microdeformation. *Biophys. J.* 71: 1680-1694.

Gimm, J.A., D.W. Knowles, L.L. Peters, S.E. Lux, and N. Mohandas. 1997. Band 3 plays a critical role in red cell membrane stability but is not required for membrane-skeletal assembly. *Biophys. J.* 72: A197. (abstract)

Peters, L.L., R.A. Shivdasani, S.C. Liu, M. Hanspal, K.M. John, J.M. Gonzalez, C. Brugnara, B. Gwynn, N. Mohandas, S.L. Alper, S.H. Orkin, and S.E. Lux. 1996. Anion exchanger (Band 3) is required to prevent erythrocyte membrane surface area loss but not to form the membrane skeleton. *Cell* 86: 917-927.

Mohandas, N., R. Winardi, D. Knowles, A. Leung, M. Parra, E. George, J. Conboy, and J.A. Chasis. 1992. Molecular basis for membrane rigidity of hereditary ovalocytosis - a novel mechanism involving the cytoplasmic domain of band 3. *J. Clin. Invest.* 89: 686-692.

Mohandas, N., and J.A. Chasis. 1993. Red cell deformability, membrane material properties and shape : regulation by transmembrane, skeletal and cytosolic proteins and lipids. *Semin. Hematol.* 30: 171-192.

Mohandas, N., and E. Evans. 1994. Mechanical properties of the red cell membrane in relation to molecular structure and genetic defects. *Annu. Rev. Biophys. Biomol. Struct.* 23: 787-818.

IgE Receptor Signaling Utilizes Specialized Membrane Domains

Barbara Baird, Kenneth A. Field *and* David Holowka

Abstract

Our recent studies have led to the hypothesis that signal transduction immediately following aggregation of the high affinity receptor for immunoglobulin E (FcϵRI) on mast cells involves the association of this receptor with specialized domains of the plasma membrane. We have evidence that these membrane domains, which are characterized by their resistance to solubilization by non-ionic detergents, mediate phosphorylation of aggregated receptors by supplying a locally high concentration of active tyrosine kinase, Lyn. Membrane domain-mediated FcϵRI activation points to a model for signal initiation that emphasizes selective protein-lipid interactions to facilitate functional coupling between proteins. This co-compartmentalization that depends on FcϵRI aggregation is a mechanism for signal regulation that is increasingly appreciated as relevant to signaling by other receptors.

Barbara Baird, Kenneth A. Field *and* David Holowka
Department of Chemistry,
Cornell University,
Ithaca, NY 14853, USA
e-mail: bab13@cornell.edu

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:147–151

Detergent-Resistant Membrane Domains

The plasma membrane comprises a large diversity of components which includes glycerophospholipids, sphingolipids, cholesterol, transmembrane proteins, and lipid anchored proteins. There is increasing evidence that these molecules are not uniformly distributed within the bilayer but are clustered to some extent by their physical properties. Protein-protein interactions provide the basis for some specialized membrane domains, e.g., focal adhesion contacts, clathrin-coated pits, or tight junctions. Recent studies demonstrate that lipid-based interactions can also organize distinct regions of the plasma membrane. Brown and Rose (1992) originally characterized a type of membrane domain that could be isolated because of its insolubility in non-ionic detergents such as Triton X-100. These detergent-resistant plasma membrane domains include flask-like structures identified as caveolae with electron microscopy. Caveolae are organized by both lipid- and protein-based inter-

actions that are facilitated by the oligomerization of the structural protein caveolin (Rothberg et al., 1992; Lisanti et al., 1993).

Cells that do not express caveolin, including mast cells and other hematopoietic cells, do not display caveolae on their surface. However, these cells do contain detergent-resistant membrane domains enriched in some of the same molecules as caveolae (Fra et al., 1994). Formation of these membrane domains is probably facilitated by favorable packing of the predominantly saturated acyl chains of certain lipids. Domains isolated from lysed cells are enriched in sphingomyelin, gangliosides, other glycolipids, and cholesterol. As shown in model membrane studies, membrane domains form in the absence of any structural proteins and exist prior to the addition of non-ionic detergents (Schroeder et al., 1994; Ahmed et al., 1997). As isolated from lysed cells by sucrose gradient ultracentrifugation, these domains are enriched in proteins linked to the plasma membrane by glycosyl-phosphatidylinositol (GPI) or by saturated fatty acids. In particular, certain Src-family tyrosine kinases, including Lyn, associate with these membrane domains, dependent on their tandem myristoyl and palmitoyl acyl chains (Shenoy-Scaria et al., 1993).

Current Working Model For FcεRI Activation in Membrane Domains

Phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of FcεRI subunits is a tightly controlled step in receptor activation. The unaggregated receptors are not phosphorylated, and they associate weakly with certain components of the membrane domains. In the resting state, the domains are probably dynamic in composition and small in size. Aggregation of FcεRI rapidly enhances receptor-domain associations, possibly because the increased valency of FcεRI aggregates leads to cooperative binding to small clusters of domain components. This causes the formation of larger, more stable membrane domains around the receptor clusters. The locally high concentration of Lyn within the domains favors FcεRI phosphorylation. Once phosphorylated, the ITAM of the β subunit of FcεRI binds directly to the SH2 domain of Lyn, and this interaction amplifies the signal, possibly by increasing the effectiveness of Lyn (Lin et al., 1996). Lyn-mediated phosphorylation of the ITAM of the γ subunit leads to recruitment, phosphorylation, and activation of the tyrosine kinase Syk, which, in turn, initiates activation of downstream signaling pathways (Scharenberg and Kinet, 1995; Beaven and Baumgartner, 1996).

The formation of membrane domains around aggregated FcεRI may facilitate additional signaling steps, including Ca^{2+} mobilization. Other proteins involved in these processes, such as tyrosine phosphatases and serine/threonine kinases and phosphatases may be enriched or excluded from the domains. Enzymes associated with the domains may have restricted access to other membrane-associated substrates. For example, lipid metabolizing enzymes that are recruited to these receptor complexes would have altered substrate access due to the local enrichment of domain-associated lipids (Pike and Casey, 1996; Hinderliter et al., 1997). In addition, these membrane domains may interact directly with the microfilament cytoskeleton which can further regulate FcεRI signaling (Pierini et al, 1996; 1997).

Experimental Evidence for FcεRI Activition in Membrane Domains

The most compelling evidence for the model of FcεRI receptor activation in membrane domains is the observation that stimulated tyrosine phosphorylation of FcεRI on cells occurs only with receptors that co-isolate with detergent-resistant membrane domains (Field et al., 1997). A large fraction of FcεRI aggregated with an optimal dose of multivalent ligand associate with the isolated domains, whereas unaggregated FcεRI do not. Furthermore, aggregation-dependent association of FcεRI with membrane domains occurs more rapidly than receptor phosphorylation, and this association occurs similarly when stimulated tyrosine phosphorylation is prevented. We have reconstituted the tyrosine phosphorylation of FcεRI in vitro with isolated membrane domains. The tyrosine phosphorylation of the β and γ receptor subunits does not occur when micellar Triton X-100 is included in the kinase assay. We found that micellar Triton extracts FcεRI from the domains, thereby decoupling the receptor from active Lyn which remains associated with the domains.

Membrane domains that form on intact cells can be fluorescently labeled with lipid analogs or with antibodies to GPI-linked proteins or gangliosides. DiI-C_{16}, a fluorescent lipid analog with saturated acyl chains, co-redistributes at the cell surface with aggregated FcεRI and together with the AA4 ganglioside derivatives and the GPI-linked protein Thy-1. The observation that the DiI-C_{16} located in large patches around aggregated receptors is less laterally mobile than that located elsewhere on the plasma membrane (Thomas et al., 1994) suggests that receptor aggregation can cause the coalescence of less fluid membrane domains, a property predicted for detergent resistant membrane domains from model studies (Schroeder et al., 1994; Ahmed et al., 1997).

Further support for the existence of membrane domains on the plasma membrane of intact cells comes from experiments in which the composition of the plasma membrane is altered by sphingomyelin liposome treatment. Confocal fluorescence microscopy reveals that this treatment promotes the formation of large membrane domains even in the absence of receptor aggregation (Holowka et al., 1996), and other experiments show that functional responses to FcεRI aggregation are enhanced under these conditions (Chang et al., 1995). Experiments with cytochalasin D (Pierini et al., 1997) and with liposome treatments (Holowka et al., 1996) indicate that the membrane domains couple to the cytoskeleton, and interference alters receptor-mediated signaling. Ongoing experiments are directed toward elucidating the molecular details underlying these results.

A central question for understanding the mechanism for coalescence of domains with aggregated FcεRI and the possible role of membrane domains in the function of this and other receptors is the structural basis for this association. A significant clue is the detergent sensitivity of the interaction. As first defined for the association of the β and γ subunits with the subunit of FcεRI by Metzger and colleagues (Kinet et al., 1985), the ratio of detergent to cell lipid is critical for maintaining interactions between aggregated FcεRI and membrane domains following cell lysis (Field et al., 1998). The working model is FcεRI associating weakly with gangliosides or similar domain components. This association could involve either the transmembrane or extracellular portions of the receptor but does not depend on the β and γ ITAMs. Our view that aggregation of FcεRI co-clusters small patches of domain components and nucleates the formation of stable, active membrane domains is consistent with current biophysical and functional data (Holowka and Baird, 1996).

Other Receptors

Remaining to be discovered is how the observations summarized here will extend to other cell surface receptors. This issue is particularly relevant for other receptors that are known to utilize Src-family tyrosine kinases in an early signaling step, such as other members of the multichain immune response receptor family. Recent reports that receptor tyrosine kinases, such as the EGF and PDGF receptors, utilize caveolae to generate local activation signals point to the possibility that compartmentalized receptor activation is a more general phenomena for mitogenic receptors (Mineo et al., 1996; Liu et al., 1996). As appreciation of the complexity of cellular signaling networks grows, localization of signaling components becomes increasingly attractive as a mechanism for the cell to interpret and respond rapidly to the many stimuli it receives.

References

Ahmed, S.N., D.A. Brown, and E. London. 1997. On the origin of sphingolipid/cholesterol rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry* 36:10944-10953.

Beaven, M.A., and R.A. Baumgartner. 1996. Downstream signals initiated in mast cells by FceRI and other receptors. *Curr. Opinion Immunol.* 8:766-772.

Brown, D.A., and J.K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68:533-544.

Chang, E.Y., Y. Zheng, D. Holowka, and B. Baird. 1995. Alteration of lipid composition modulates Fc-epsilon-RI signaling in RBL-2H3 cells. *Biochemistry* 34:4376-4384.

Field, K.A., D. Holowka, and B. Baird. 1997. Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. *J. Biol. Chem.* 272:4276-4280.

Field, K.A., D. Holowka, and B. Baird. 1998. Structural basis for the association of FcεRI with detergent-resistant membrane domains. Submitted for publication.

Fra, A.M., E. Williamson, K. Simons, and R.G. Parton. 1994. Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem.* 269:30745-30748.

Hinderliter, A.K., A.R.G. Dibble, R.L. Biltonen, and J.J. Sando. 1997. Activation of protein kinase C by co-existing diacylglycerol-enriched and diacylglycerol-poor lipid domains. *Biochemistry* 36: 6141-48.

Holowka, D., and B. Baird. 1996. Antigen-mediated IgE receptor aggregation and signaling: a window on cell surface structure and dynamics. *Annu. Rev. Biophys. Biomol. Struct.* 25:79-112.

Holowka, D, C. Hine, and B. Baird. 1996. Alterations in cellular lipid composition affect the interactions of aggregated FceRI with $p53/56^{lyn}$ and the microfilament cytoskeleton. *FASEB J.* 10:A1214.

Kinet, J.-P., G. Alcaraz, A. Leonard, and H. Metzger. 1985. Dissociation of the receptor for immunoglobulin E in mild detergents. *Biochemistry* 24:4117-4124.

Lin, S., C. Cicala, A.M. Scharenberg, and J.-P. Kinet. 1996. The FcεRIβ subunit functions as an amplifier of FcεRIγ-mediated cell activation signals. *Cell* 85:985-995.

Lisanti, M.P., Z.L. Tang, and M. Sargiacomo. 1993. Caveolin forms a hetero-oligomeric protecomplex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae. *J. Cell. Biol.* 123:595-604.

Liu, P., Y. Ying, Y.G. Ko, and R.G.W. Anderson. 1996. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J. Biol. Chem.* 271:10299-10303.

Mineo, C., G.L. James, E. . Smart, and R.G.W. Anderson. 1996. Localization of epidermal growth factor-stimulated ras/raf-1 interaction to caveolae membrane. *J. Biol. Chem.* 271:11930-11935.

Pierini, L., D. Holowka, and B. Baird. 1996. FcεRI-mediated association of 6-μm beads with RBL-2H3 mast cells results in exclusion of signaling proteins from the forming phagosome and abrogation of normal downstream signaling. *J. Cell. Biol.* 134:1427-1439.

Pierini, L., N.T. Harris, D. Holowka, and B. Baird. 1997. Evidence supporting a role for microfilaments in regulating the coupling between poorly-dissociable IgE-FcεRI aggregates and downstream signaling pathways. *Biochemistry* 36:7447-7456.

Pike, L.J., and L. Casey. 1996. Localization and turnover of phosphatidylinositol 4,5 bisphosphate in caveolin-enriched membrane domains. *J. Biol. Chem.* 271: 26453-56.

Rothberg, K.G., J.E. Heuser, W.C. Donzell, Y.-S. Ying, J.R. Glenney, and R.G.W. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell* 68:673-682.

Scharenberg, A.M., and J.-P. Kinet. 1995. Early events in mast cell signal transduction. *Chem. Immunol.* 61:72-87.

Schroeder, R., E. London, D. Brown. 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl. Acad. Sci. USA* 91:12130-12134.

Shenoy-Scaria, A.M., L.K. Gauen, J. Kwong, A.S. Shaw, and D.M. Lublin. 1993. Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. *Mol. Cell. Biol.* 13:6385-6392.

Thomas, J., D. Holowka, B. Baird, and W.W. Webb. 1994. Large-scale co-aggregation of fluorescent lipid probes with cell surface proteins. *J. Cell Biol.* 125:795-802.

Rapid Activation of the Na^+,K^+-pump - Mechanisms and Functional Significance

Torben Clausen *and* Ole Bækgaard Nielsen

Abstract

In a wide variety of tissues (skeletal muscle, heart, smooth muscle, nerve cells, pancreas and kidney), hormones induce a rapid increase in the rate of active Na^+,K^+-transport. In skeletal muscle, this was shown to lead to a decrease in intracellular Na^+, indicating that the affinity for intracellular Na^+ had increased. This activation of the Na^+,K^+-pump seems to be mediated by cAMP and protein kinases. During excitation, active Na^+,K^+-transport was found to increase up to 22-fold within 10 sec. This was also associated with a decrease in intracellular Na^+. Moreover, due to the electrogenicity of the Na^+,K^+-pump, acute activation leads to hyperpolarization. In muscles where contractility had been inhibited by exposure to high extracellular K^+, activation of the Na^+,K^+-pump with hormones or by repeated electrical stimulation restored contractile force within 5-10 min. This force recovery could be prevented by ouabain. In conclusion, the Na^+,K^+-pump is essential for the restoration and maintenance of excitability during continued exercise. This function of the Na^+,K^+-pump is considerably amplified by the potential to undergo rapid activation.

Torben Clausen *and* Ole Bækgaard Nielsen
Department of Physiology
University of Aarhus
DK-8000 Århus C, Denmark
e-mail: tc@fi.aau.dk

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:153–158

The transport of ions, amino acids and sugars across the plasma membrane is mediated by specific transport systems. This allows for selectivity and at the same time defines the transport capacity by the number of transport systems per unit of membrane area. It can be assumed that the theoretical maximum rate of transport in a tissue is a function of the number of transporters multiplied by the maximum turnover number of each transporter.

Transport capacity is likely to reflect the physiological need for performing a given type of transport. Thus the capacity for performing active Na^+,K^+-transport can be estimated by measuring the concentration of Na^+,K^+-ATPase. Among a wide variety of human tissues, from erythrocytes

to brain cortex, the concentration of Na^+,K^+-ATPase expressed as pmol/g tissue wet wt. has been found to vary over a 160,000-fold range (Clausen, 1997). This shows that the requirement for active Na^+,K^+-transport varies considerably and indicates that each tissue regulates the concentration of Na^+,K^+-ATPase, allowing its cells to meet the challenges arising from local changes in Na^+,K^+-distribution. Skeletal muscle is excited to contract by a rapid influx of Na^+. This together with the subsequent rapid efflux of K^+ leads to a net gain of Na^+, a net loss of K^+, depolarization and eventually a loss of excitability. Thus, the very process of excitation is the basis for a progressive loss of excitability (Juel, 1986; Balog and Fitts, 1996; Nielsen and Clausen, 1996; Nielsen and Overgaard, 1996). Moreover, the total pool of skeletal muscle cells in the body is so large that during intense exercise, the net loss of K^+ from the working muscles within 60 sec induces a doubling of plasma K^+ in arterial blood, sufficient to cause cardiac arrest if it took place under resting conditions (Medbø and Sejersted, 1990).

Thus, during intense exercise, there is an urgent need for rapid restoration of Na^+,K^+-gradients and membrane potential - not only in the working muscle cells, but also in several other cells of the body. To this end, muscle cells contain a high concentration of Na^+,K^+-ATPase and therefore have a large spare capacity for active Na^+,K^+-transport. For this to play an important role in the maintenance of the Na^+,K^+-gradients during work, the Na^+,K^+-ATPase must undergo prompt and substantial activation at the onset of contractile activity. Indeed, it was recently demonstrated that in isolated rat skeletal muscle, electrical stimulation induces a rapid increase in the rate of net Na^+ extrusion (Everts and Clausen, 1994). Following 10 sec of high frequency stimulation, net Na^+ extrusion increased up to 22-fold above the resting rate, reaching full activation of all available Na^+,K^+-pumps (Nielsen and Clausen, 1997). More importantly, a substantial part of this activation was shown to be independent of an increase in intracellular Na^+ ($[Na^+]_i$), and as shown below in muscles undergoing isometric contractions, electrical stimulation was found to lead to a substantial decrease in intracellular Na^+, which was maintained for up to 30 min after the cessation of stimulation. At constant $[Na^+]_i$, an increase in the rate of active Na^+,K^+-transport may in principle take place in the following ways:

1. by upregulation of the concentration of transporters in the plasma membrane
2. by translocation of transporters from an intracellular or membraneous pool to the plasma membrane
3. activation due to increased maximum turnover number of the transporters already located in the plasma membrane
4. activation due to increased affinity of the transporter for the transported agent

Fig. 1 exemplifies some major aspects of the acute and long-term regulation of active Na^+,K^+-transport in skeletal muscle. Long-term changes in the transport capacity are generally established by de novo synthesis of transporters and their subsequent insertion in the plasma membrane. These processes take place over hours and days and are obviously too slow to meet urgent needs for a rapid increase in the rate of transport. More expedient mechanisms are required to solve this problem. For a rapid increase in active Na^+,K^+-transport two mechanisms have been proposed - translocation and activation.

Insulin-induced translocation of the glucose transporter Glut 4 from a separate (possibly intracellular) pool to the plasma membrane is well-documented. There is some evidence that in skeletal muscle insulin may also induce translocation of Na^+,K^+-ATPase from an intracellular pool to the plasma membrane (for a review, see Ewart and Klip, 1995). The size of the intracellular pool is not defined, however, and due to the very low recovery of Na^+,K^+-ATPase from the tissue it cannot be ascertained whether the fractions isolated are representative. Moreover, studies on intact rat muscle, where the total pool of Na^+,K^+-ATPase located in the plasma membrane could be quantified gave no evidence for insulin-induced translocation of the enzyme to the plasma membrane (Clausen and Hansen, 1977).

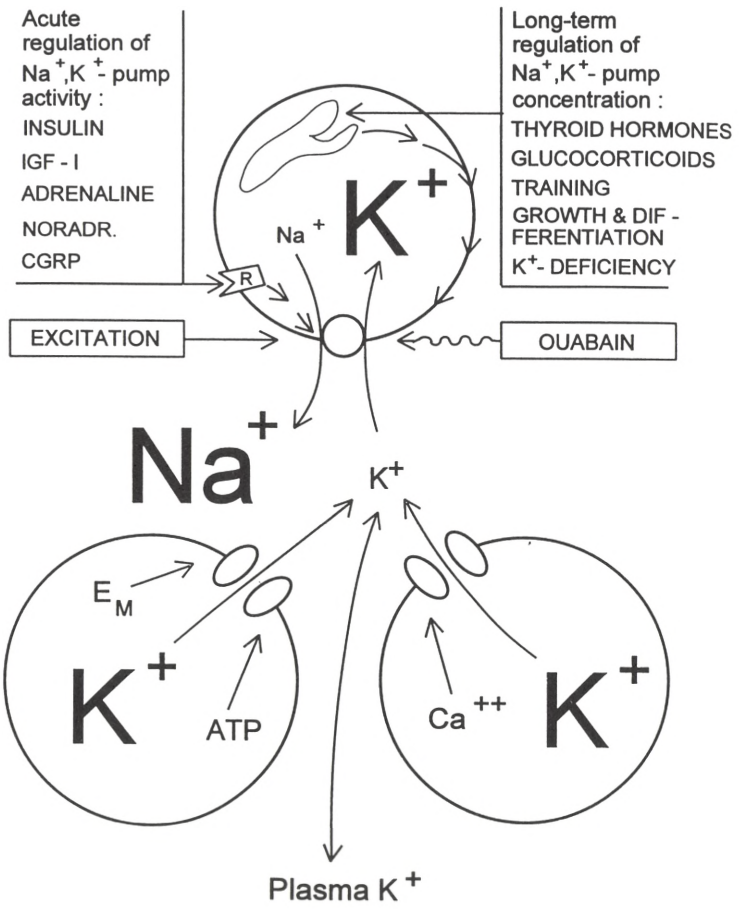


Figure 1. Some major aspects of the acute and long-term regulation of active Na^+,K^+-transport in skeletal muscle.

An acute increase in the activity of Na^+,K^+-ATPase already located in the plasma membrane may be achieved by increasing the maximum turnover number or the affinity of the transporter for intracellular Na^+. Several studies have shown that in muscle cells insulin produces a decrease in intracellular Na^+ (for a review, see Clausen, 1986), indicating that the stimulating effect of insulin on active Na^+,K^+-transport is the result of increased affinity for intracellular Na^+. More recently, insulin was found to reduce $[Na^+]_i$ also in renal tubular cells (Feraille et al., 1994).

Several other hormones have been found to stimulate the Na^+,K^+-pump in skeletal muscle (Clausen, 1996), in isolated cardiac myocytes (Desilets and Baumgarten, 1986) and other tissues. It is important that in such experiments, intracellular Na^+ decreases to values below the control level, indicating that the stimulation of the Na^+,K^+-pump cannot be related to increased influx of Na^+. Adrenaline-induced stimulation of the Na^+,K^+-pump was also shown to lead to a decrease in the activity of intracellular Na^+ in isolated rat and human muscle (Ballanyi and Grafe, 1988). As shown in Fig. 2, insulin, insulin-like growth factor I (IGF-I), adrenaline, noradrenaline and calcitonin gene related peptide (CGRP) all induce a marked decrease in intracellular Na^+ in isolated rat soleus muscle. Dibutyryl 3',5' cAMP has the same effect, and there is further evidence that 3',5'cAMP mediates the stimulating effect of the

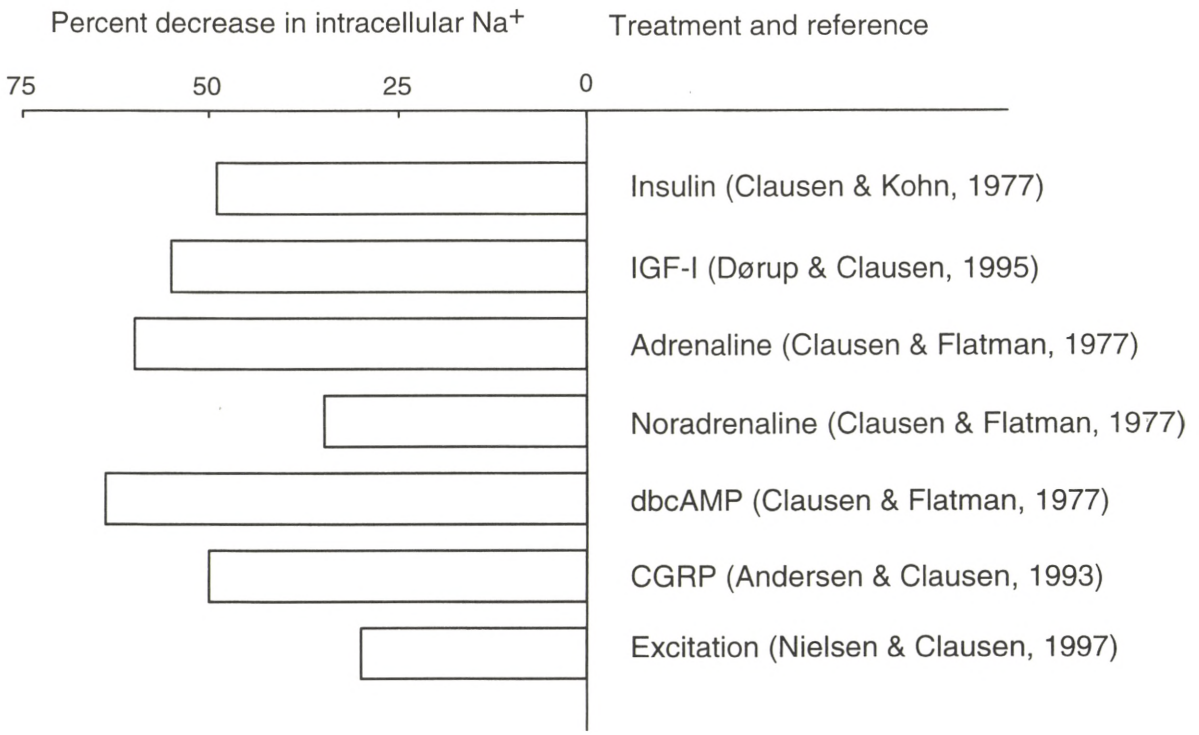


Figure 2. Effects of insulin, insulin-like growth factor I (IGF-I), adrenaline, noradrenaline, dibutyryl cyclic AMP (dbcAMP) and electrical stimulation (60 Hz for 30 sec, followed by 10 min rest) on $[Na^+]_i$ in isolated rat soleus muscle. Data were obtained from the publications indicated by references.

catecholamines and CGRP (Clausen and Flatman, 1977; Andersen and Clausen, 1993). In cells cultured from shark rectal glands, 3',5'cAMP induces stimulation of the Na^+,K^+-pump. This leads to a decrease in intracellular Na^+ (Lear et al., 1992), indicating that this is a general mechanism.

Taken together, the observations presented in Fig. 2 indicate that there is a general mechanism for rapid activation of the Na^+,K^+-pump, leading to a decrease in intracellular Na^+. The hormonal activation takes place within minutes and seems to be mediated by second messengers and protein kinases. The excitation-induced stimulation of the Na^+,K^+-pump, however, may reach maximum within 10 sec. We assume that these rapid changes reflect conformational modifications of the Na^+,K^+-pump, allowing it to function with a higher affinity for intracellular Na^+. This is analogous to the observation that the Ca^{++}-ATPase located in the plasma membrane exists in two interconvertible functional states, one with a considerably higher affinity for intracellular Ca^{++} than the other (Scharff and Foder, 1993).

The molecular mechanisms underlying the rapid activation of the Na^+,K^+-pump are poorly understood. We need more information about how the Na^+,K^+-pump might be influenced by 3',5'cAMP, protein kinases, rapid changes in membrane potential or the opening of Na^+-channels. A model for the plasma membrane must include mechanisms for a rapid increase in the rate of active Na^+,K^+-transport that is independent of a rise in $[Na^+]_i$.

Implications of Activation of the Na^+,K^+-pump:

Due to the electrogenicity of the Na^+,K^+-pump, an acute activation leads to hyperpolarization. This would allow restoration of excitability in muscles exposed to high extracellular K^+ ($[K^+]_o$). Indeed, in isolated rat soleus muscles which had been inhibited by exposure to a $[K^+]_o$ of 10-12.5 mM, the addition of hormones inducing stimulation of the Na^+,K^+-pump was found to produce hyperpolarization and a marked force recovery. This force recovery was completely suppressed by ouabain and closely correlated to the concomitant stimulation of ^{86}Rb uptake and the decrease in intracellular Na^+ (Clausen et al., 1993). These experiments strongly suggest that during exercise, activation of the Na^+,K^+-pump by the elevated plasma level of catecholamines is important for the maintenance of excitability and contractile performance in skeletal muscle. The much more rapid activation of the Na^+,K^+-pump associated with excitation allows the muscle cells to restore excitability at the moment where this is most needed. Recently, it was shown that in the isolated rat soleus muscle, intensified electrical stimulation could prevent the inhibitory effect of high $[K^+]_o$ on force development (Clausen et al., 1996).

Contrary to earlier assumptions, the Na^+,K^+-pump is more than a slow mechanism for gradual compensation of excitation-induced rundown of Na^+,K^+-gradients. The new concept is that the Na^+,K^+-pump restores and maintains excitability by undergoing prompt and sometimes large-scale activation.

References

Andersen, S.L.V., and T. Clausen. 1993. Calcitonin gene-related peptide stimulates active Na^+-K^+ transport in rat soleus muscle, *Am. J. Physiol.* 264:C419-C429.

Ballanyi, K., and P. Grafe. 1988. Changes in intracellular ion activities induced by adrenaline in human and rat skeletal muscle, *Pflügers Arch.* 411:283-288.

Balog, E.M., and R.H. Fitts. 1996. Effects of fatiguing stimulation on intracellular Na^+ and K^+ in frog skeletal muscle, *J. Appl. Physiol.* 81:679-685.

Clausen, T. 1986. Regulation of active Na^+-K^+ transport in skeletal muscle, *Physiological Reviews* 66:542-580.

Clausen, T. 1996. Long- and short-term regulation of the Na^+-K^+ pump in skeletal muscle, *News in Physiol. Sci.* 11:24-30.

Clausen, T. 1997. The clinical and therapeutic significance of the Na^+,K^+-pump. Submitted for publication.

Clausen, T., S.L.V. Andersen, and J.A. Flatman. 1993. Na^+-K^+ pump stimulation elicits recovery of contractility in K^+-paralysed rat muscle, *J. Physiol.* 472:521-536.

Clausen, T., and J.A. Flatman. 1977. The effect of catecholamines on Na-K transport and membrane potential in rat soleus muscle, *J. Physiol.* 270:383-414.

Clausen, T., and O. Hansen. 1977. Active Na,K-transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes, *J. Physiol.* 270:415-430.

Clausen, T., and P.G. Kohn. 1977. The effect of insulin on the transport of sodium and potassium in rat soleus muscle, *J. Physiol.* 265:19-42.

Clausen, T., O.B. Nielsen, and L. Hilsted. 1996. Excitation-induced force recovery in K^+-inhibited muscle, *The Physiologist* 39:A58 (abstr.).

Desilets, M., and C.M. Baumgarten. 1986. Isoproterenol directly stimulates the Na^+-K^+ pump in isolated cardiac myocytes, *Am. J. Physiol.* 251:H218-H225.

Dørup, I., and T. Clausen. 1995. Insulin-like growth factor I stimulates active Na^+-K^+ transport in rat soleus muscle, *Am. J. Physiol.* 268:E849-E857.

Everts, M.E., and T. Clausen. 1994. Excitation-induced activation of the Na^+-K^+ pump in rat skeletal muscle, *Am. J. Physiol.* 266:C925-C934.

Ewart, H.S., and A. Klip. 1995. Hormonal regulation of the Na^+,K^+-ATPase: mechanisms underlying rapid and sustained changes in pump activity, *Am. J. Physiol.* 269:C295-C311.

Feraille, E., M.L. Carranza, M. Rousselot, and H. Favre. 1994. Insulin enhances sodium sensitivity of Na-K-ATPase in isolated rat proximal convoluted tubule, *Am. J. Physiol.* 267:F55-F62.

Juel, C. 1986. Potassium and sodium shifts during in vitro isometric muscle contraction, and the time course of the ion-gradient recovery, *Pflügers Arch.* 406:458-463.

Lear, S., B.J. Cohen, P. Silva, C. Lechene, and F.H. Epstein. 1992. cAMP activates the sodium pump in cultured cells of the elasmobranch rectal gland, *J. Am. Soc. Nephrol.* 2:1523-28.

Medbø, J.I., and O.M. Sejersted. 1990. Plasma potassium changes with high intensity exercise, *J. Physiol.* 421:105-122.

Nielsen, O.B., and T. Clausen. 1996. The significance of active Na^+,K^+ transport in the maintenance of contractility in rat skeletal muscle, *Acta Physiol. Scand.* 157:199-209.

Nielsen, O.B., and K. Overgaard. 1996. Ion gradients and contractility in skeletal muscle: the role of active Na^+,K^+ transport, *Acta Physiol. Scand.* 156:247-256.

Nielsen, O.B., and T. Clausen. 1997. Regulation of Na^+,K^+-pump activity in contracting rat muscle, *J. Physiol.* 503:571-581.

Scharff, O., and B. Foder. 1993. Regulation of cytosolic calcium in blood cells, *Physiol. Rev.* 73:547-582.

Relationships Between Protein Domains and Lipid Monolayers in Membrane Fusion

Fredric S. Cohen, Grigory B. Melikyan,
Judith M. White *and* Robert A. Lamb

Abstract

The hemagglutinin (HA) of influenza virus is the prototypic viral fusion protein. The possible roles of each domain of HA in fusion are presented. Specifically, it is proposed that the ectodomain causes hemifusion, the transmembrane domain causes fusion pore formation, and the cytoplasmic tail causes pore flickering. Lipids must also participate in fusion. Pores are created in stable hemifusion diaphragms by increasing the spontaneous curvature of inner monolayers of membranes to be more positive, but further increase in spontaneous curvature does not promote pore growth. In contrast, increasing spontaneous curvature of inner leaflets does promote pore enlargement for wild type HA, demonstrating that there is a lipid component to pore growth.

Fredric S. Cohen *and* Grigory B. Melikyan
Department of Molecular Biophysics and Physiology,
Rush Medical College
Chicago, IL 60612, USA
e-mail: fcohen@rpslmc.edu

Robert A. Lamb
Northwestern University,
Department of Biochemistry,
Molecular Biology and Cell Biology,
and Howard Hughes Medical Institute
Evanston, IL 60208, USA

Judith M. White
Department of Cell Biology,
University of Virginia,
Charlottesville, VA 22908, USA

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:159–165

Introduction

Biological membrane fusion occurs when two distinct membranes merge into one, allowing mixing of their constituent lipids and integral membrane proteins, and formerly separated aqueous compartments become joined, allowing mixing of their contents through a fusion pore. It has long been hypothesized that between membrane binding and initiation of a fusion pore is a key intermediate step, termed hemifusion (Palade, 1975). Hemifusion is defined as the continuity of initially

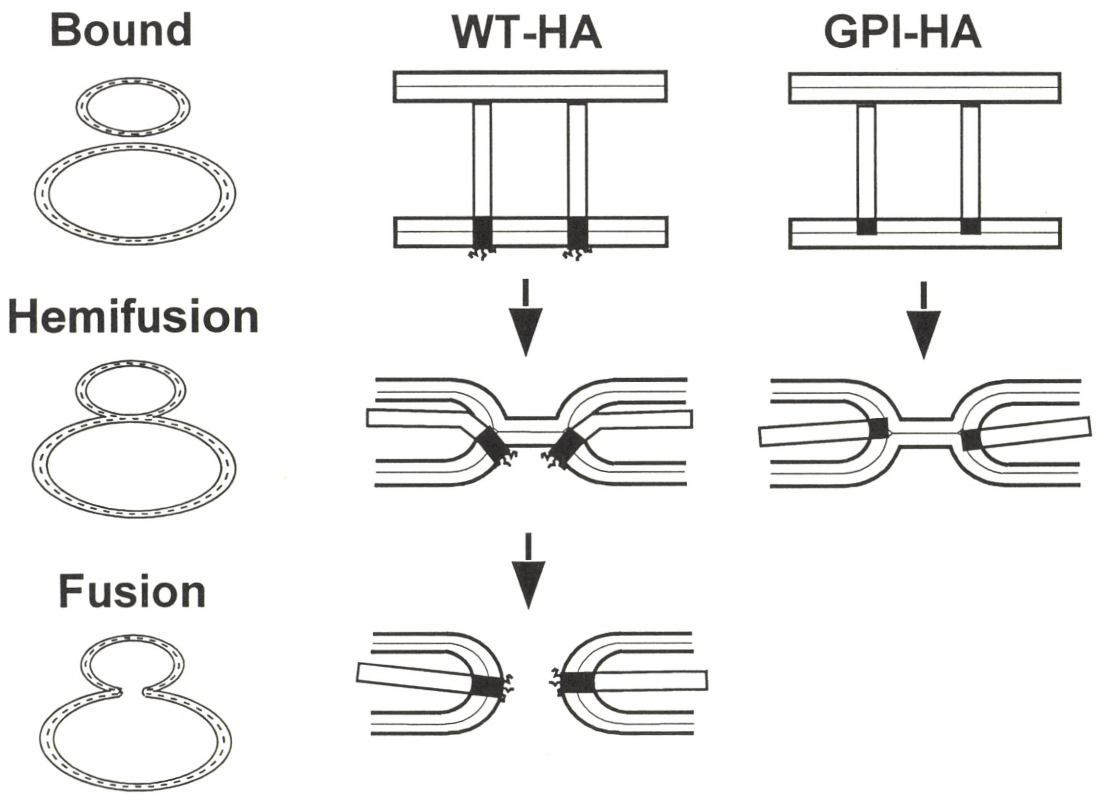


Figure 1. Schematic illustration of the membrane fusion process involving a stage of hemifusion.

contacting (for cellular fusion, outer) membrane monolayers, while noncontacting (inner) monolayers remain distinct but apposed and form what is called a hemifusion diaphragm (Fig. 1, left-hand panel). Hemagglutinin (HA) of influenza virus is the best characterized fusion protein (Wiley and Skehel, 1987; Bullough et al., 1994; Hernandez et al., 1996). In this paper, we review evidence from our laboratories to argue that separate domains of HA in particular, and possibly for fusion proteins in general, control sequential steps in fusion, including hemifusion.

HA is responsible for both binding and fusion. Influenza virus is internalized within cells by endocytosis and the low pH of the endosomes triggers fusion by causing conformational changes in HA (Gaudin et al., 1995). The fusion that occurs in this natural environment is modeled by expressing HA on cell surfaces, binding these cells to target membranes, and inducing fusion by lowering pH. HA is assembled from three identical monomers, each synthesized as a single polypeptide chain. Each monomer consists of about 550 amino acids (the precise number depends upon the strain) that can be divided into three domains: the ectodomain of some 515 amino acid residues, the transmembrane (TM) domain of some 27 residues, and the intraviral domain of 10-11 residues (Fig. 2). The intraviral domain is located in the cytoplasm when HA is expressed in cells, hence is referred to as the cytoplasmic tail (CT). The ectodomain is located in the extracellular space and HA-expressing cells interact extracellularly with their target membranes.

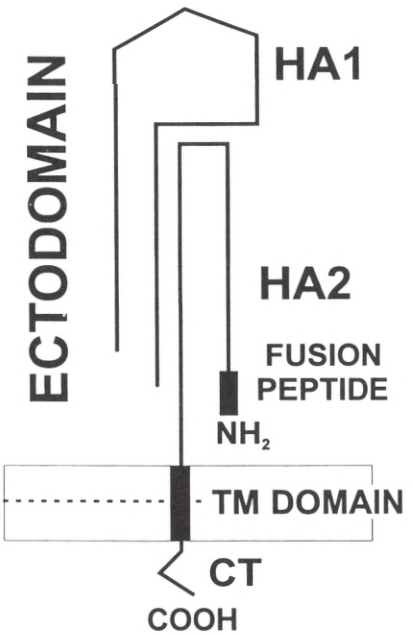


Figure 2. Hemagglutinin (HA) fusion protein of influenza virus.

The Ectodomain of HA Induces Hemifusion

The ectodomain of HA has been molecularly engineered to anchor to membranes by a glycosyl linkage to a phospholipid (i.e. it is GPI-coupled) rather than attached to a TM domain (Kemble et al., 1993). Using this GPI-HA construct, we showed that hemifusion resulted without the subsequent formation of fusion pores (Kemble et al., 1994; Melikyan et al., 1995). We conceive that the process of hemifusion occurs as follows: The two distinct outer membrane leaflets come into contact and merge, allowing their lipids to mix. The high curvature of the initial connecting structure (known as a "stalk") causes these merging leaflets to move outward from the point of contact. With these leaflets cleared from the former contact point, the inner leaflets are now able to appose each other, forming a new lipid bilayer membrane, referred to as a "hemifusion diaphragm." The aqueous compartments are now separated by only a single bilayer. Any integral membrane proteins remain in the undisturbed portion of their original membranes because the portion of the TM domain that spans an inner leaflet is not able to enter the inner leaflet of its hemifused partner; inner leaflets remain unaltered and distinct (Fig. 1). The extracellular portion and CT of these proteins continue to reside in their respective aqueous spaces. At this stage hemifusion is complete. From the standpoint of lipid rearrangement, all that remains in order for fusion to be accomplished is the formation of a pore in the hemifusion diaphragm (HD). Since the GPI-HA construct, which isolates the behavior of the ectodomain, shows the ectodomain of HA is capable of inducing hemifusion, we can expect that the ectodomain of wild type also induces hemifusion.

The CT of HA Controls Pore Flickering, but not Pore Formation

We know that the CT is not required for fusion: virus constructed to be void of the CT of HA (CT-minus) still produces fully infectious particles (Simpson and Lamb, 1992; Jin et al., 1994; 1996). Furthermore, cells expressing CT-minus fuse both to red blood cells (RBCs) and to planar membranes with the same kinetics as wild type HA (WT HA) (Melikyan et al., 1997b). Generally, when pores first form they flicker open and closed before fully opening, a common behavior of biological fusion pores . We have found that the CT controls flickering (Melikyan et al., 1997b): For CT-minus HA, pore flickering occurred twenty times less frequently than for WT. In WT HA, the CT is palmitoylated on conserved cysteines. Palmitoyls on the CT of HA are essential for significant amounts of pore flickering: mutating these cysteines to prevent palmitoylation also greatly reduced the degree of pore flickering. In other words, a palmitoylated CT promotes pore closing, but does not affect pore opening. Since the CT does not aid fusion and the ectodomain only yields hemifusion, the TM domain must be essential for full fusion.

The Linkage Model of Fusion

What would account for the observation that the GPI-HA construct terminates in stable hemifusion while HA with an intact TM domain causes full fusion? It is known that upon exposure to low pH, the ectodomain of each monomer of HA dramatically reconfigures. In the case of WT HA, the massive conformational changes of the ectodomain in response to low pH should cause the small TM domain to which it is attached to move. Because HA should be surrounding the rim of the HD it creates, we conjecture that ectodomain movement pushes the TM domain into the HD as it is forming. Any forced insertion of the TM domain would disturb the HD: the TM domain would no longer be in its natural membrane-spanning orientation. The stresses created would be relieved if the lipids reconfigured around the TM domain into a new single bilayer, thereby reestablishing the TM domain's energetically favored orientation. For this to occur, a fusion pore would have to form (Fig. 1, WT-HA). Because the central conjecture of this model is that conformational changes of the ectodomain are linked to movements of the TM domain, we refer to it as the "linkage model" (Melikyan et al., 1995) In contrast to WT HA, for GPI-HA the lipid anchoring the ectodomain is free to move throughout the continuous outer leaflets upon hemifusion, thus the HD is not disrupted and fusion pores do not form (Fig. 1, GPI-HA, right-hand panels).

The linkage model has a common-sense logic in that function of each HA domain follows naturally from topology: in terms of cellular membranes, fusion proceeds outside-to-inside sequentially; each HA domain acts on the portion of the membrane to which it is in immediate proximity – the ectodomain merges outer leaflets, the TM domain spans and disrupts inner leaflets, and the CT, inside the cell, acts only after initial pore formation. Moreover, since we know the TM domain is linked to the ectodomain and that the ectodomain is moving extensively, it would seem imperative that the TM domain must also be moving to some degree; the TM domain is positioned just outside the HD, in the precise location one would expect for a structure that would be disrupting an HD.

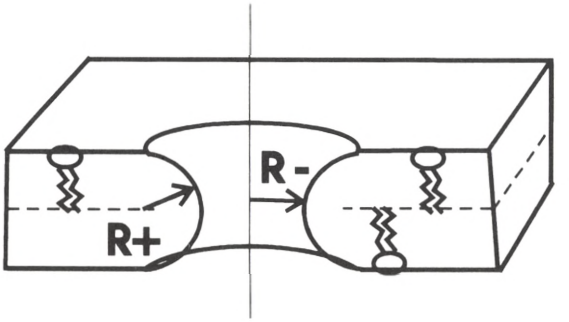


Figure 3. A lipidic pore has both negative (R_-) and positive (R_+) curvature.

Increasing Positive Spontaneous Curvatures of Inner Leaflets Promotes Pore Formation within Hemifusion Diaphragms

In biological fusion, the role of the lipids as well as the proteins must be considered: at a minimum there is a change in their configuration as two bilayers merge into one. The manner by which lipids transiently leave their bilayer configuration is central to the fusion process. Any rearrangements that lipids undergo have to involve elastic energy which is required to bend the lipids out of the curvature of their original leaflets (Helfrich, 1973). A lipidic pore within an HD has a positive and negative curvature (Fig. 3). The positive curvature is expected to dominate, based on a straightforward application of surface geometry (Kozlov et al., 1989). Several laboratories have studied the consequences of altering spontaneous curvature of outer leaflets in protein-mediated fusion systems (Günter-Ausborn et al., 1995; Chernomordik et al., 1995; Shangguan et al., 1996), but because of their inaccessibility, inner leaflets – of which an HD is comprised – had not been studied in this manner. We surmounted this problem by adding, to solution, membrane permeable cationic agents (MPCAs) that are surface active. In this simple manner we were able to gain access to inner leaflets. These positively-charged micelle-forming agents preferentially insert into inner leaflets (Sheetz and Singer, 1974; Steck, 1989) (because inner leaflets are more negatively charged than outer ones) and promote formation of positive curvature structures (Hornby and Cullis, 1981).

GPI-HA-cells were hemifused to RBCs. The effect of adding MPCAs was the creation of pores that were highly targeted to the HD. We showed that fusion was caused by the positive spontaneous curvature agents acting directly upon inner leaflets (Melikyan et al., 1997a). Because the HD should be devoid of integral membrane proteins, the MPCAs could promote pore formation within lipid bilayers by a mechanism similar to that in an HD. In fact, we showed that the ability of MPCAs to bend into a positive curvature in planar bilayers directly paralled their ability to induce full fusion from a state of hemifusion. If in fact a fusion pore is a basically lipidic structure, then it is reasonable to conjecture that the TM domain of HA may induce pore formation through control of the spontaneous curvature of lipids of inner leaflets.

Positive Spontaneous Curvature of Inner Leaflets Promotes the Growth of Pores Induced by WT HA

We also showed that increasing the positive spontaneous curvature of inner leaflets promotes enlargement of pores formed by the intact WT HA. By keeping pH too high or temperature too low (i.e. "suboptimal" fusogenic conditions), we were able to induce lipid continuity, measured by spread of a fluorescent lipid dye from RBCs to HA-cells, without spread of aqueous dye loaded into the RBCs. We refer to this pattern of dye spread with HA-cells as "stunted fusion." This pattern could be due to hemifusion, or due to small pores that did not enlarge. We have shown by dye transfer experiments that stunted fusion is distinct from stable hemifusion: MPCAs promoted transfer of aqueous dye in stunted fusion with almost an order of magnitude lower concentration than was required for the same result with GPI-HA cells. We also showed that when MPCAs were added to GPI-HA cells that were hemifused to RBCs, more pores formed with increasing concentration of MPCA, but the average pore size did not increase. In contrast, for stunted fusion the average size of pores (perhaps formed by the MPCA) gradually enlarged as the concentration of MPCA was increased. These results indicate that pore growth is not only under control of the fusion protein, but under lipid control as well. The more positive the spontaneous curvature of the inner leaflet, the more readily a pore enlarges. Thus, these MPCA experiments combined with those using GPI-HA provide evidence for the concept that the two lipid leaflets perform different roles in the fusion process: outer leaflets are responsible for hemifusion; inner leaflets control pore formation and enlargement.

References

Bullough, P.A., F.M. Hughson, J.J. Skehel, and D.C. Wiley. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371:37-43.

Chernomordik, L.V., M.M. Kozlov, and J. Zimmerberg. 1995. Lipids in biological membrane fusion. *J. Membr. Biol.* 146:1-14.

Gaudin, Y., R.W.H. Ruigrok, and J. Brunner. 1995. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. *J. Gen. Virol.* 76:1541-1556.

Günter-Ausborn, S., A. Praetor, and T. Stegmann. 1995. Inhibition of influenza-induced membrane fusion by lysophosphatidylcholine. *J. Biol. Chem.* 270:29279-29285.

Helfrich, W. 1973. Elastic properties of lipid bilayers: Theory and possible experiments. *Zeitschrift fur Naturforschung* 28:693-703.

Hernandez, L.D., L.R. Hoffrman, T.G. Wolfsberg, and J.M. White. 1996. Virus-cell and cell-cell fusion. *Annu. Rev. Cell Dev. Biol.* 12: 627-661.

Hornby, A.P. and P.R. Cullis. 1981. Influence of local and neutral anaesthetics on the polymorphic phase preferences of egg yolk phosphatidylethanolamine. *Biochim. Biophys. Acta* 647:285-292.

Jin, H., G.P. Leser, and R.A. Lamb. 1994. The influenza virus hemagglutinin cytoplasmic tail is not essential for virus assembly or infectivity. *EMBO J.* 13: 5504-5515.

Jin, H., K. Subbarao, S. Bagai, G.P. Leser, B.R. Murphy, and R.A. Lamb. 1996. Palmitylation of influenza virus hemagglutinin (H3) is not essential for virus assembly or infectivity. *J. Virol.* 70:1406-1414.

Kemble, G.W., Y.I. Henis, J.M. White. 1993. GPI- and Transmembrane-Anchored Influenza Hemagglutinin Differ in Structure and Receptor Binding Activity. *J. Cell Biol.* 122:1253-1265.

Kemble, G.W., T. Danieli, and J.M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* 76:383-391.

Kozlov, M.M., S.L. Leikin, L.V. Chernomordik, V.S. Markin, Yu.A. Chizmadzhev. 1989. Stalk mechanism of membrane fusion. *Eur. Biophys. J.* 17:121-129.

Melikyan, G.B., J.M. White, and F.S. Cohen. 1995. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* 131:679-691.

Melikyan, G.B., S.A. Brener, D.C. Ok, and F.S. Cohen. 1997a. Inner, but outer, membrane leaflets control the transition from glycosylphosphatidylinosiiol-anchored influenza hemagglutinin-induced hemifusion to full fusion. *J. Cell Biol.* 136:995-1005.

Melikyan, G.B., H. Jin, R.A. Lamb, and F.S. Cohen. 1997b. The role of the cytoplasmic tail region of HA in formation and growth of fusion pores. *Virology* 235:118-128.

Palade, G.E. 1975. Intracellular aspects of the process of protein synthesis. *Science* 189: 347-358.

Simpson, D.A. and R.A. Lamb. 1992. Alterations to influenza virus hemagglutinin cytoplasmic tail modulate virus infectivity. *J. Virol.* 66:790-803.

Shangguan T.D., D. Alford, and J. Bentz. 1996. Influenza virus-liposome lipid mixing is leaky and largely insensitive to the material properites of the target membrane. *Biochemistry* 35:4956-4965.

Sheetz, M.P. and S.J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA* 71:4457-4461.

Steck, T.L. 1989. Red cell shape. *In:* Cell Shape: Determinants, Regulation and Regulatory Role, pgs. 205-246. Eds. Stein, W.D. and Bronner, F. Academic Press, Inc., San Diego, CA.

Wiley, D.C. and J.J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Ann. Rev. Biochem.* 56:365-394.

Wilson, I.A., J.J. Skehel, and D.C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289:366-373.

Transmembrane Lipid Trafficking: Facts and Speculations

Philippe F. Devaux

Abstract

The transmembrane distribution of phospholipids in eukaryotic cells is under the control of specific proteins or "flippases". These proteins either permit the rapid equilibration of phospholipids between both bilayer halves or accumulate specific lipids on one side and, hence, create lipid domains. The role of flippases during blood coagulation and apoptosis is well established. At a more speculative level it can be inferred that flippases, because they can generate membrane invaginations, are implicated in endocytosis. Finally, by modulating the surface tension, they could regulate the activity of other membrane proteins.

Philippe F. Devaux
Institut de Biologie Physico-Chimique
F-75005 Paris, France
e-mail: devaux@ibpc.fr

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:167–173

Introduction

All biological membranes contain a mixture of many different lipids. For example, it has been estimated that about 400 chemically different lipids coexist in the erythrocyte membrane. Although functional membranes are in a fluid state which *a priori* favours lipid mixing, there are many experiments which prove the existence of transverse and lateral lipid segregations in biological membranes, most likely due to lipid-protein interactions. The life-time, size and origin of lateral domains in fluid biomembranes are often matters of controversies. By contrast, stable transmembrane segregation of lipids in the plasma membrane of eukaryotic cells is a well established fact that has been carefully evaluated. Briefly, aminophospholipids (phosphatidylserine, PS and phosphatidylethanolamine, PE) as well as phosphoinositides are essentially located in the inner monolayer while the choline-containing phospholipids (phosphatidylcholine, PC and sphingomyeline, SM) as well as glycolipids are mainly located in the outer monolayer.

This asymmetry, first discovered in human erythrocytes, seems to be a ubiquitous property of animal cells. Inner membranes, as well, are probably asymmetrical although the evidence are less

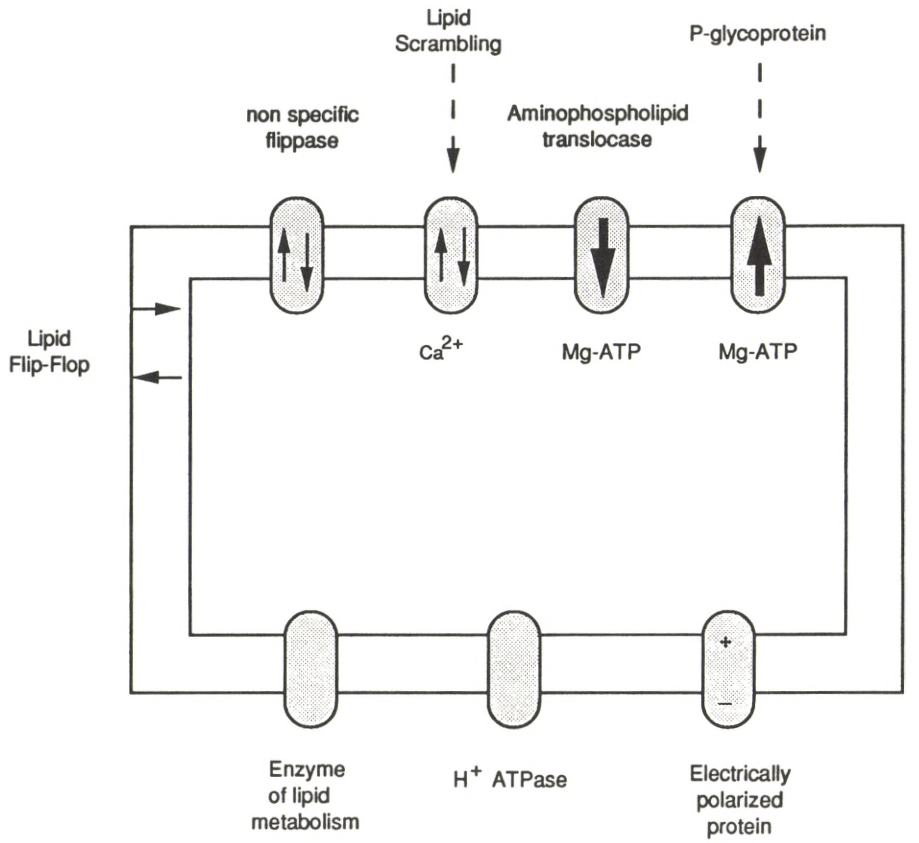


Figure 1. Schematic representation of the various proteins involved in transmembrane orientation of lipids.

compelling. Sonication of a lipid mixture with different head groups gives rise to asymmetrical bilayers because of the high curvature of small vesicles. However, transverse lipid segregation in biological systems is essentially due to the activity of proteins, some of which are enzyme involved in the synthesis of phospholipids, other proteins called "flippases" are transport-proteins that catalyze the exchange of lipids in both directions or transport lipids selectively from one leaflet to the other. In the latter case, ATP hydrolysis is required as in the case of ion carriers since such a process maintains a membrane in an out-of-equilibrium situation. Note that the stability of the asymmetrical distribution of phospholipids is facilitated by the very slow spontaneous flip-flop of phospholipids which is of the order of several hours or even several days (Devaux, 1991; Devaux, 1993; Devaux and Zachowski, 1994; Williamson and Schlegel, 1994; Diaz and Schroit, 1996).

Figure 1 is a schematic representation of the various type of "flippases" which have been reported in the literature. They comprise proteins that accumulate specific lipids and proteins that relax an asymmetrical situation by allowing the rapid exchange of lipids. Unfortunately only a few

of the transport proteins whose existence has been postulated, were purified to homogeneity (Tang et al., 1996) and none of them have been characterized structurally. Nevertheless, successful reconstitution experiments have been performed (Auland et al., 1994; Comfurius et al., 1996). References giving the main data concerning these proteins can be found in the reference list below. The objective of the present communication is not to discuss the experimental evidence of flippases proteins but rather to summarize the biological implications of these transmembrane movements.

Consequences of in Vivo Transmembrane Lipid Traffic

Lipid translocation and cellular traffic

In several cases it is clear that the translocation of lipids permits their subsequent transport to a different membrane within the cell. For example, PC is synthesized on the lumenal side of the ER, it then flips rapidly to the cytosolic face from where it can be shuttled to the plasma membrane or to the mitochondria by a PC exchange protein. Other lipids are accumulated by an active translocase on the external side of the plasma membrane in order to be exported out of a cell. PC in particular is extracted from the outer monolayer of the canicular membrane by bile salts. In the case of multidrug resistance, it is generally assumed that the amphiphilic character of the drugs transported by the P-gp from the inner to the outer monolayer of the plasma membrane implies automatically their solubilisation in the blood stream.

Lipid translocation and asymmetry

Does the existence of lipid pumps, such as the aminophospholipid translocase, suffice to explain the stable asymmetrical distribution of phospholipids in plasma membrane of eukaryotes? This is often assumed implicitly. Yet, there is a serious problem because the outward transport of choline containing lipids (PC and SM), if it exist, is much less efficient than the inward transport of PS and PE. There are several reports of an outward transport of PC due to proteins of the MDR family in specialized membranes like canalicular membranes (Ruez and Gros, 1994; van Helvoort et al., 1996). There is also indication that PC outward transport in red cells is faster than the steady state inward transport of the same lipid, however, the efficiency of this outward flux of PC is much slower than that of the inward flux of PS and PE. A consequence is that if lipids with a totally random distribution are sorted between the two bilayer halves with the machinery existing in red cells, the membrane will vesiculate and probably collapse. This phenomenon which is related to the "bilayer coupled effect" (Sheetz and Singer, 1974) can be demonstrated by insertion of a small percentage of amphiphilic drugs in the outer or inner monolayers of red cells. Similarly manipulation of the transmembrane distribution of lipids in liposomes causes important shape changes (Farge and Devaux, 1992; Mathivet et al., 1996). However *in vivo* the membrane asymmetry is not generated from a random distribution of lipids. Membranes of a new cell come from the division of a mother cell containing already asymmetrical membranes. In other words, asymmetry has to be reajusted progressively as new lipids are synthesized. In the latter case a difference in the kinetics of inward and outward movements may be acceptable. Moreover part of the asymmetry comes from the transmembrane orientation of the enzymes that synthesize the lipids in organelles, not in the plasma membrane. For example, it is admitted that glycosphingolipids are synthesized on the lumenal leaflet of Golgi membranes, from where they are transported to the plasma membrane by fusion of vesicles derived from the Golgi. Thus, glycosphingolipids are directed to the plasma membrane with

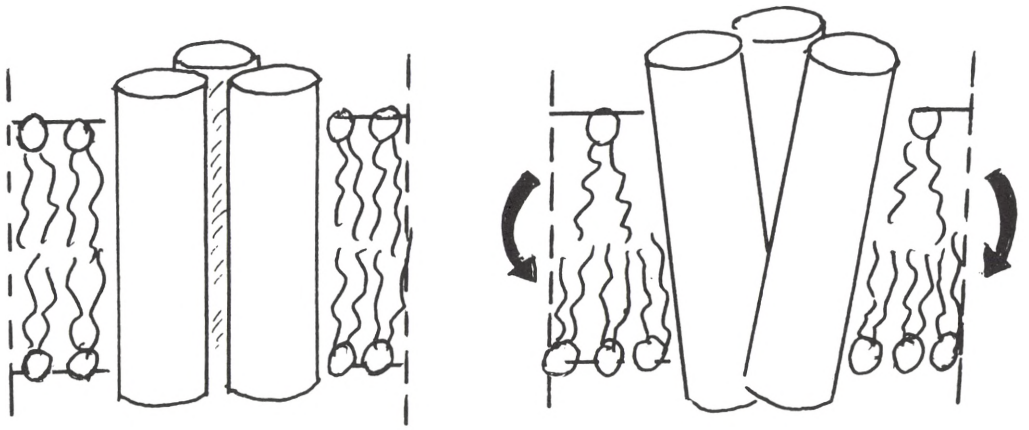
the proper transmembrane orientation. This requires the free diffusion of the precursor lipids in the Golgi membrane (Buton et al., 1997). In conclusion it is perhaps not necessary to have exact compensation of inward and outward phospholipid transports by lipid-translocators as long as the membrane is never far from its steady state lipid distribution.

Asymmetric Membranes: some Biological Advantages

The difference in lipid composition between each monolayers is in fact necessitated by the asymmetrical role of all cell membranes. For example the outer surface of the plasma membrane of an eukaryotic cell contains specific receptors sites, among which are glycolipids, but it should not be charged otherwise it would trap many plasma proteins. On the contrary, the inner surface contains charged lipids such as PS and PA (phosphatidic acid) to which cytosolic extrinsic proteins (G proteins, annexins) can bind but there is no glycolipids. During the cell life-time a change in the transmembrane distribution can take place to trigger a new biological event. For example, lipid scrambling in aged cells or apoptotic cells permits PS exposure on the outer monolayer and, hence, the recognition of the senescent red cells by PS receptors on macrophages. Similarly stimulation of platelets triggers the passage of PS towards the outer monolayer of platelet membranes and provides plasma prothrombinase with a PS interface needed for the conversion of prothrombin into thrombin (Williamson and Schlegel, 1994; Diaz and Schroit, 1996).

Lipid Translocation and Membrane Bending

In other cells, there is an intense flippase activity which is not justified *a priori* by the export or import of lipids. In the latter case, the asymmetrical transfer of lipids between bilayer leaflets could be used for membrane bending, a process which has been studied in liposomes (Farge and Devaux, 1992). I proposed several years ago that the lipid pumps could be responsible for the formation of membrane invaginations and, hence, be directly associated with the endocytic process (Devaux and Zachowski, 1994). Several reports indicate that, indeed, the aminophospholipid translocase activity is high in cells where there is a high endocytic activity. Recent work by Farge has shown that increasing artificially the concentration of PS, i.e. the translocase substrate, results in increased endocytosis rates in K562 cells (Farge, 1995). Note that an active outward transport of PC or SM from inside to outside would lead to the loss of membrane by shedding of vesicles in the plasma, an event which generally does not happen except in very special cases. Stimulated platelets do shed vesicles: it could be due to an active outward transport of lipids in the platelet plasma membrane.



Lipid Translocation and Surface Tension

The formation of an asymmetrical membrane with an excess of lipids on one of the monolayers (or with lipids with larger head groups) can cause another physical modification than membrane bending. Indeed, a mismatch between each monolayer creates surface tension which is detectable when the spontaneous curvature associated with the difference in lipid density of the two monolayers differs significantly from the actual curvature imposed in particular by the closure of the vesicle. That is often the case with small vesicles for which the curvature is high (Farge and Devaux, 1993). Increase in surface tension can inhibit surface undulations which constitute a repulsive force between two bilayers in close vicinity. In the absence of undulations (and of repulsive electrostatic interactions) two bilayers attract themselves by van der Waals interactions. The effect of surface tension on the aggregation of liposomes has indeed been observed (Mathivet et al., 1996). This process could be important in the preliminary step of membrane fusion *in vivo*. We can speculate also about the possible role of surface tension in the modulation of protein transconformation or as a modulator of membrane viscosity.

An Hypothetical Regulation Procedure

From the above remarks it is clear that a strict regulation of outward and inward lipid traffic is necessary to avoid unwanted membrane invaginations and/or surface tension, at least it is necessary to control the magnitude of these physical events which accompany the reorientation of lipids. As more lipid translocators are discovered, it may seem more difficult to conceive a general regulation procedure. Yet, a simple way of control could be the inhibition of the lipid translocators by the surface tension itself, see Figure 2. This could explain the difficulty that we have experienced in trying to obtain proteoliposomes with a translocase activity in LUVs with a diameter of approximately 100 nm (Auland et al., 1994). Related experiments were carried out by Cullis and his collaborators who showed that it is possible to translocate specific phospholipids through LUVs' membranes which were submitted to a transmembrane pH gradient. They showed that in order to

translocate 10% phospholipids (such as phosphatidic acid or phosphatidylglycerol) from one leaflet to the other, the LUVs had to be incubated at 60°C for half an hour whereas the translocation of a small percentage (1%) can be achieved at 20°C in a few minutes (Mui et al., 1995). This saturation effect can be explained by the building of a surface tension. Thus, lipid flipping by proteins could be regulated by the surface tension.

Conclusions

Transmembrane asymmetry forms stable separated domains of lipids in membranes. The exchange of lipids between the two pools are under the control of proteins that can accumulate against a gradient or open energy barriers to let the lipid flow. The transfer of lipids between the two pools can be a process facilitating the traffic of lipids within the cell, in particular from one membrane to the other, but it can also be a way to modify the properties of a membrane or to signal to the outside world a change within the cell. Thus, the lipid asymmetry can serve several purposes. It then becomes apparent that having a significant variety of lipids offers the possibility to use this mechanism to communicate different messages or fulfil different functions by controlling the flip-flop of specific phospholipids. Some of the ideas put forward here implicate that the translocation of lipids could be more important for eukaryotic cells than for prokaryotic cells. Therefore one is tempted to suggest that the development of flippases proteins was an important step between prokaryotes and eukaryotes.

***

Work supported by grants from the Centre National de la Recherche Scientifique (UPR 9052) and the Université Paris 7.

References

Auland, M.E., B.D. Roufogalis, P.F. Devaux and A. Zachowski. 1994. Reconstitution of ATP-dependent aminophospholipid translocation in proteoliposomes. *Proc. Natl. Acad. Sci. USA* 91:10938-10942.

Buton, X., K.N.G. Burger, P. Fellmann, P.F. Devaux and M. Seigneuret. 1997. Transbilayer movement of monohexosyl sphingolipids in endoplasmic reticulum and Golgi membranes. Submitted.

Comfurius, P., P. Williamson, E.F. Smees, R.A. Schlegel, E.M. Bevers and R.F.A. Zwaal. 1996. Reconstitution of phospholpid scramblase activity from human blood platelets. *Biochemistry* 35:7631-7634.

Devaux, P.F. 1991. Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* 30:1163-1173.

Devaux, P.F. 1993. Lipid transmembrane asymmetry and flip-flop in biological membranes and in lipid bilayers. *Cur. Opin. Struct. Biol.* 3:489-494.

Devaux, P.F. and A. Zachowski. 1994. Maintenance and consequences of membrane phospholipid asymmetry. *Chem. Phys. Lipids* 73:107-120.

Diaz, C. and A.J. Schroit. 1996. Role of translocases in the generation of phosphatidylserine asymmetry. *J. Membrane Biol.* 151: 1-9

Farge, E. and P.F. Devaux. 1992. Shape change of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids. *Biophys. J.* 61:347-357.

Farge, E. and P.F. Devaux. 1993. Size dependant response of liposomes to phospholipid transmembrane redistribution: from shape change to induced tension. *J. Phys. Chem.* 97:2958-2961.

Farge, E. 1995. Increased vesicles endocytosis due to an increase in the plasma membrane phosphatidylcholine concentration. *Biophys. J.* 69:2501-2506.

Mathivet, L., S. Cribier and P.F. Devaux. 1996. Shape change and physical properties of giant phospholipid vesicles prepared in the presence of an electric field. *Biophys J.* 70:1112-1121.

Mui, B.L.S., H.G. Dobereiner, T.D. Madden and P.R. Cullis. 1995. Influence of transbilayer area asymmetry on the morphology of large unilamellar vesicles. *Biophys. J.* 69:930-941.

Ruetz, S. and P. Gros. 1994. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell* 77:1071-1081.

Sheetz, M.P. and S.J. Singer. 1974. Biological membranes a bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Nal. Acad. Sci. USA* 71:4457-4461.

Tang, X., M.S. Halleck, R.A. Schlegel and P. Williamson. 1996. A novel subfamily of P-type ATPase with aminophospholipid transporting activity. *Science* 272:1495-1497.

van Helvoort, A., A.J. Smith, H. Sprong, I. Fritzsche, A.H. Schinkel, P. Borst and G. van Meer. 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* 87:507-512.

Williamson, P. and R.A. Shlegel. 1994. Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. *Mol. Memb. Biol.* 11:199-216.

Membrane Negative Spontaneous Curvature as an Ancient Signal for Cell Growth

Paavo K.J. Kinnunen

Abstract

Biomembranes have been described as platforms which integrate and control entire metabolic pathways in a cooperative manner, the activities of the proteins being dependent on the physical (phase) state of the given membrane, the latter further being controlled by the liquid crystalline properties of the constituent lipids. Accordingly, there is a correlation between the physiological state of the cell (or cell organelle in a eukaryote) and the physical state of the membrane (Kinnunen et al., 1994; Kinnunen, 1996a). A good example is provided by the control of growth in prokaryotes and eukaryotes (Kinnunen, 1996b). In brief, a unifying feature in rapidly growing cells appears to be the increase in the content of lipids which in isolation form inverted non-lamellar phases (INLs). On a mechanistic level the activities of membrane proteins in these cells can be controlled by the lateral pressure profile which in addition to possible direct effects on the conformation of integral proteins can also induce the attachment of peripheral membrane proteins by the so-called extended lipid anchorage. Similarly, INLs may also induce peripheral, secondary interactions for integral membrane proteins.

Paavo K.J. Kinnunen
Department of Medical Chemistry
Institute of Biomedicine
University of Helsinki, POB 8
Helsinki, FIN-00014 Finland
email: Paavo.Kinnunen@Helsinki.Fi

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:175–178

Inverted Non-lamellar (INL) Phases

Both prokaryote and eukaryote cells contain in their membranes substantial amounts of lipids forming, in isolation and at proper conditions (e.g. temperature, presence of ions, protons, proteins, and impurities) inverted non-lamellar phases (INL), such as hexagonal phase HII and inverted cubic phases (Tate et al., 1991). Notable examples of INLs are phosphatidylethanolamine (PE), in particular its plasmalogen form, diacylglycerol (DAG) containing unsaturated acyl chains, and cardiolipin, the latter in the presence of protons or divalent cations. We will here refer to these lipids as INLs. These lipids have a smaller polar head group when compared to the relative size of their

hydrophobic part and, accordingly, at the free energy minimum they spontaneously adopt 3-D structures with negative curvature. When present in sufficiently small quantities in mixed membranes with lipids forming lamellar phases, INLs increase the tendency of the bilayer for adopting negative curvature, even though the membrane remains lamellar. Such membranes are frustrated and there is increasing packing strain towards the interior of the hydrocarbon phase of the bilayer. In contrast, the packing closer to the bilayer surface becomes more loose. In other words, compared to a relaxed bilayer, the free volume distribution within the bilayer has changed. The magnitude of the pressure difference in the interior of the bilayer and the surface can be considerable (Cantor, 1997). A parameter Q can be calculated, giving the free energy difference for the membrane in frustrated and relaxed states, i.e. in lamellar and non-lamellar states, the latter corresponding to the organization after adoption of the spontaneous negative curvature.

INLs and Cellular Growth

In eukaryotes the contents of INLs vary in different tissues as well as in different organelles. Likewise, their content varies in the cell cycle (for recent review, see Kinnunen, 1996b). More specifically, the content of PE increases towards the end of the G1 phase of cell cycle, preceeding the entry of the cell into the S-phase, where the duplication of the cellular macromolecular machineries, such as replication of DNA, takes place. DAG is used in eukaryotes as a lipidic second messenger for cellular proliferation, and is generated from both phosphatidylcholine (PC) and -inositol (PI) by specific phospholipases C (PLC) which become activated in signalling cascades following the activation of plasma membrane growth factor receptors by their agonists. Increasing contents of DAG are present also in ras-transformed cells. In mutants at restrictive temperatures, the contents of DAG remain low but increase at permissive temperatures. Overexpression of PC-PLC results in the transformed phenotype. Notably, increasing the content of DAG in cells by choline deficiency results in epigenetic transformation, i.e. in the absence of mutation. Accordingly, there is strong evidence linking both normal as well as malignant growth of eukaryotes to increasing contents of INL and thus an increase in Q.

As demonstrated by Lindblom and his coworkers (e.g. Lindblom and Rilfors, 1989) optimal growth conditions for the mycobacterium *Acholeplasma laidlawii* require their plasma membrane to contain INL, i.e. to have a proper negative spontaneous curvature and thus proper value for Q. This may be achieved at appropriate temperature, ions, or proper lipids or membrane-partitioning additives. Likewise, recent data for *E. coli* indicate that no specific lipid structure such as PE is necessary but membrane negative spontaneous curvature in general is needed (see Kinnunen, 1996b, for a review). Correlation between lipid packing and the activity of one of the rate-limiting enzymes of lipid synthesis in *A. laidlawii* has recently been demonstrated *in vitro* (Karlsson et al., 1996) and is discussed in more detail by Wieslander in the present volume.

To conclude, it is argued that INLs and more specifically their manifestation in a particular physical property of the membrane, given by Q, represent an ancient signal activating cellular growth both in eukaryotes and prokaryotes. Yet, exceeding growth-promoting values for Q causes destabilization of the membrane and ultimately their transition into non-lamellar phases, thus resulting in cell death. Efficient feedback mechanisms (synthesis and degradation) are required for maintaining the correct membrane lipid composition yielding under given conditions (e.g. temperature, pressure, presence of membrane partitioning 'impurities'), both membrane stability as well as correct signalling for growth.

To this end, it important to notice that Q can be regulated independently in the different or-

ganelle membranes of eukaryote cells and thus can be used to activate or inactivate specific metabolic pathways independently. Yet, in the S-phase Q should increase in all organelles harboring machineries needed for replication. Aberrant control of the content of INL will result in malignant transformation and cancer.

INLs and Lipid-Protein Interactions

In both eukaryotes as well as prokaryotes, INLs activate enzymes needed for replication as well as acting in cascades promoting growth (Kinnunen, 1996a). Prominent examples of peripheral proteins activated by INLs are protein kinase, CTP:phosphocholine cytidyl transferase, dnaA protein, and cytochrome c. The molecular-level mechanisms have remained poorly understood. We have propagated the concept of INL causing, due to the strain in the membrane, anchoring of proteins to lipid surfaces by so-called extended lipid anchorage (Kinnunen et al., 1994; Rytömaa and Kinnunen, 1995). In this mechanism, one of the acyl chains of a lipid becomes intercalated within a hydrophobic cavity of a protein, while the other chain remains in the bilayer. Accordingly, there is reduction in Q and hydrophobic lipid-protein interaction is established in the absence of penetration of the protein into the membrane. The extent of this interaction is regulated by factors controlling the overall magnitude of Q of the membrane and may further be linked to specific lipid headgroup-proteins as well as specific lipid acyl chain-protein interactions. In this regard, the role of hydroxy-fatty acids and eicosanoids is of particular interest.

Towards the Functioning Biomembrane

The modern view of biomembranes emphasizes coupling between their organization and function (Mouritsen and Kinnunen, 1996). The supramolecular membrane assemblies of proteins and lipids exist on different length- and timescales and represent both:

(i) spontaneously forming self-organizing assemblies due to intermolecular forces at thermodynamic equilibrium as well as

(ii) dissipative non-equilibrium structures, maintained by energy input.

These organizates are highly dynamic and apt to regulation by a number of membrane binding ligands such as hormones and growth factors, metabolites, ions, pH, drugs, proteins, as well as membrane potential, osmotic forces, pressure, temperature, and hydration (Kinnunen, 1991). As an inherent feature of all liquid crystalline materials is their ability to undergo phase changes, and accompanying these phase changes are alterations in the physical state of the membrane. Inherent to phase transitions are also changes in the lateral distribution of the membrane constituents, as recently demonstrated (Jutila and Kinnunen, 1997).

The central issue advocated by the present author is that life has adopted these phase changes for the regulation of both metabolism as well as replication of cells, with phase transitions in the membranes and the intracellular polymers corresponding to changes in the physiological states of the cell, such as those represented by apoptosis and the distinct phases in the cell cycle.

References

Cantor, R.S. 1997. Lateral pressures in cell membranes: a mechanism for modulation of protein function. *J. Phys. Chem.* 101: 1723-1726.

Jutila, A., and P.K.J. Kinnunen. 1997. Novel features of the main transition of dimyristoylphosphatidylcholine bilayers revealed by fluorescence spectroscopy. *J. Phys. Chem.* 101: 7635-7640.

Karlsson, O., M. Rytömaa, A. Dahlqvist, P.K.J. Kinnunen, and Å. Wieslander. 1996. Correlation between bilayer lipid dynamics and activity of the diglucosyldiacylglycerol synthase from Acholeplasma laidlawii membranes. *Biochemistry* 35: 10094-10102.

Kinnunen, P.K.J. 1991. On the principles of functional ordering in biological membranes. *Chem. Phys. Lipids* 57: 375-399.

Kinnunen, P.K.J. 1996a. On the molecular level mechanisms of peripheral protein/membrane interactions induced by lipids forming non-lamellar phases. *Chem. Phys. Lipids* 81: 151-166.

Kinnunen, P.K.J. 1996b. On the mechanisms of the lamellar→hexagonal HII phase transition and the biological significance of the HII propensity. *In:* Nonmedical Applications of Liposomes. Lasic D.D. and Y. Barenholz, editors. CRC Press, Boca Raton, FL. 153-171.

Kinnunen, P.K.J., A. Koiv, J.Y.A. Lehtonen, M. Rytömaa, and P. Mustonen. 1994. Lipid dynamics and peripheral interactions of proteins with membrane surfaces. *Chem. Phys. Lipids* 73: 181-207.

Lindblom, G., and L. Rilfors. 1989. Cubic phases and isotropic structures formed by membrane lipids - possible biological relevance. *Biochim. Biophys. Acta* 988: 222-256.

Mouritsen O.G., and P.K.J. Kinnunen. 1996. Role of lipid organization and dynamics for membrane functionality. *In:* Biological Membranes. A Molecular Perspective from Computation and Experiment. Merz, K.M. and B. Roux, editors. Birkhäuser. 465-504.

Rytömaa M., and P.K.J. Kinnunen. 1995. Reversibility of the binding of cytochrome c to liposomes. Implications for lipid-protein interactions. *J. Biol. Chem.* 270: 3197-3202.

Seddon, J.M. 1990. Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids. *Biochim. Biophys. Acta* 1031: 1-69.

Tate, M.W., E.F. Eikenberry, D.C. Turner, E. Shyamsunder, and S.M. Gruner. 1991. Nonbilayer phases of membrane lipids. *Chem. Phys. Lipids* 57: 147-164.

Luminal Ion Channels Involved in Isotonic Secretion by Na^+-Recirculation in Exocrine Gland-acini

Jakob Balslev Sørensen, Morten Schak Nielsen,
Robert Nielsen *and* Erik Hviid Larsen

Abstract

According to the novel 'Na^+-recirculation theory' isotonic secretion is accomplished by processing of the fluid in two energy-requiring steps: The first step involves laterally placed Na^+/K^+-pumps, transporting Na^+ into the lateral intercellular space (LIS) making it hypertonic with respect to the bathing solution. This drags water by osmosis from the serosal side, raising the hydrostatic pressure of the LIS, which will cause a convective flow of a hypertonic solution to pass into the lumen of the gland. The second step involves re-uptake of Na^+ by the gland cells via Na^+-selective luminal ion-channels, likewise energized by lateral Na^+/K^+-pumps, making the luminal solution isotonic. Since the junctional complex of the gland is cation-selective Cl^- flows through the cells, being secreted by apical Cl^--channels, while luminal K^+-channels maintain the apical membrane-potential at a set-point value. Here we demonstrate, for the first time, the co-existence of Na^+, Cl^-, and K^+-channels in the luminal membrane of an exocrine gland. By applying whole-cell patch clamp, we verified that Cl^--channels are activated by adrenaline, causing cell depolarization and a concomitant rise in the conductance. At the same time, the transport number for Na^+ was increased, indicating the activation of Na^+-selective channels. The existence of all three kinds of channels was verified by patch-clamp on the luminal membrane. Cl^--channels were small ATP-dependent 8 pS channels activated by cAMP, resembling the CFTR-type Cl^--channel. The K^+-channels were voltage-activated with a conductance of ~30 pS. Na^+-channels had a conductance of 5 pS at the spontaneous membrane potential and the current-voltage relationships reversed at 103 mV depolarization from the spontaneous membrane-potential. We conclude that Cl^-, K^+, as well as Na^+-channels are found in the luminal membrane of frog skin glands. The Na^+-recirculation theory constitutes a framework where the presence of these ion channels is reconciled with isotonic fluid secretion.

Jakob Balslev Sørensen, Morten Schak Nielsen,
Robert Nielsen *and* Erik Hviid Larsen
August Krogh Institute
DK-2100 Copenhagen Ø, Denmark
e-mail: EHLarsen@aki.ku.dk

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:179–191

Introduction

The so-called 'leaky epithelia' share the capacity for transporting large amounts of ions and water in isotonic proportions in either absorptive or secretory direction. The concept of 'solute-coupled water transport' indicates that the transport is divided into an energy-consuming process (i.e., the vectorial transport of ions) and a dissipative process (i.e., osmosis). Models of isotonic transport generally differ where the coupling between the two processes is assumed to take place (in the lateral intercellular spaces (LIS): 'the standing gradient hypothesis', in the cell: 'transcellular osmosis', or inside specialized transport proteins: 'osmotic engines'). Here we suggest based on the novel '*Na^+-recirculation theory*' (Ussing and Eskesen, 1989; Ussing et al., 1996) that active, vectorial transport of ions may take place simultaneously in both directions (*i.e.* both inward and outward). The forward transport takes place between the cells (via the LIS) and drives water by osmosis, whereas the backward transport goes through the cells and returns part of the transported ions to the LIS. This recirculation of transported ions serves to bring the net osmolality of the otherwise hypertonic transportate back to isotonic proportions. Since both processes are active this theory states that achieving isosmolality is an active, energy-consuming process and not based on simple dissipation. Whether the net transport takes place in the inward or the outward direction is determined by properties of two membranes lining the LIS towards the serosal and mucosal side, respectively.

The Model of Na^+-recirculation in the Gland

A. Structural features of the model

The existence of a Na^+-recycling loop in the gland was suggested by Ussing and Eskesen (1989) and further developed by Ussing et al. (1996). The model is depicted in Fig. 1. The basic features of this model are:

1. The plasma membrane of the transporting cell is divided into three regions: The basal membrane, the lateral membrane facing the LIS, and the luminal membrane. Thus the so-called 'basolateral' membrane has been divided into two functionally distinct domains.

2. Two barriers between the cells are responsible for this division: The junctional complex and a hypothesized barrier at the level of the basement membrane.

3. The Na^+/K^+-pumps present in the lateral membrane energize both the forward and backward ion transport, and will thus pump Na^+ into the LIS. Parallel K^+-channels serve to recycle K^+.

4. The basal membrane is furnished with a $Na^+/K^+/2Cl^-$-cotransporter which will bring Cl^- into the cell in an electroneutral fashion, thereby raising cell-$[Cl^-]$ above electrochemical equilibrium. Basal Cl^- and K^+-channels are also present of which the last-mentioned have been identified by patch-clamping (Andersen et al., 1995).

5. As a distinctive feature of this model, the luminal membrane is supposed to posses both Cl^--, Na^+-, and K^+-selective ion channels all of which are active during secretion. Of these, the Cl^--channel serves primary Cl^--secretion, whereas the Na^+-channel is responsible for Na^+-recycling through the gland cells. The K^+-channel probably serves to stabilize the apical membrane-potential.

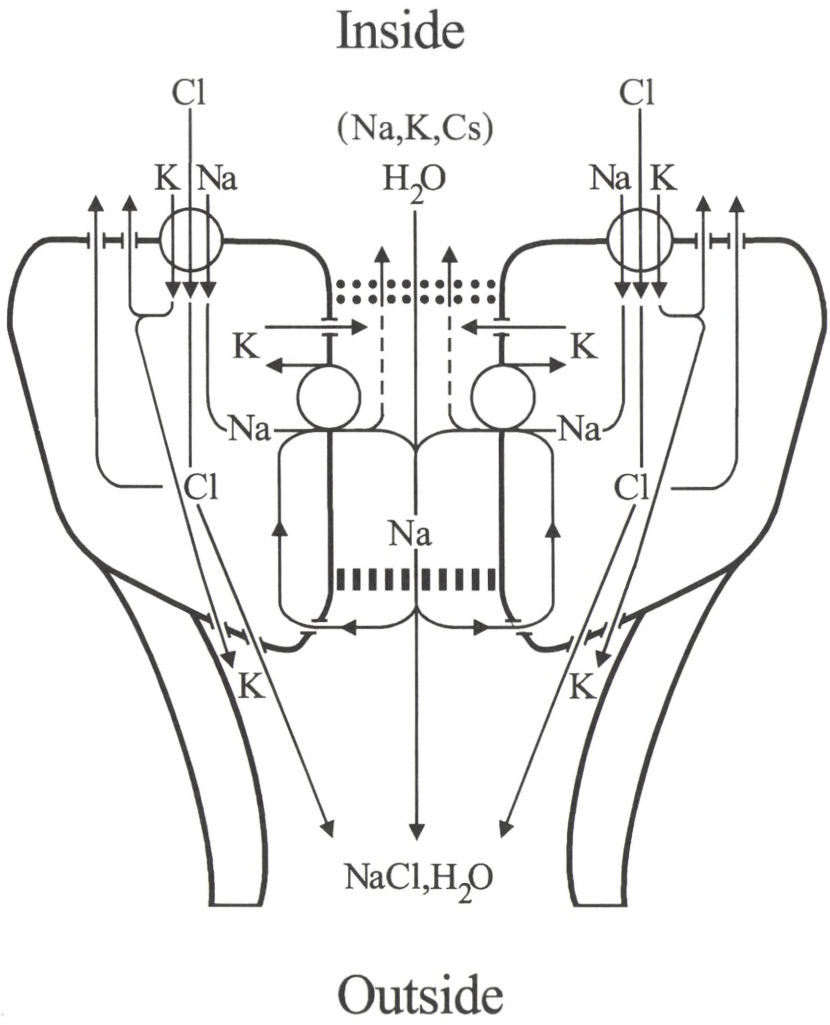


Figure 1. Model of frog skin gland maintaining a flow of isotonic fluid from 'inside' (= blood side) to 'outside' by way of Na^+-recirculation. Two identical gland cells are shown, lining the lateral intercellular space. The function of the lateral intercellular space and the concerted working of the lateral Na^+/K^+ pumps, the basal $Na^+/K^+/2Cl^-$ co-transporter, and the ion channels of the basal, lateral and apical membranes for producing an isotonic secretion are explained in detail in the text. From Ussing et al. (1996).

B. Dynamic features of the model

The frog skin glands can be stimulated to carry out stationary secretion for hours using noradrenalin (Eskesen and Ussing, 1989), the β-agonist isoproterenol (Thompson and Mills, 1983) or prostaglandin E_2 (Bjerregaard and Nielsen, 1987). According to the model, stimulation by cAMP-inducing substances will open up the luminal Cl^--channels and allow Cl^- to exit down its electrochemical gradient. For a while this will set up a transepithelial voltage-gradient, which will serve to drag Na^+ and water via the LIS. At this point of time, the gland model may agree more or less with the Silva model of the shark rectal gland (Silva et al., 1977). However, after about 30-60 minutes, the transepithelial potential gradient disappears and the glands continue to secrete in the absence of transepithelial voltage, pressure, osmotic, or chemical gradients (Ussing et al., 1996). The model explains how this may take place. Activation of the Na^+/K^+-pump will pump Na^+ into the LIS with Cl^- following probably via the basal barrier. The resulting raised NaCl-concentration of the LIS will drag water by osmosis into the LIS from the serosal bath. This allows a hydrostatic pressure build-up in the LIS, which will cause a filtrate of the LIS-solution to pass through the junctional membrane into the gland lumen by convection. An important point at this stage is to realize the conditions under which the directional flow will be set up in the outward and not the inward direction. Intuitively, if water is dragged *into* the LIS via the basement membrane and leaks *out* of the junctional membrane, the condition required is that the reflection-coefficients of the two membranes, σ^{BM} and σ^{JM}, respectively, must satisfy the condition $\sigma^{BM} > \sigma^{JM}$. Indeed, it can be shown that this is both a necessary and sufficient condition for net secretion (Larsen *et al.*, 1997).[1]

The composition of the filtrate which leaves the LIS and enters the gland lumen depends on the $[NaCl]_{LIS}$ and the σ^{JM}_{NaCl} in such a way, that $J_{NaCl}/J_v = [NaCl]_{LIS} \cdot (1 - \sigma^{JM}_{NaCl})$, where J_{NaCl} is the flux of NaCl and J_v is the water flux.[2] Since $[NaCl]_{LIS} > [NaCl]_{bath}$ due to the activity of the Na^+/K^+-pump, under a range of conditions the emergent fluid is expected to be hypertonic. This necessitates the appearance of a recirculation-loop for Na^+, where Na^+ is subjected to reuptake into the cells to recycle through the pump. The reuptake-pathway was hypothesized to be apically located Na^+-channels (Ussing and Eskesen, 1989; Ussing et al., 1996), but in principle any transport mechanism for Na^+-reuptake will do. Thus, active Na^+-secretion by the pump sets up conditions for the dragging of water via a low-resistance pathway (the LIS) into the gland lumen, where the tonicity is then regulated by returning Na^+ via a high-resistance pathway (the cells).

C. Flux-ratio analysis of the frog skin glands shows the presence of an 'invisible' part of the Na^+-flux

Ussing and Lind (1996) discovered that flux-ratio analysis of $^{134}Cs^+$ can be used as an accurate measure for paracellular ion-flow. This results from the fact that $^{134}Cs^+$ is irreversibly trapped in the epithelial cells upon entry. The appearing fluxes at the opposite side of the epithelium must therefore result from a stricktly paracellular route of permeation. Using the Cs^+-trapping method Ussing et al. (1996) showed that the flux-ratio of $^{134}Cs^+$ (J^{out}/J^{in}) was significantly above unity (range 3.25-11) when measured in the stationary phase of secretion under thermodynamic equilibrium conditions. The force dragging Cs^+ through the paracellular route was estimated to be 30-60 mV. This force can only be explained by solvent drag caused by a substantial directional water flow between the cells and thus reveals the secretory activity of the glands in the absence of an external

[1] In fact, by reversing the inequality, one can transform the model into an absorbing state. A similar model with the opposite inequality has been applied to the absorptive small intestine (Larsen et al., 1997).

[2] For simplicity we here consider the general case where both Na^+ and Cl^- flow through the junction, thus NaCl is treated as an uncharged species. In reality for the gland, experimental evidence suggests that the Cl^--flux proceeds via the gland cells, whereas Na^+ flows via the junction. Thus, only Na^+ is subjected to recirculation and the Cl^--flux must be regulated to match the remaining (not recirculated) part of the Na^+.

driving force. Further, Ussing et al. (1996) showed that the flux-ratio of Na^+ (J^{out}/J^{in}) was significantly less than would be expected from a purely paracellular permeation of this ion along the same route as Cs^+. The argument is that for convective ion flow, we expect that since $D_{Cs}/D_{Na} \approx 1.5$ the flux-ratios for Na^+ and Cs^+ obey

$$[J^{out}_{Na}/J^{in}_{Na}] = [J^{out}_{Cs}/J^{in}_{Cs}]^{1.5}. \quad (1)$$

Actually, if the Na^+/K^+-pumps are placed as in Fig. 1, we would even expect the inequality '>' to hold since Cs^+can not substitute for Na^+ in the pump. Regardless, Ussing and coworkers consistently found that the flux-ratio for Na^+ was significantly lower than the right side of Eq. (1). This means that a significant fraction of the Na^+-flux must return to the solution of origin, making part of the Na^+-flux in effect 'invisible'. The recirculated flux of Na^+ was estimated to be 80% of the total Na^+-flux in the LIS *i.e.* each Na^+-ion would be recirculated on average 4 times before escaping to the outside solution. Using $^{42}K^+$, Nielsen and Nielsen (1994) also showed that the flux-ratio (J^{out}/J^{in}) for K^+ was significantly larger than expected from a passive distribution of this ion. This was confirmed by comparison with the Cs^+-flux-ratio (Ussing et al., 1996). It would seem, therefore, that K^+ is subject to active secretion by the cells. Thus, Cl^-, Na^+, and K^+ channels should all be present in the luminal membrane of the gland.

Identifying Luminal Ion Channels in the Gland

The frog skin glands constitute an ideal model-epithelium for the study of isotonic secretion. The glands constitute a large acinus and a very short duct, making the final secretion very close to the primary secretion. With the Na^+-absorption of the epithelium blocked by amiloride, the activity of a large number of gland acini can be studied in isolation, using tracer-technology or electrical measurements. Furthermore, by stimulation with noradrenalin or prostaglandin E_2 the glands enter a stationary phase of secretion which can persist for hours. Recently, a preparation of isolated epithelium with intact glands *in situ* stripped of connective tissue was developed (Andersen et al., 1995), which allows patch-clamp and ion imaging technology to be applied to the glands. In the first publication using this preparation a population of 'maxi' K^+-channels was identified in the basal membrane (Andersen et al., 1995). We set out to study electrical properties of individual gland cells and the luminal ion channels involved in secretion. Sheets of epithelia with intact glands were prepared using serosal exposure to crude collagenase, followed by a hydrostatic pressure head of 10-25 cm H_2O. A piece of epithelium was mounted in a chamber with the serosal side up and perfused at the serosal, but not at the mucosal side.

A. Whole-cell properties of secretory acinar cells

As a starting point, we performed patch-clamp in the whole-cell configuration to study the secretory behaviour of the entire cell (Sørensen and Larsen, 1997a). By using permeabilization of the patch by the poreforming antibiotic nystatin, we were able to study the membrane potential, V_c (by clamping the pipette-current to 0) and the conductance (by clamping the pipette-voltage to values between -90 and +90 mV in 10 mV steps) in cells with a relatively unperturbed intracellular environment. The unstimulated (resting) gland cells had membrane-potentials of -65 mV, governed by the equilibrium potential for K^+, as shown by substitution of K^+ for Na^+ in the bath, which yielded a linear dependence of V_c on log(bath-[K^+]) with a slope of 31 mV/decade (*i.e.* the transport-number for K^+ would be 31/58 = 0.53). Perfusion of the bath with a low-[Cl^-] (8 mM) or a low-[Na^+] (3 mM) solution hardly changed the membrane-potential (transport-numbers 0.01 and 0.05, respectively).

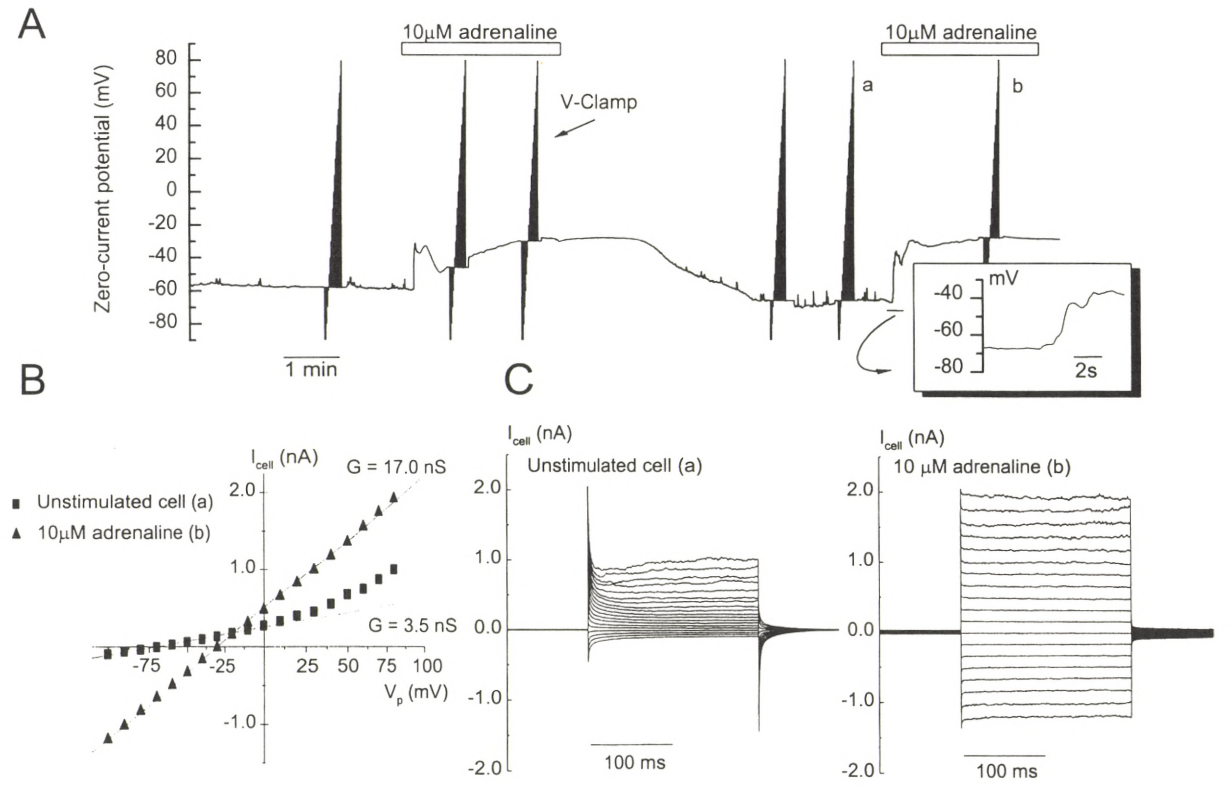


Figure 2. Nystatin-permeabilized whole-cell experiment on gland cell. The glands were perfused with NaCl-Ringer (containing, in mM: 110 NaCl, 3.7 KCl, 3 Na-acetate, 10 glucose, 5 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, pH=7.4) and patched with a high-K^+ pipette-solution (containing, in mM: 30 KCl, 10 NaCl, 90 K-gluconate, 5 TES, 1 EGTA, 10 glucose, 1 $MgCl_2$, 0.38 $CaCl_2$, 0.1 mg/ml nystatin, pH=7.2 with KOH, total $[K^+]$=126 mM). *A.* Voltage-trace from a whole-cell experiment. The pipette was clamped to zero current by the amplifier (EPC-9) in order to monitor the voltage. Periodically, the pipette-voltage was clamped at values between -90 and +90 mV in 10 mV steps ('V-Clamp') from the spontaneous membrane-potential for measurement of the current-voltage relationship. Application of 10 μM adrenaline caused a very rapid (note inset) depolarization, which was fully reversible. *B.* Steady-state current-voltage relationships obtained at markers a and b (see *A*). The conductance (G) was found as the slope of the regression-line for points at $\leq$ 0 mV for the unstimulated condition and for all points after stimulation. *C.* Individual current-traces for the voltage-pulses at a and b (see *A*). Note the activation at depolarized potential by the unstimulated cell. All voltages were corrected for the liquid junction potential (10 mV referenced to the pipette) between pipette and bath.

Stimulation of the gland by $10\,\mu M$ adrenaline caused a fast (seconds) depolarization to $V_c = -32$ mV (Fig. 2) which was sustained in the presence of agonist. This was accompanied by an increase of cell conductance by a factor of 2-15. The current-voltage relationship in the stimulated cell rectified at most slightly in the outward direction, whereas non-stimulated cells often had a strongly outwardly rectifying current-voltage curve due to the presence of voltage-activated K^+-channels.[3]. In the stimulated state, the transport number for Cl^- now increased to 0.27, shown by a substantial (but very variable) depolarization upon lowering of bath-[Cl^-], whereas transport numbers for K^+ and Na^+ were 0.15 and 0.11, respectively. Thus upon stimulation, the gland activated Cl^--channels (Fig. 1). This increase in the Cl^--conductance caused a secondary decrease in the transport-number for K^+. The increase in the transport-number for Na^+ from 0.05 to 0.11 in face of the increased total conductance is interesting, since this could be taken to indicate that Na^+-channels had also been activated substantially, an important and discriminatory property of the Na^+-recirculation model. However, this interpretation would be premature, since the change in V_c upon Na^+-removal could be secondary to effects on Na^+-dependent co- and counter-transporters ($Na^+/Ca^{2+}-$, Na^+/H^+-exchangers and $Na^+/Cl^-/(K^+)$-cotransporters of differing stochiometry). We therefore tested the Na^+-channel blocker amiloride on activated cells, which caused a hyperpolarization in 2/3 of the cells investigated. This hyperpolarization could be interpreted as representing blocking of Na^+-selective channels, but again the results were not conclusive, since we had to use high concentrations of amiloride ($100\,\mu$M) for the effect to appear. This overlaps with the sensitivity of the Na^+/H^+-exchanger for amiloride and could therefore again be explained by secondary effects of amiloride. Using the flourescent dye Fura-2 for measurement of intracellular Ca^{2+} and the pH-sensitive dye BCECF, we were able to monitor effects on these parameters by perturbation of bath-Na^+. These (unpublished) results showed that stimulation by adrenaline caused a transient increase in cell-Ca^{2+} from 75 nM to a peak of 540 nM followed by a plateau-level of 160 nM, but left the pH relatively unaffected (pH=7.55 before and pH=7.50 after stimulation). Na^+-removal was ineffective in raising cell-Ca^{2+} in both stimulated and unstimulated cells, whereas it resulted in a slight acidification (from pH=7.50 to pH=7.37) under stimulated conditions. However, the application of $100\,\mu$M amiloride did not affect cell-pH[4] under either condition. These negative results together with the increase in the Na^+-transport number argue for the appearance of a Na^+-conductance in stimulated cells.

B. Single-channel properties of luminal ion-channels

To verify directly the presence of the suggested ion channels in the luminal membrane, we undertook the task of patching the luminal membrane. This was possible by slitting up the acinus using a discarded patch-clamp pipette. A fresh pipette could then be used for going through the slit and forming a patch on the apical membrane.

In *cell-attached* patches, application of $2\,\mu$M isoproterenol, $12.5\,\mu$M forskolin, or 0.5 mM dibutyryl-cAMP and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) activated small Cl^--channels with a conductance of 8.3 pS at the membrane potential (Sørensen and Larsen, 1997b). The current-voltage (i/V-) relationship for the channel followed the Goldman-Hodgkin-Katz equation for passive electrodiffusion with a reversal potential very close to 0 mV pipette potential. This means that following maximal activation using cAMP-inducing substances, the equilibrium potential for Cl^- coincided with the membrane potential (within the error of measurement). This was also the conclusion of whole-cell studies.

[3]The presence of these channels can explain why the transport numbers in this study did not add to unity: A perturbation of the membrane potential in either direction would be antagonized by the activation/inactivation of K^+-channels.

[4]That the dye was able to measure changes in cell-pH was shown by applying an ammonium-prepulse to the loaded cells.

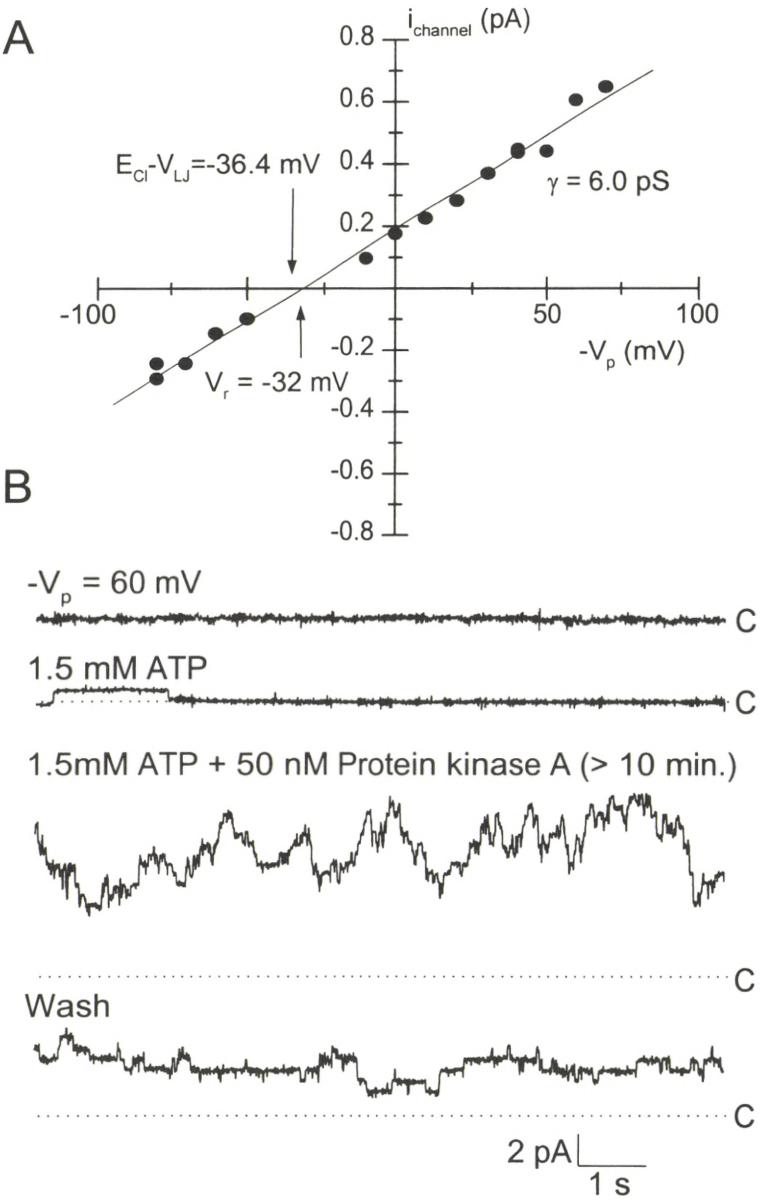


Figure 3. Excised inside-out patch from the luminal membrane of the gland patched with NaCl-Ringer in the pipette (see legend to Fig 2) and exposed to an intracellular solution containing 25 mM Cl^- on the cytoplasmic side (composition, in mM: 15 NaCl, 105 gluconic acid, 10 TRIS-HCl, 105 TRIS(hydroxymethyl)aminomethane, 5 HEPES, 1 EGTA, 0.371 $CaCl_2$, 1.1 $MgCl_2$, pH=7.2). *A.* Current-voltage relationship for the channel. The conductance (γ) and the reversal-potential (V_r) were identified by linear regression analysis. The equilibrium potential for Cl^- corrected for the junction potential at the reference electrode ($E_{Cl} - V_{LJ}$) compares well with V_r, confirming Cl^--selectivity of the channel. Voltages are given as the negative of the pipette-potential (V_p), *i.e.* as $-V_p$. *B.* Current traces for the patch held at 60 mV and exposed to intracellular solution alone, or after the sequential addition of 1.5 mM ATP and 50 nM catalytic subunit of cAMP-dependent protein kinase. Finally, the patch was washed with intracellular solution alone. 'C' denotes the current with all channels closed.

The channel was also studied in *excised inside-out* patches, where it exhibited fast run-down in the absence of ATP. Fig. 3 shows a patch where application of ATP induced a single channel, whereas incubation with the catalytic subunit of cAMP-dependent protein kinase activated several channels. The activation could be reversed by removing ATP. The i/V-relationship shows the Cl^--selectivity of these $\sim$ 6 pS channels in this case. The activation in cell-attached or inside-out configuration by cAMP and the ATP-dependence of the channel in the phosphorylated state together with the low single-channel conductance constitute a hallmark for the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^--channel. The message for CFTR as well as the protein itself has been localized to frog skin glands in *Xenopus* by Engelhardt et al. (1994). Thus CFTR Cl^--channels constitute the route of cAMP-dependent Cl^--secretion in glands of frog skin, resemblant of the shark rectal gland (Gögelein et al., 1987, Marshall et al., 1991).

In cell-attached or inside-out patches (stimulated or resting) K^+-channels were also often noted - often in the same patches as the small Cl^--channel. The K^+-channel was voltage-dependent and activated by membrane depolarisation from the spontaneous membrane potential (corresponding to 0 mV pipette potential). The channel in Fig. 4 had a chord conductance of 28 pS at 50 mV (voltage referenced to the pipette), which clearly distinguishes it from the basal 'maxi' K^+-channel, which has a conductance of $\sim$ 200 pS (Andersen et al., 1996). Due to the voltage-dependence, only points at $\geq$ 0 mV could be obtained on the i/V-curve. This voltage-dependent activation would make the channel ideally suited for maintaining the apical membrane-potential at some set-point value.

In resting cells, Na^+-channels were frequently found in the cell-attached configuration (Fig. 5, and Sørensen and Larsen, 1997b). These had a relatively fast kinetics with openings lasting in the order of 10-100 ms, which distinguish them from the slowly gating heterologous expressed α,β,γ-ENaC (*E*pithelial *Na C*hannel)-type channel (Canessa et al., 1994). The mean conductance at 0 mV pipette potential was 5.1 pS and the i/V-relationship reversed at 103 mV depolarization with respect to the spontaneous V_c. The study of these channels was hampered by a fast run-down in cell-attached patches. The Na^+-channels were found separately, or together with K^+- and/or Cl^--channels.

Concluding Remarks

The model in Fig. 1 is considered a working hypothesis at this time. Distinguishing properties of the model (e.g. the lateral placing of Na^+/K^+-pumps, the two barriers between the cells, the apical Na^+-entry pathways, the elevated NaCl-concentration of the LIS during secretion) constitute solid, testable predictions which can be addressed using established methods. The preparation of glands stripped of connective tissue, but still attached to the epithelium, seems an ideal preparation for investigating an isotonic secreting epithelium at all levels of organisation. So far, the patch-clamp experiments on the glands have placed the presupposed ion channels of Fig. 1 in the luminal membrane. Thus both Cl^--, Na^+- and K^+-channels are present in the luminal membrane of a secretory epithelium. Curiously, this means that similar ion-channels are present in a secretory epithelium and in several absorptive epithelia, such as the toad skin epithelium (Larsen et al., 1987, Harvey and Larsen, 1993, Sørensen and Larsen, 1996) and the airways (Haws et al., 1992, Russo et al., 1992, Voilley et al., 1994, Burch et al., 1995) which also express both CFTR-like Cl^--channels and Na^+-channels. In the absorptive airway-epithelium the function of the secretory Cl^--channel is generally unknown, even though severe dysfunction results from its absence (cystic fibrosis). Instead, it is supposed to function in the regulation of apical Na^+-channels in an anta-

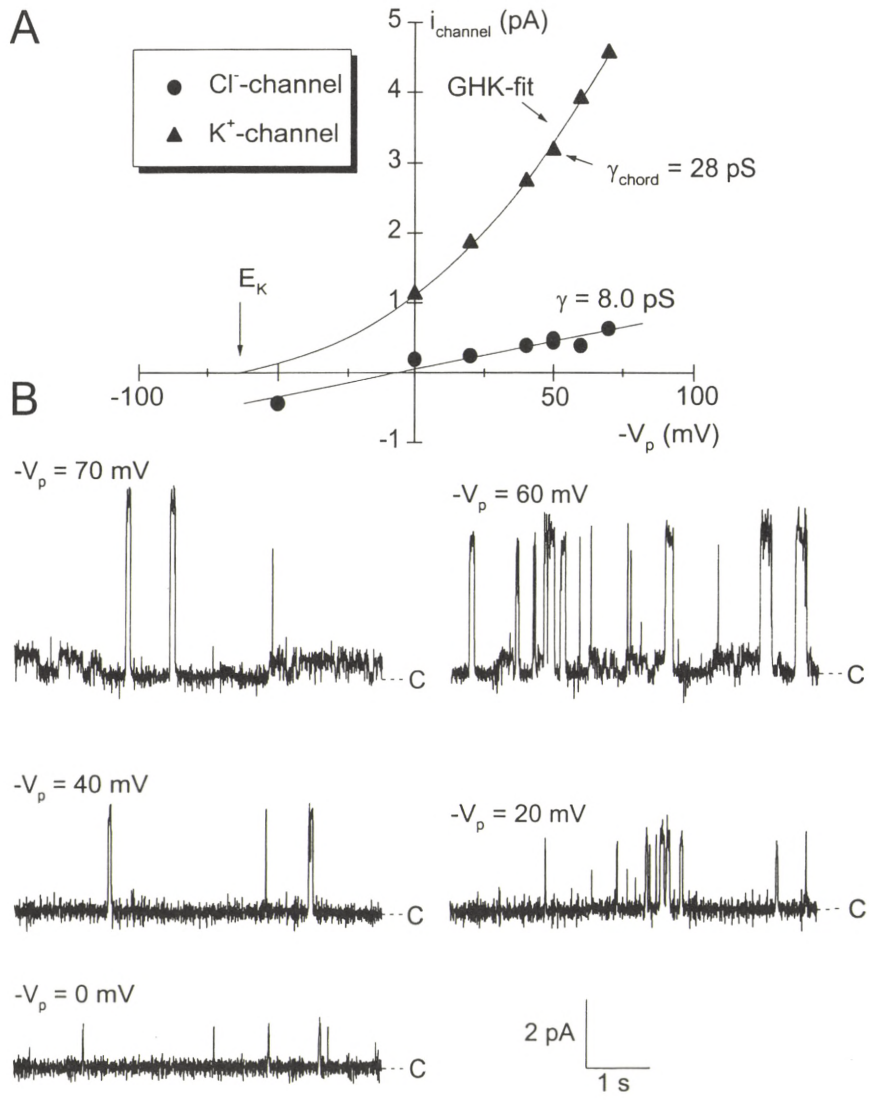


Figure 4. Cell-attached patch on the luminal membrane of a gland cell stimulated by $2\mu M$ isoproterenol. NaCl-Ringer in the pipette. *A*. Current-voltage relationship for two different types of channels in the same patch. Filled circles show currents through 8 pS Cl^--channels of the same type as shown in Fig. 3. Filled triangles show currents through a K^+-channel, which was only noted at $-V_p \geq 0$mV. The i/V-relationship for the Cl^--channel was fitted by linear regression, whereas the i/V-relationship for the K^+-channel was fitted by the Goldman-Hodgkin-Katz equation for passive electrodiffusion of K^+:

$$i_K = P_K \frac{(V_c - V_P)F^2\,([K]_c - [K]_o \exp(-F(V_c - V_p)/RT))}{RT\;\;(1 - \exp(-F(V_c - V_p)/RT))}$$

where we fixed the cellular and outer K^+-concentrations $[K]_c$ and $[K]_o$ to 140 and 3.7 mM, respectively, and fitted with the permeability for K^+ (P_K) and the spontaneous membrane-potential (V_c) as free parameters. The best fit (line) yielded $P_K = 1.64 \times 10^{-13}\text{cm}^3 s^{-1}$ and V_c=-28.2 mV. This yields a reversal potential for K^+ currents at $-V_p = -63$ mV. The chord conductance at 50 mV was calculated using this value. *B*. Current traces at different potentials. Note the small Cl^--channels at 60 and 70 mV.

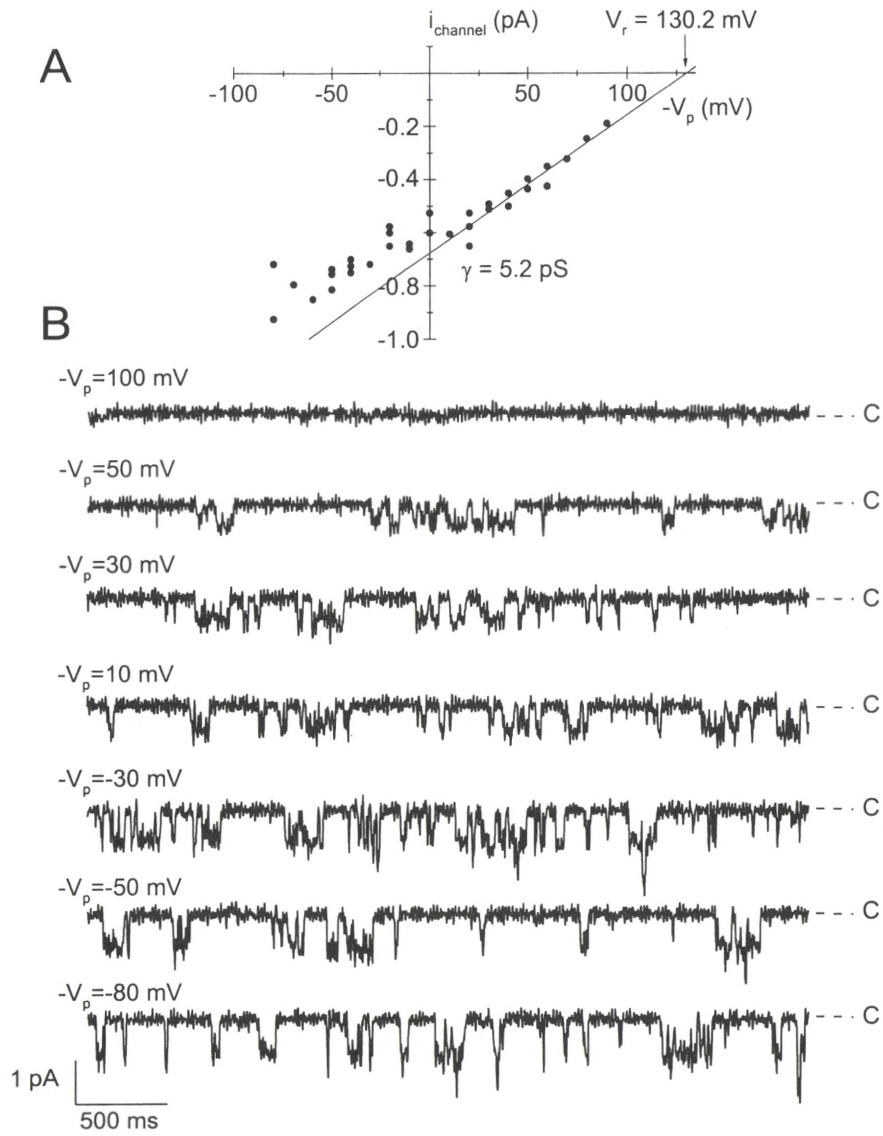


Figure 5. Cell-attached patch on the luminal membrane of an unstimulated gland cell with NaCl-Ringer in the pipette. *A*. Current-voltage relationship of Na^+-selective channel. Each point shows the amplitude of one full opening. Line shows linear regression to points $-V_p > 0\,\text{mV}$ and yields the single-channel conductance (γ) and the reversal-potential (V_r). *B*. Current-traces of Na^+-channels at different potentials. Note the relatively fast kinetics.

gonistic fashion: Upregulation of CFTR Cl^--channel-activity results in diminished Na^+-channel activity (Knowles et al., 1983, Willumsen and Boucher, 1991; Stutts et al., 1995). The frog skin glands present the interesting possibility of investigating the same type of interaction in a secretory epithelium, where the function of Cl^--channels is well understood, but the function of a Na^+-channel unassigned in all previous secretory models. A prediction from Fig. 1 is that both channels should be active simultaneously, indicating an opposite mode of interaction (if any). So far, this question is still open, since the luminal ion channels have only been studied in the resting and acutely stimulated state, under conditions where the large current flowing through Cl^--channels would probably obscure Na^+-channel activity. Thus, upregulation of Na^+-channels may appear in the long run as the epithelium enters the state of stationary secretion under equilibrium conditions.

References

Andersen H.K., V. Urbach, E.V. Kerkhove, E. Prosser, and B.J. Harvey. 1995. Maxi K^+ channels in the basolateral membrane of the exocrine frog skin gland regulated by intracellular calcium and pH. *Pflügers Arch.* 431: 52-65.

Bjerregaard H.F., and R. Nielsen. 1987. Prostaglandin E_2-stimulated glandular ion and water secretion in isolated frog skin (*Rana esculenta*). *J. Membrane Biol.* 97: 9-19.

Burch, L.H., C.R. Talbot, M.R. Knowles, C.M. Canessa, B.C. Rossier, and R.C. Boucher. 1995. Relative expression of the human epithelial Na^+ channel subunits in normal and cystic fibrosis airways. *Am. J. Physiol.* 269: C511-C518.

Canessa, C.M., L. Schild, G. Buell, B. Thorens, I. Gautschi, J.-D. Horisberger, and B.C. Rossier. 1994. Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* 367: 463-467.

Engelhardt, J.F., S.S. Smith, E. Allen, J.R. Yankaskas, D.C. Dawson, and J.M. Wilson. 1994. Coupled secretion of chloride and mucus in skin of *Xenopus laevis*: possible role for CFTR. *Am. J. Physiol.* 267: C491-C500.

Eskesen, K., and H.H. Ussing. 1989. Transport pathways for Na^+ and Br^- (Cl^-) in noradrenaline-stimulated frog skin (*Rana temporaria*). *Acta Physiol. Scand.* 136: 535-546.

Gögelein, H., E. Schlatter, and R. Greger. 1987. The "small" conductance chloride channel in the luminal membrane of the rectal gland of the dogfish (*Squalus acanthias*). *Pflügers Arch.* 409: 122-125.

Harvey, B.J., and E.H. Larsen. 1993. Sodium and chloride currents in single mitochondria-rich cells of amphibian skin. *J. Physiol.* 459: 241P.

Haws, C., M.E. Krouse, Y. Xia, D.C. Gruenert, and J.J. Wine. 1992. CFTR channels in immortalized human airway cells. *Am. J. Physiol.* 263: L692-L707.

Knowles, M., M.J. Stutts, A. Spock, N. Fisher, J.T. Gatzy, and R.C. Boucher. 1983. Abnormal ion permeation through cystic fibrosis respiratory epithelium. *Science* 221: 1067-1070.

Larsen, E.H., S. Nedergaard, and H.H. Ussing. 1997. Sodium recirculation and isotonic transport. Manuscript submitted.

Larsen, E.H., H.H. Ussing, and K.R. Spring. 1987. Ion transport by mitochondria-rich cells in toad skin. *J. Membrane Biol.* 99: 25-40.

Marshall, J., K.A. Martin, M. Picciotto, S. Hockfield, A.C. Nairn, and L.K. Kaczmarek. 1991. Identification and localization of a dogfish homolog of human cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 266: 22749-22754.

Nielsen, M.S., and R. Nielsen. 1994. K^+ is actively secreted in the frog skin glands. *Acta Physiol. Scand.* 115: P31.

Russo, R.M., R.L. Lubman, and E.D. Crandall. 1992. Evidence for amiloride-sensitive sodium channels in alveolar epithelial cells. *Am. J. Physiol.* 262: L405-L411.

Silva, P., J. Stoff, M. Field, L. Fine, J.N. Forrest, and F.H. Epstein. 1977. Mechanism of chloride secretion by shark rectal gland: role of Na^+-K^+-ATPase in chloride transport. *Am. J. Physiol.* 233: F298-F306.

Stutts, M.J., C.M. Canessa, J.C. Olsen, M. Hamrick, J.A. Cohn, B.C. Rossier, and R.C. Boucher 1995. CFTR as a cAMP-dependent regulator of sodium channels. *Science* 269: 847-850.

Sørensen, J.B., and E.H. Larsen. 1996. Heterogeneity of chloride channels in the apical membrane of isolated mitochondria-rich cells from toad skin. *J. Gen. Physiol.* 108: 421-433.

Sørensen, J.B., and E.H. Larsen. 1997a. Whole-cell patch-clamp studies of exocrine glands from frog skin (*Rana esculenta*). *FASEB Journal* 11: A306.

Sørensen, J.B., and E.H. Larsen. 1997b. Co-existence of Cl^-- and Na^+-channels in the apical membrane of exocrine gland acini from frog skin (*Rana esculenta*). *Proceedings of The Physiological Society Meeting in London, January 1998*, 10p.

Thompson, I.G., and J.W. Mills. 1983. Chloride transport in glands of frog skin. *Am. J. Physiol.* 244: C221-C226.

Ussing, H.H., and K. Eskesen. 1989. Mechanism of isotonic water transport in glands. *Acta Physiol. Scand.* 136: 443-454.

Ussing, H.H., and F. Lind. 1996. Trapping of $^{134}Cs^{+}$in frog skin epithelium as a function of short circuit current. *Kidney International* 49: 1568-1569.

Ussing, H.H., F. Lind, and E.H. Larsen. 1996. Ion secretion and isotonic transport in frog skin glands. *J. Membrane Biol.* 152: 101-110.

Voilley, N., R. Lingueglia, G. Champigny, M.-G. Mattei, R. Waldmann, M. Lazdunski, and P. Barbry. 1994. The lung amiloride-sensitive Na^{+}-channel: biophysical properties, pharmacology, ontogenesis, and molecular cloning. *Proc. Natl. Acad. Sci. USA* 91: 247-251.

Willumsen, N.J., and R.C. Boucher. 1991. Sodium transport and intracellular sodium activity in cultured human nasal epithelium. *Am. J. Physiol.* 261: C319-331.

Active Membranes

Jaques Prost

Abstract

The question is addressed as to how non-equilibrium conditions control the large-scale properties of membranes.

Jaques Prost
Institut Physico-Chimie Curie
F-75231 PARIS Cédex 05, France
email: Jacques.Prost@curie.fr

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:193–194

Morphology and fluctuations of membranes around equilibrium have been extensively studied for several decades.

First, fluid membranes have been investigated, then multicomponents membranes, and sophisticated in-plane order, such as hexatic order, have been considered. Very interesting notions, such as the "crumpling" transition, and the entropic repulsive force, spontaneous curvature, gaussian curvature, etc, have emerged from these studies, and have been tested experimentally.

Whereas the concepts, developed for describing membranes close to equilibrium, are certainly useful for understanding some important properties of biological membranes, they do not retain a very important feature, namely the out-of-equilibrium activity taking place on, or in the vicinity of the membrane. This activity is, in general, very specific like in transport by pumps or channels, or surface polymerization of filaments like actin, proteins synthesis, exocytosis, endocytosis, etc. Considering this specificity, it might well be that no general law governing these membranes could be expected. This is, as a matter of fact, probably true for the short-scale behavior.

Under such circumstances, the first sensible attitude is to study individual problems, one at a time, as was done, for instance, with ion channels using patch clamp techniques. We know however, from our past experience in many other fields of research, that even when local processes are complex and specific, large-scale properties may obey simple generic rules. A natural question to raise is then: in what way does a membrane, in which local chemistry takes place, differ on large scales, from a membrane close to equilibrium? There are certainly many aspects to this question: one, very basic and practically unaddressed, concerns the sta-

tistical ensemble in which the membrane should be described. The constant number of molecules ensemble valid for the description of vesicles, is obviously inappropriate for real biological membranes. The constant tension ensemble, valid for black films in the presence of a plateau border, is probably closer to reality since there is a large surface reservoir in the endoplasmic reticulum.

However, exchanges in the cell are regulated "actively", and what is maintained constant is not clear. On a "semi-local" scale, the usual notions of tension, curvature modulus, etc., are probably still correct. The most salient difference with situations close to equilibrium, comes from the fact that the noise acting on the membrane now does not satisfy the fluctuation-dissipation theorem. "Chemical noise" has two components, one stemming from the transient nature of the chemical process (which we call shot noise), another one stemming from concentration fluctuations of the noise sources (e.g. protein concentration fluctuations).

We discuss several consequences resulting from the existence of this noise; in particular, we show how membranes can "recognize" the presence of a nearby wall and change its fluctuation amplitude accordingly (Prost et al., 1996; Prost et al., 1998). We also discuss the nature of a new instability, which arises when the noise sources redistribute in response to membrane curvature (Ramaswany et al., in preparation).

We eventually evaluate orders of magnitude corresponding to experimental situations (Manneville et al., in preparation), and discuss the biological relevance of these considerations.

References

Prost, J., and R.B. Bruinsma. 1996. Shape fluctuations of active membranes. *Europhys. Letts.* 33:321-326

Prost, J., J.B. Manneville, and R.B. Bruinsma. 1998. Fluctuation-magnification of non-equilibrium membranes near a wall. *Eur. Phys. J. B* 1:465-480.

Ramaswany, S., J. Toner, and J. Prost. In preparation.

Manneville, J.B., P. Bassereau, and J. Prost. In preparation.

Membrane Bilayer Properties in *Acholeplasma laidlawii* are Sensed and Set by the Lipid-synthesizing Enzymes

S. Berg, O. Karlsson, L. Li, S. Vikström *and* Å. Wieslander

Abstract

In membranes of the small prokaryote *Acholeplasma laidlawii* (i) a constant surface charge density, given by the anionic lipids, (ii) similar phase equilibria, close to a potential bilayer-nonbilayer transition, and (iii) a nearly constant spontaneous curvature, are metabolically maintained for the lipid bilayer under a variety of conditions *in vivo*. This involves extensive changes in the amounts and types of bilayer- and nonbilayer-prone glucolipids synthesized. Analyses of the three first, consecutively acting enzymes in the glucolipid metabolic pathway show that the major metabolic responses of the living cell can be mimicked by the enzymes in reconstituted amphiphile-enzyme aggregates. The enzymes respond to a proper lipid surface charge, certain activator lipids, domain formation, and the spontaneous curvature, and it is concluded that they are the sole sensors and mediators for the maintenance of lipid bilayer packing properties in the cell membrane.

S. Berg, L. Li, O. Karlsson
S. Vikström *and* Å. Wieslander
Department of Biochemistry
Umeå University
Umeå, SE-90187 Sweden
email: awi@chem.umu.se

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:195–200

No Models

We are still a long way from a detailed, structural understanding for the involvement of the many different membrane lipids in various cellular processes. Exept for the metabolic maintenance of a "melted" (liquid-crystalline) state for the hydrocarbon chains of biological lipid bilayers, essentially no physico-chemical models have been proposed to rationalize the typical differences in polar lipid composition, and the related inter-membrane flow, that occur for the many membranes in the eukaryotic cell. The reasons for this are several, e.g. the large number of lipids and synthesis pathways, several genes/enzymes for many individual lipid synthesis reactions, the localization of enzymes to different organelle membranes, plus the technical problems of manipulating and purifying various specific membranes from eukaryotic cells, respectively.

Acholeplasma laidlawii Membrane

A bilayer organization, and the essential character of the liquid-crystalline (LC) state for cell growth, was early shown for the small and simple prokaryote *Acholeplasma laidlawii*. However, this parasitic organism lacks efficient metabolic means to regulate or adjust the gel to LC transition of the lipid bilayer since it can only synthesize saturated (SFA), but not unsaturated (UFA) fatty acyl chains for polar lipid synthesis. Still, these cells can grow with a large variety of bilayer lipid acyl chains, yielding from ~14 to almost ~20 carbons in average chain length (C_n), but with restrictions in the extent of chain unsaturation tolerated in the short and long C_n range, respectively (Wieslander et al., 1995). This causes an allowed span between different membranes for the thickness of the hydrophobic bilayer region, from ~2.3 nm to ~3.1 nm, with little influence of the membrane proteins, according to chain order profiles determined by NMR (Thurmond et al., 1994). However, experimental variation of the C_n and SFA/UFA parameters yield large changes in the amounts of individual polar lipids, as well as between the classes of phospholipids, phosphoglucolipids and glucolipids synthesized.

Similar Charge Density and Phase Equilibria

Extensive analyses of the physico-chemical properties of the individual lipids, and more or less *in vivo*-like mixtures thereof, have revealed a correlation between physiological composition (regulation) and properties of the lipids (summarized in Rilfors et al., 1993; Wieslander and Karlsson, 1997). A constant surface charge density (potential) was maintained by the varying amounts of anionic lipids synthesized as a function of increased chain unsaturation (lipid lateral area). Concomitantly, the amounts of the major nonlamellar-prone (reversed cubic/hexagonal phase) lipid monoglucosyldiacylglycerol (MGlcDAG) decreased, whereas the lamellar-forming diglucosyl derivative (DGlcDAG) increased, see Fig. 1. Based on arguments of "molecular shape" it was proposed that the metabolic balance between MGlcDAG and DGlcDAG aimed at maintaining a certain "bilayer stability" (or instability), i.e. similar phase equilibria, close to a potential lamellar- nonlamellar transition for the membrane lipid bilayer. Evidence for this was gained from studies, where the different but established effects of certain hydrocarbons, alcohols, detergents and steroids on amphiphile phase equilibria, correlated strongly with the metabolic responses (i.e. MGlcDAG/DGlcDAG amounts synthesized) from *A. laidlawii* to the presence of these foreign additives in the membrane bilayer. An elaborate fine-tuning by these regulatory mechanisms was also established. MGlcDAG, although always being made by the cells, will have its phase equilibria progressively shifted towards the lamellar phase by decreasing C_n and increasing chain saturation, respectively (Lindblom et al., 1993). However, at such conditions other, more potent nonbilayer derivatives are successively made or increased in amounts, assisting MGlcDAG in its critical "packing" function. These lipids are the precursor DAG, and variants of the two glucolipids with an extra, third acyl chain, i.e. MAMGlcDAG and MADGlcDAG (*-label in Fig. 1.) (Wieslander et al., 1995; Andersson et al., 1996).

For amphiphiles in general, increased chain unsaturation and decreased C_n, lead to a decreased average chain order (at constant temperature). However, in *A. laidlawii* the metabolic variation of the MGlcDAG amounts as a function of UFA and C_n changes seems to have a stronger impact on chain order, with increased order correlating with increased MGlcDAG amounts (and decreased C_n).

This is due to the small polar head of MGlcDAG allowing for a denser lateral packing of the lipids with a concomitant increase in chain order, as was shown for different MGlcDAG/DGlcDAG mixtures (Eriksson et al., 1991), similar to other lipid mixtures with contrasting packing properties (e.g. nonbilayer DOPE/bilayer DOPC). Hence, chain order is not kept constant in the *A. laidlawii* bilayer by the lipid metabolism.

Constant Spontaneous Curvature

The presence of MGlcDAG, and the other nonbilayer-prone lipids (see above) with small polar headgroups in relation to their more (laterally) bulky acyl chains, is obviously regulated to maintain certain packing properties as is evident from the similar (temperature) closeness to a bilayer-nonbilayer transition for all *in vivo* lipid mixtures. The imbalance in lateral forces yields a spontaneous curvature for the two monolayers of the bilayer, tending to curl outward (concavely) from each other. This bending force is considered large enough to be able to affect the conformation of proteins. In *A. laidlawii* the radius of the spontaneous curvature (R_0) is actively kept constant by the lipid metabolism as seen for *in vivo* lipid mixtures, where the % UFA chains and C_n in the lipids were varied in a systematic manner, by metabolic adjustments in MGlcDAG (and other nonbilayer lipid) amounts (Österberg et al., 1995). Likewise, the lamellar to nonlamellar phase transition, but not the melting one, occurred at fairly similar temperatures. The R_0 for unsaturated MGlcDAG and DGlcDAG species was 1.7 and 12.3 nm, similar to DOPE (2.1 nm) and DOPC (9.6 nm), respectively (saturated DGlcDAG and PC are bound to have substantially larger R_0). However, R_0 values for the *in vivo* mixtures only varied between 5.8 and 7.3 nm over the series, a remarkable constancy unders these circumstances (Österberg et al., 1995).

Enzymes Sense and Set Bilayer Properties

At the executing level these regulatory mechanisms in *A. laidlawii* must involve a direct sensing of bilayer packing properties by the lipid-synthesizing enzymes, or by specific "sensors" communicating proper signal to activate enzyme genes or to modify activities of the enzymes, respectively. A sensing of indirect effects, like for instance lipid domain formation, must also be considered. The membrane phospholipids and glucolipids are consecutively made in two competing pathways from a common phosphatidic acid (PA) precursor. Purification and reconstitution of the three first enzymes of the glucolipid pathway (Table 1 and enzyme 1, 2 and 3 in Fig. 1) in mixed-micelles and liposome bilayers have revealed that the regulatory mechanisms (cf. above) most likely rely solely on the "sensing" and kinetic properties of the synthesizing enzymes. The synthase enzyme for the major nonbilayer-prone lipid MGlcDAG (DAG-glucosyltransferase = enzyme 2) demands a critical fraction of negatively charged lipids for activity (Karlsson et al., 1994; 1997). This is coupled to a conformational change of the enzyme, as analyzed by proteolytic resistance (Li et al., 1997). Chain length of the DAG substrate, the latter made from PA by a PA-phosphatase (= enzyme 1) (Berg and Wieslander, 1997), but not curvature or phase equilibria, is important for activity. PA and DAG precursors do not accumulate normally. Hence, the MGlcDAG synthase (but not the PA phosphatase) is probably a main site for the lipid surface charge regulation, balancing the two

Table 1. Properties of *A. laidlawii* lipid-synthesizing enzymes.

	PA phosphatase	MGlcDAG synthase	DGlcDAG synthase
In vivo substrates	PA	DAG & UDP-Glc	MGlcDAG & UDP-Glc
In vivo products	DAG & PI	MGlcDAG & UDP	DGlcDAG & UDP
Purification	near homogeneity	homogeneity	near homogeneity
Cofactors	not detected	Mg^{2+}	Mg^{2+}
Membrane assoc.	integral protein	hydrophobic + electrostatic	prob. integral protein
Substrate specificity	low	lipid chain variants	lipid chain variants
Activator	not detected	anionic lipids	PG

pathways, and activation of this enzyme is brougth by all the anionic lipids, see Fig. 1. The following, single glucosylation of MGlcDAG to yield the major bilayer-forming DGlcDAG (MGlcDAG-glucosyltransferase = enzyme 3) only occurs in the presence of substantial amounts of a specific activator lipid (in a cooperative fashion), i.e. phosphatidylglycerol (PG) from the other, phospholipid pathway, see the Fig. 1. (Dahlqvist et al., 1995). *In vivo* these two lipids are coordinately synthesized on an amount basis. Amounts of PG activator needed *in vitro* depend on chain order, bilayer curvature and phase equilibria, i.e. the fractions and nonbilayer propensities of several additives tested. Variations in synthesis rates for both glucosyltransferases *in vitro* are in accordance with, and sufficient for, the recorded *in vivo* changes of MGlcDAG and DGlcDAG amounts needed for the compensatory curvature adjustments due to the inflicted membrane perturbations (Dahlqvist et al., 1995). A domain formation of the activator PG, induced experimentally by an activator-PG/bilayer-matrix chain length mismatch, could superseed the curvature effects for the DGlcDAG synthase and strongly enhance the glucosyltransferase activity at low activator (PG) amounts (Karlsson et al., 1996). Hence, a local enrichment of the activator lipid may be a fast, potential second mechanism for regulating DGlcDAG synthesis (from MGlcDAG), which may override the primary curvature effects. Preliminary data also indicate that certain ions can stimulate this enzyme substantially, yielding a coupling to cell energy metabolism.

General Validity

Following these studies, analogous mechanism for maintaining similar lamellar-to-nonlamellar phase equilibria have been vizualized in several other, not related bacteria (with no glucolipids), e.g. the Gram-negative *Pseudomonas fluorescens* and *Escherichia coli*, and the Gram-positive *Bacillus megaterium* plus two *Clostridium* species, and are likely to occur in many more by arguments of similarity in lipid composition. For several of these species the regulation takes place at a specific (soluble) enzyme in acyl chain synthesis (elongation), whereas for others it involves changes in polar headgroup composition or even at both these levels. Hence, similar principles operate in various organisms, but with different tools for the metabolic execution. Likewise, the high content of nonbilayer-prone lipids in several eukaryotic organelle membranes (e.g. the endoplastic reticulum, mitochondria and chloroplasts) indicates that in these bilayers a certain spontaneous curvature must indeed be present and actively maintained.

Several integral membrane proteins, and

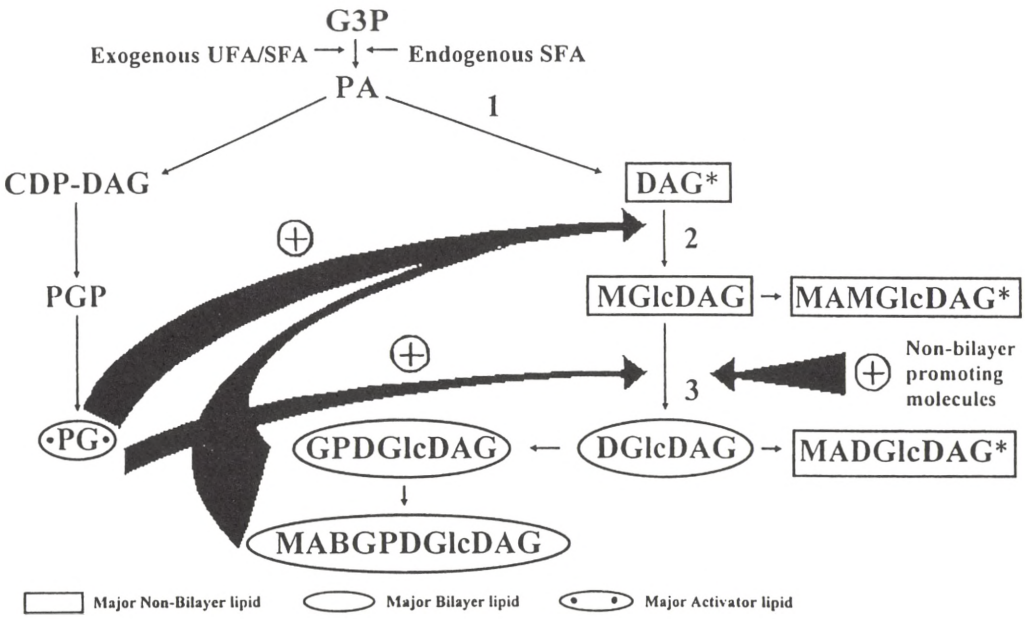


Figure 1. Connected pathways for the biosynthesis of membrane lipids in *Acholeplasma laidlawii* A-EF22. →, enzymatic step; filled, thick arrows indicate (different) enzyme activation by influence (contact?) of anionic lipids or PG (for enzyme 2 and 3, respectively); and influence of curvature and chain order on enzyme 3. Lipids boxed all form nonbilayer/nonlamellar aggregates, but with varying propensities; lipids with an asterisk may accumulate under certain conditions, especially when the phase equilibria of MGlcDAG are shifted from nonlamellar towards lamellar phases by more saturated or shorter acyl chains. Lipids encircled form lamellar phases. The glucolipid pathway is initiated by enzyme 1, a PA-phosphatase producing DAG; enzyme 2 is the MGlcDAG synthase, and enzyme 3 the following DGlcDAG synthase. These three are all very minor, integral membrane proteins (≈ 23, 40 and 41 kDa). Enzyme 2 and 3 are both glucosyltransferases (lipid plus UDP-glucose substrates).

temporarily membrane-associated soluble proteins, have been shown to depend on similar lipid packing properties as the two *A. laidlawii* glucolipid synthases described here, i.e. lipid surface charge and the spontaneous curvature. Interesting examples are the rate-limiting step in the eukaryotic phospholipid synthesis, i.e. CTP:phosphocholine cytidylyltransferase and protein kinase C; both of these have important regulatory functions in the cell.

***

These studies have been financially supported by the NFR and the K. and A. Wallenberg Foundation.

References

Andersson, A.-S., L. Rilfors, M. Bergvist, S. Persson, and G. Lindblom. 1996. New aspects on membrane lipid regulation in *Acholeplasma laidlawii* A and phase equilibria of monoacyldiglucosyldiacylglycerol. *Biochemistry* 35: 11119-11130.

Berg, S., and Å. Wieslander. 1997. Purification of a phosphatase which hydrolyzes phosphatidic acid, a key intermediate in glucolipid synthesis in *Acholeplasma laidlawii* A membranes. *Biochim. Biophys. Acta* 1330: 225-232.

Eriksson, P.-O., L. Rilfors, Å. Wieslander, A. Lundberg, and G. Lindblom. 1991. Order and dynamics in mixtures of membrane glucolipids from *Acholeplasma laidlawii* studied by 2H NMR. *Biochemistry* 30: 4916-4924.

Dahlqvist, A., S. Nordstrøm, O.P. Karlsson, D.A. Mannock, R.N. McElhaney, and Å. Wieslander. 1995. Efficient modulation of glucolipid enzyme activities in membranes of *Acholeplasma laidlawii* by the type of bilayer matrix lipids. *Biochemistry* 34: 13381-13389.

Karlsson, O.P., A. Dahlqvist, and Å. Wieslander. 1994. Activation of the membrane glucolipid synthesis in *Acholeplasma laidlawii* by phosphatidylglycerol and other anionic lipids. *J. Biol. Chem.* 269: 23484-23490.

Karlsson, O.P., M. Rytömaa, A. Dahlqvist, P. Kinnunen, and Å. Wieslander. 1996. Correlation between bilayer lipid dynamics and acticity of the diglucosyldiacylglycerol synthase from Acholeplasma laidlawii membranes. *Biochemistry* 35: 10094-10102.

Karlsson, O.P., A. Dahlqvist, S. Nordström, and Å. Wieslander. 1997. Lipid dependence and basic kinetics of the purified monoglucosyldiacylglycerol synthase from membranes of *Acholeplasma laidlawii*. *J. Biol. Chem.* 272: 929-936.

Li, L., O.P. Karlsson, and Å. Wieslander. 1997. Activating amphiphiles cause a conformational change of the 1,2-diacylglycerol 3-glucosyltransferase from *Acholeplasma laidlawii* membranes according to proteolytic digestion. *J. Biol. Chem.* 272: 29602-29606.

Lindblom, G., J.B. Haukson, L. Rilfors, B. Bergenståhl, Å. Wieslander, and P.-O. Eriksson. 1993. Membrane lipid regulation in *Acholeplasma laidlawii* grown with saturated fatty acids. Synthesis of a triacyl glucolipid not forming liquid crystalline structures. *J. Biol. Chem.* 268: 16198-16207.

Rilfors, L., Å. Wieslander, and G. Lindblom. 1993. Regulation and physical properties of the polar lipids in *Acholeplasma laidlawii*. *In* Mycoplasma Cell Membranes (Rottem, S., and Kahane, I., eds.), pp. 109-166, Plenum Publishing Corporation.

Thurmond, R., A. Niemi, G. Lindblom, Å. Wieslander, and L. Rilfors. 1994. Membrane thickness and molecular ordering in *Acholeplasma laidlawii* studied by 2H NMR spectroscopy. *Biochemistry* 33: 13178- 13188.

Wieslander, Å., S. Nordström, A. Dahlqvist, L. Rilfors, and G. Lindblom. 1995. Membrane lipid composition and cell size of *Acholeplasma laidlawii* strain A are strongly influenced by lipid acyl chain length. *Eur. J. Biochem.* 227: 734-744.

Wieslander, Å., and O.P.Karlsson. 1997. Regulation of lipid synthesis in Acholeplasma laidlawii. *In* Lipid Polymorphism and Membrane Properties (R. Epand, ed.), Current Topics in Membranes 44: 517- 540.

Österberg, F., L. Rilfors, Å. Wieslander, G. Lindblom, and S. Gruner. 1995. Lipid extracts from membranes of *Acholeplasma laidlawii* A grown with different fatty acids have a nearly constant spontaneous curvature. *Biochim. Biophys. Acta* 1257: 18-24.

Model Membrane Systems as Drug Delivery Vehicles

Pieter R. Cullis *and* David B. Fenske

Abstract

Model membrane systems have been developed primarily to provide simplified versions of biological membranes, which allow the properties of individual components to be studied in detail. Model systems have provided a great deal of information on the conformation of lipids in membranes, the rates and type of motions undergone by individual lipid molecules, and the types of polymorphic phases preferred by lipids in isolation and in mixtures. This has led to many insights concerning the roles of lipids in membranes, such as the relationship between non-bilayer phases and membrane fusion (Cullis and De Kruijff, 1979; Bloom et al., 1991; Siegel, 1993). However, model membranes and the techniques used to generate them have considerable utility in their own right for drug delivery applications. This utility is straightforward to illustrate. An ideal drug delivery system will exhibit several characteristics: (i) a small size (<100 nm diameter) combined with reasonably long circulation lifetimes to be able to access disease sites such as tumours, (ii) efficient loading with a biologically active agent, and (iii) fusogenic properties that allow the contents of the carrier to be delivered into target cells. All of these properties can be satisfied by utilizing our knowledge of model membrane systems.

Pieter R. Cullis *and* David B. Fenske
Department of Biochemistry & Molecular Biology
University of British Columbia
Vancouver, B.C., V6T 1Z3 Canada
e-mail: pcullis@inexpharm.com

Biol. Skr. Dan. Vid. Selsk. 1998, **49**:201–208

Size and Circulation Lifetime

Many model membrane studies have involved multilamellar vesicles (MLVs), large multilamellar lipid dispersions with diameters on the order of microns (Cullis and De Kruijff, 1979; Bloom et al., 1991). While excellent systems for biophysical studies involving magnetic resonance and fluorescence techniques, MLVs are unsuitable for examining many fundamental membrane properties, such as lipid asymmetry and membrane permeability. These types of studies require unilamellar vesicles, which can be formed in sizes ranging from 20 nm to several microns by a variety of techniques (Hope et al., 1993; Bloom et al., 1991). The extrusion technique, a procedure developed to make large unilamellar vesicle (LUV) model membrane systems (Hope et al., 1993), requires only hydra-

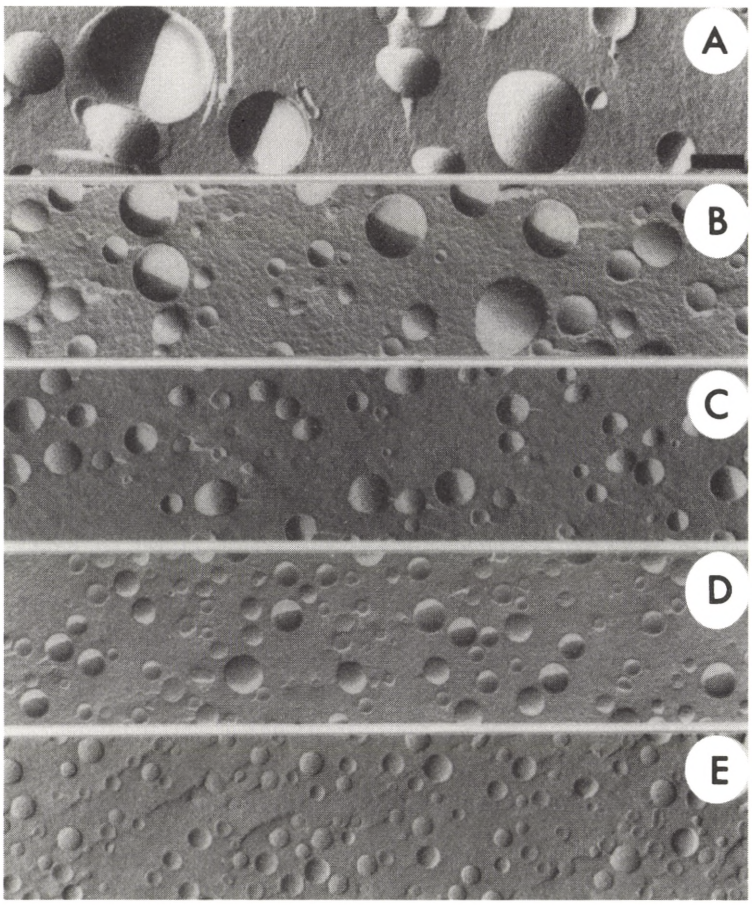


Figure 1. Freeze-fracture electron micrographs of egg phosphatidylcholine LUVs prepared from MLVs (freeze-and-thawed) passed 20 times through polycarbonate filters of various pore sizes. The pore sizes of the filters employed were (A) 400 nm, (B) 200 nm, (C) 100 nm, (D) 50 nm, and (E) 30 nm. The bar in panel A represents 150 nm and all panels represent the same magnification. Reproduced from Hope et al. (1986) with permission.

tion of the lipid and extrusion under medium pressures through polycarbonate filters with pore size of 200 nm or smaller. Vesicles as large as 400 nm diameter can be made, but some of these are not unilamellar. The procedure is quick (15 minutes) and yields a homogeneous population of LUVs in the size regime ideal for drug delivery applications (100 nm diameter). Vesicles larger than this are cleared more rapidly by the reticuloendothelial system, and are less readily taken up into cells by endocytosis, whereas vesicles much smaller have an internal volume that is too small to transport sufficient quantity of drug. Freeze-fracture electron micrographs of LUVs ranging in size from 30 to 400 nm are shown in Figure 1.

The requirement for reasonably long circulation lifetimes can be satisfied in two additional ways. The first involves the use of lipid compositions such as saturated phosphatidylcholines or sphingomyelins, in combination with cholesterol in (nearly) equimolar concentration. This lipid composition, which corresponds to the lipids found on the outer monolayer of eukaryotic membranes such as the erythrocyte membrane (Op Den Kamp, 1979), binds relatively small amounts of serum proteins, the presence of which triggers uptake

by fixed and free macrophages in the circulation (Chonn et al., 1992). The second method of increasing circulation times is to attach hydrophilic polymers, such as polyethylene glycol (PEG), to the liposome surface. As little as 5 mol% of a phosphatidylethanolamine-PEG construct incorporated into an LUV provides a steric barrier to plasma proteins which can greatly increase circulation time (Gabizon and Papahadjopoulos, 1988).

Loading of Drugs into LUVs in Response to Transmembrane pH Gradients

The requirement for efficient loading of the drug delivery system can also be satisfied using techniques developed for model membrane systems. The first method for loading LUVs followed logically from the development of a model membrane system exhibiting asymmetric transbilayer distributions of lipids, such as is observed in biological membranes. It was reasoned that transmembrane pH gradients (ΔpH) should result in asymmetric transbilayer distributions of lipids that are weak acids or weak bases, a prediction that was borne out for lipids such as fatty acids, stearylamine, and some phospholipids (phosphatidic acid and phosphatidylglycerol) (Hope et al., 1989). This suggested that other lipophilic weak bases, a class which includes a large proportion of commonly used drugs, would also migrate across lipid bilayers in the presence of a ΔpH (Cullis et al., 1997). The mechanism for uptake is illustrated in Figure 2, which represents the addition of a drug such as doxorubicin (a weak base with a $pK_a = 8.6$) to LUVs exhibiting a ΔpH (inside acidic). For external pH values near neutrality, the drug will exist in a mixture of neutral and charged forms. The neutral forms are membrane permeable and can diffuse into the vesicle interior where they are protonated and trapped (due to the impermeability of the charged form). It is straightforward to show that at equilibrium $[\text{Drug}]_{in}/[\text{Drug}]_{out}=[H^+]_{in}/[H^+]_{out}$, and thus for a pH gradient of 3 units the internal drug concentration will be 1000 times higher than the external. Conditions can be achieved for LUVs with an acidic interior where essentially 100% uptake and retention of drugs (such as the major anticancer drugs doxorubicin and vincristine) can be attained at drug-to-lipid ratios which are much higher than can be achieved by other techniques (Mayer et al., 1993; Cullis et al., 1997). As shown in Figure 3, essentially complete uptake of doxorubicin into 200 nm egg phosphatidylcholine/cholesterol LUVs can be achieved in less than 5 minutes.

Other methods for drug loading have been described, which are also based on the generation of a transmembrane pH gradient. For example, LUVs exhibiting a transmembrane ammonium sulfate gradient develop secondary pH gradients, which form as a quantity of neutral ammonia leaks out of the vesicle, leaving its protons behind, acidifying the interior. This pH gradient can then drive drug uptake (Haran et al., 1993). An alternative loading strategy is based on drug-uptake driven by pH gradients generated by the addition of a suitable ionophore to LUVs containing an appropriate entrapped metal ion (Fenske et al., 1998). For example, the addition of nigericin to LUVs containing K_2SO_4 leads to generation of a ΔpH and rapid uptake of anticancer drugs such as vincristine, or of antibiotics such as ciprofloxacin. Similar results are obtained for the ionophore A23187 in conjunction with LUVs containing entrapped $MnSO_4$. These applications follow from early studies by Deamer et al. (1972), who demonstrated the ionophore-mediated generation of a pH gradient in small unilamellar vesicles.

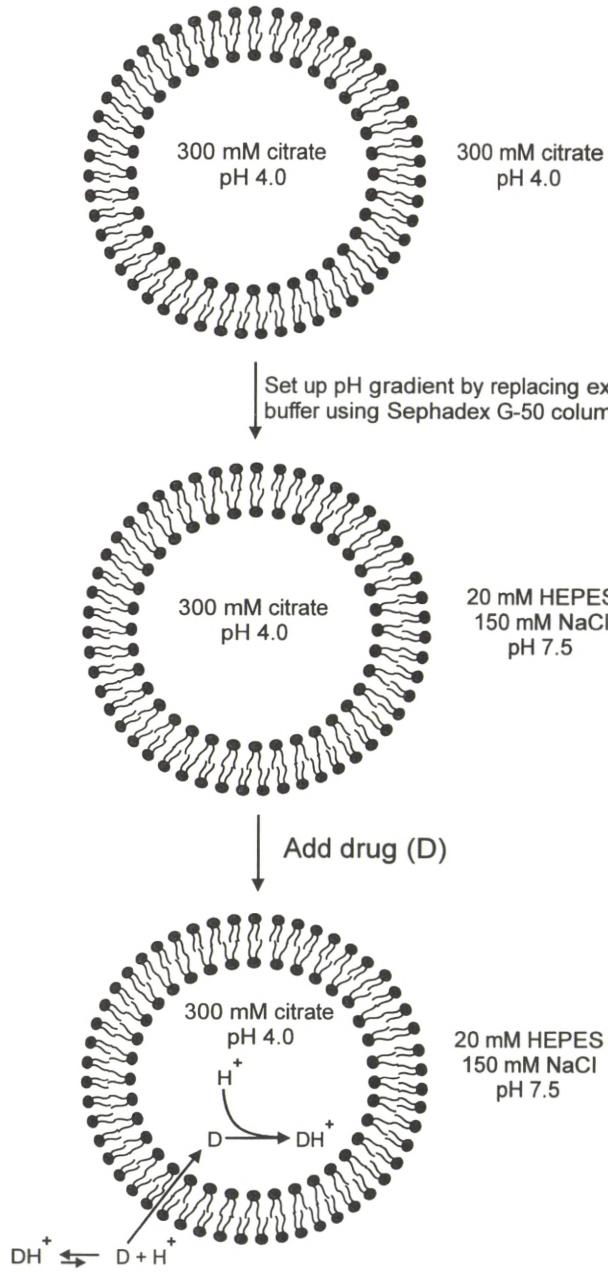


Figure 2. Diagrammatic representation of drug loading in response to a transmembrane pH gradient. The lipid mixture is hydrated and subsequently extruded in 300 mM citrate pH 4.0 (top). A pH gradient is established by passage of the LUVs down a column of Sephadex G-50 hydrated in 20 mM HEPES 150 mM NaCl pH 7.5 (center). The remote loading process involves addition of drug to the LUVs exhibiting the pH gradient. The external drug exists in neutral and protonated forms, and it is the former which can diffuse across the membrane (down its concentration gradient). Once inside the vesicle, the drug is protonated and trapped, resulting in an extremely low internal concentration of neutral drug, which therefore continues to drive uptake.

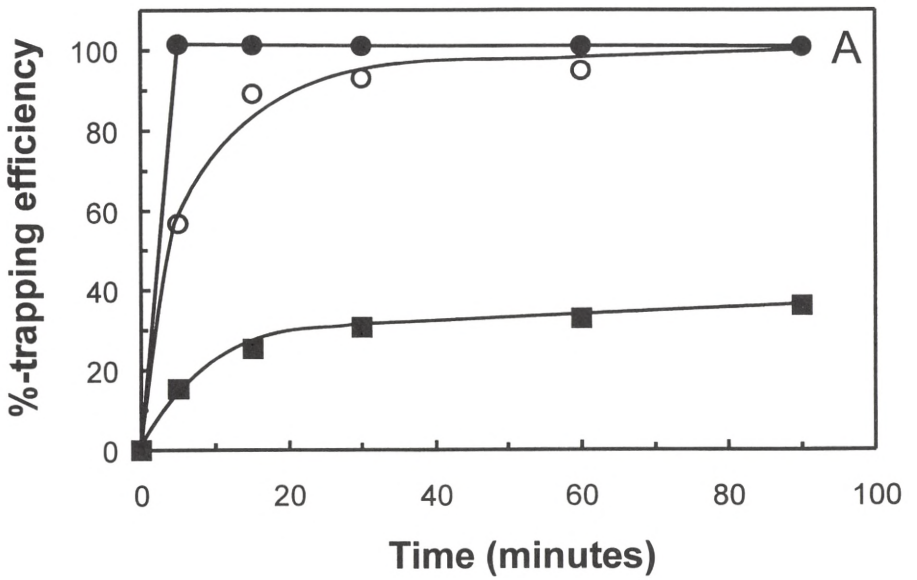


Figure 3. Uptake of doxorubicin into 200 nm EPC/cholesterol (55:45 mol/mol) LUVs exhibiting a transmembrane pH gradient (pH 4 inside, 7.8 outside). Doxorubicin was added to LUVs (drug-to-lipid ratio = 0.3 wt:wt) equilibrated at 21°C (closed squares), 37 °C (open circles), and 60°C (closed circles). Note that rapid and complete uptake only occurs in fluid membranes. The trapping efficiency was calculated as the percentage of the drug initially in the exterior medium which was accumulated into the LUVs. Reproduced from Mayer et al. (1989) with permission.

In the case of large "biopharmaceutical" drugs such as plasmids for gene therapy, loading techniques developed for model membrane systems can also be applied. For example, recent work has shown that incubation of plasmids with cationic lipids results in hydrophobic entities which are soluble in organic solvent. In order to entrap this construct within a lipid based system it was found that a variant of the detergent dialysis procedure used for reconstituting membrane proteins in vesicles (Kagawa and Racker, 1971) can be employed. The resulting DNA delivery systems are small (<100 nm diameter) and stable in biological fluids (J.J. Wheeler, unpublished results).

Fusogenic Properties of Drug Delivery Systems

The requirement for fusogenicity for intracellular delivery of carrier contents can be approached in two ways. First, it is well known from model membrane studies that the presence of "non-bilayer" lipids such as unsaturated phosphatidylethanolamines (PE) can dramatically enhance the ability of lipid bilayers to fuse (Cullis and De Kruijff, 1979; Cullis et al., 1990; Siegel, 1993). Second, as can also be shown employing model membrane systems, fusion between vesicles is enhanced by electrostatic interactions. Vesicles containing negatively charged lipids such as fatty acids or phosphatidic acid can be induced to fuse by the addition of Ca^{2+} (Cullis et al., 1997), and vesicles containing positive charge can be induced to fuse

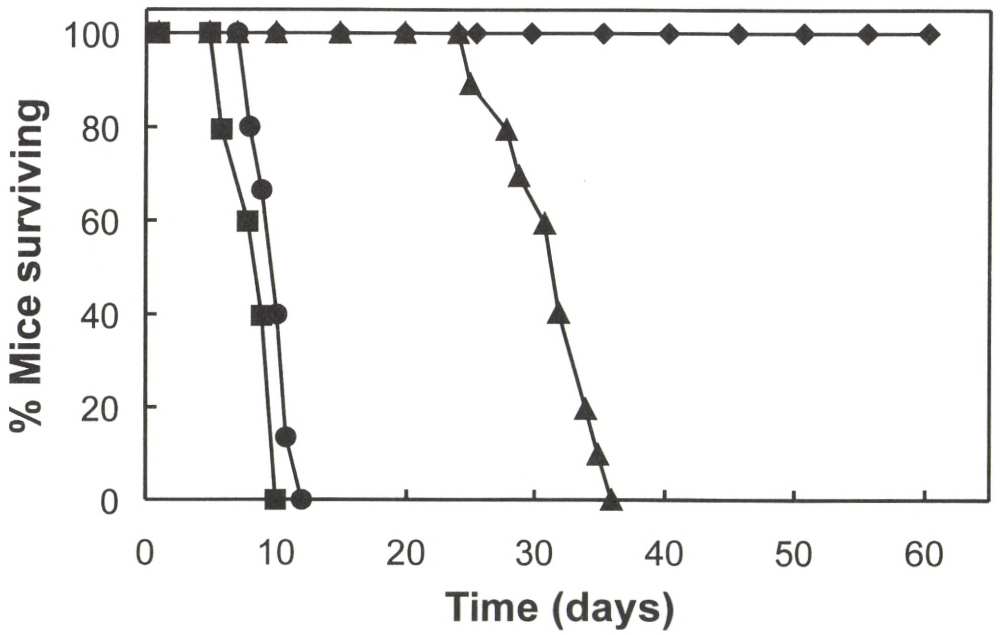


Figure 4. Influence of drug encapsulation and intravesicular pH on the efficacy of vincristine encapsulated in DSPC/Chol LUVs against P388 tumors. BDF1 mice bearing peritoneal ascitic P388 tumors were untreated (squares) or were treated with free vincristine (circles) or LUVs composed of DSPC/Chol containing encapsulated vincristine and with an intravesicular pH of either 4.0 (triangles) or 2.0 (diamonds). Reproduced from Boman et al. (1995) with permission.

with those possessing negative charge (Bailey and Cullis, 1997). As the plasma membrane of cells exhibits a negative charge, including a positive charge in the delivery vesicle can enhance fusion. For example, vesicles composed of dioleoylphosphatidylethanolamine (DOPE) and N,N-dimethyl-N,N-di-9-cis-octadecenylammonium chloride (DODAC) (the latter possessing a permanent positive charge) in a 1:1 molar ratio will fuse with erythrocyte membranes (Bailey and Cullis, 1997). A problem with these and other tactics to improve intracellular delivery, however, is that these sytems will tend to fuse with the first cells that they encounter, rather than after arrival at a disease site. This property can be corrected, at least in part, by coating the vesicle with a polyethylene glycol (PEG) coating, originally developed to engender longer circulation lifetimes for LUV systems (Gabizon and Papahadjopoulos, 1988). However, this also has the effect of inhibiting fusion with cells after arrival in target tissue. In order to render these systems fusogenic at these later times, we have developed PEG coatings that dissociate from the carrier at well defined rates, thus inhibiting fusion immediately after injection, but rendering them more fusogenic at later times. This is accomplished by varying the length of the fatty acyl chains in the lipid anchor of the PEG-lipid construct (Holland et al., 1996).

Two applications of these lipid-based delivery systems concern delivery of anticancer drugs such as vincristine and delivery of plasmids containing therapeutic genes for gene therapy applications. With regard to vincristine, delivery systems with appropriate retention and drug payout characteristics provide a remarkable improvement in efficacy in animal tumour models (Boman et al., 1995; Chonn and Cullis, 1995). A particularly striking example of this is shown in Figure 4, where the influence of drug encapsulation and intravesicular pH on vincristine efficacy is illustrated for BDF1 mice bearing ascitic peritoneal P388 tumours. A

lower internal pH results in improved vincristine retention in the LUV. The survival time of mice treated with free vincristine was only slightly better than for those receiving no treatment (10-12 days). Encapsulation of vincristine in LUVs with an internal pH of 4 more than tripled the survival time, and a further reduction of the internal pH to 2 resulted in 100% survival over a period of 60 days. These survival results correlate directly with the retention characteristics of the drug delivery systems.

While still in the early phases of research, plasmid delivery systems have now been developed which lead to expression of marker genes in a variety of tissues *in vivo*, suggesting their utility for gene therapy applications.

Summary

In summary, the techniques used for the generation of model membranes, and the results derived from model membrane studies, have played a direct role in the development of drug delivery systems. The extrusion technique provided a quick and reliable method for obtaining stable populations of vesicles with diameters ideal for drug delivery applications. The accumulation of lipophilic weak bases within LUVs in response to transmembrane pH gradients permitted the development of loading techniques that exhibited high levels of uptake and excellent retention in vivo. The use of highly ordered lipid compositions (saturated phospholipids and cholesterol) and/or inclusion of PEG-lipids greatly increased LUV circulation lifetimes, allowing accumulation of vesicles at sites of disease. Finally, the inclusion of fusogenic lipids increased the intracellular delivery of liposome contents to target cell populations. Future advances in the development of drug delivery systems for both conventional anticancer drugs and genetic drugs (e.g., plasmid DNA and antisense oligonucleotides) will undoubtedly depend on continued research using model membrane systems.

References

Bailey, A.L., and P.R. Cullis. 1997. Membrane fusion with cationic liposomes: effectsof target membrane lipid composition. *Biochemistry* 36:1628-1634.

Bloom, M., E. Evans, and O.G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Quart. Rev. Biophys.* 24:293-397.

Boman, N.L., M.B. Bally, P.R. Cullis, L.D. Mayer, and M.S. Webb. 1995. Encapsulation of vincristine in liposomes reduces its toxicity and improves its anti-tumour efficacy. *J. Liposome Research* 5:523-541.

Chonn, A., and P.R. Cullis. 1995. Recent advances in liposomal drug delivery systems. *Curr. Opin. Biotech.* 6:698-708.

Chonn, A., S.C. Semple, and P.R. Cullis. 1992. Association of blood proteins with LUVs in vivo: relation to circulation lifetimes. *J. Biol. Chem.* 267:18759-18765.

Cullis, P.R., and B. De Kruijff. 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* 559:399-420.

Cullis, P.R., M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer, and D.B. Fenske. 1997. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. *Biochim. Biophys. Acta* 1331:187-211.

Cullis, P.R., C.P. Tilcock, and M.J. Hope. 1990. Lipid Polymorphism. *In*: Membrane Fusion (J. Wilschut and D. Hoekstra, eds.), Marcel Dekker, N.Y., pp. 35-64.

Deamer, D.W., R.C. Prince, and A.R. Crofts. 1972. The response of fluorescent amines to pH gradients across liposome membranes. *Biochim. Biophys. Acta* 274:323-335.

Fenske, D.B., K.F. Wong, E. Maurer, N. Maurer, J.M. Leenhouts, N. Boman, L. Amankwa, and P.R. Cullis. 1998. Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients. Manuscript in preparation.

Gabizon, A., and D. Papahadjopoulos. 1988. Liposome formulations with prolonged circulation time in blood

and enhanced uptake by tumours. *Proc. Nat. Acad. Sci. USA* 85:6949-6953.

Haran, G., R. Cohen, L.K. Bar, and Y. Barenholz. 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. *Biochim. Biophys. Acta* 1151:201-215.

Holland, J.W., C. Hui, P.R. Cullis, and T.D. Madden. 1996. Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry* 35:2618-2624.

Hope, M.J., M.B. Bally, L.D. Mayer, A.S. Janoff, and P.R. Cullis. 1986. Generation of multilamellar and unilamellar phospholipid vesicles. *Chem. Phys. Lipids* 40:89-107.

Hope, M.J., T.E. Redelmeier, K.F. Wong, W. Rodrigueza, and P.R. Cullis. 1989. Phospholipid assymetry in large unilamellar vesicles induced by transmembrane pH gradients. *Biochemistry* 28:4182-4187.

Hope, M.J., R. Nayar, L.D. Mayer, and P.R. Cullis. 1993. Reduction of liposome size and preparation of unilamellar vesicles by extrusion techniques. Liposome Technology Vol. 1 (G. Gregoriadis, Ed.), pp. 123-133.

Kagawa, Y., and E. Racker. 1971. Reconstitution of vesicles catalyzing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* 246:5477-5487.

Mayer, L.D., T.D. Madden, M.B. Bally, and P.R. Cullis. 1993. pH gradient mediated drug entrapment in liposomes. Liposome Technology Vol. 2 (G. Gregoriadis, Ed.), pp. 27-44.

Mayer, L.D., L.C.L. Tai, D.S.C. Ko, D. Masin, R.S. Ginsberg, P.R. Cullis, and M.B. Bally. 1989. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. *Cancer Res.* 49:5922-5930.

Op Den Kamp, J.A.F. 1979. Lipid asymmetry in membranes. *Ann. Rev. Biochem.* 48:47-71.

Siegel, D.P. 1993. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* 65:2124-2140.

Index

α-helix, 26, 28, 68
α-helix packing, 120
β-sheet, 26, 28

Acholeplasma laidlawii, 198
actin-binding protein, 40
adhesion domains, 36
adhesion plaques, 36
adrenaline, 157
alamethicin, 29, 89–91
alkanes, 105
alpha2-macroglobulin receptors, 140, 142
aminophospholipid, 169
aminophospholipid translocase, 171, 172
aminophospholipid transporter, 129
analytical ultracentrifugation, 87
anchoring residues, 96
annular sites, 119
apocytochrome c, 26
apoptosis, 179
area-difference elasticity, 62
aromatic amino acids, 82, 105
association domains, 59
atomic force microscopy, 54

bacteriorhodopsin, 52, 85, 87, 131, 136
band 3 protein, 141, 142, 146
bending elasticity, 33–36, 38, 74, 75, 92
bending fluctuations, 29, 34
bending force, 199
beta barrel, 86
bilayer couple, 38
bilayer curvature, 52
bilayer deformation, 78, 120
bilayer deformation energy, 80, 82, 91
bilayer fluidity, 78
bilayer insertion, 104
bilayer stability, 11
bilayer thickness, 39, 117, 118, 120, 136
Born repulsion, 124
Brownian motion, 23
budding, 63–64

c-Src, 123
C-type inactivation, 110
Ca^{2+}-ATPase, 117–121
capillary condensation, 75
cardiolipin, 27, 177
caveolae, 125
caveolin, 150
cell adhesion, 33, 34
cell cycle, 178
cell shape, 33
cell-attached patches, 187
channels, 79, 195
charybdotoxin, 125
chemical noise, 196
chemotaxis, 16
cholesterol, 17, 39, 70, 131, 150
choline-containing phospholipids, 169
clathrin cages, 64
clathrin pits, 38
cluster formation, 44
clustering, 68
coherence length, 131
collective behavior, 68
compositional fluctuations, 50
compositional heterogeneity, 43–45
compressibility, 92
computer-simulation, 50, 59
conformational carriers, 79
conformational equilibrium, 69
contact angle, 35
contact curvature, 35
convective ion flow, 185
cooperative structure, 45
corrals, 38
correlation
    long-range, 44
Coulombic attraction, 124
current-voltage relationship, 187
curvature elasticity, 33–36, 38, 74, 75, 92
cytochalasin B, 40
cytochalasin D, 40, 142
cytochrome c, 26

cytochrome oxidase, 27
cytoskeletal corrals, 39
cytoskeleton, 34, 38, 40, 63, 140, 142, 143

Danielli-Davson model, 7
density fluctuations, 44, 50
diacylglycerol, 44, 45, 53, 177
diffusion
    confined, 140
diglucosyldiacylglycerol synthase, 53
dilation elasticity, 63
dimerization constant, 80
dioleoylphosphatidylcholine, 117
dipalmitoylphosphatidylcholine, 68
dipalmitoylphosphatidylethanolamine, 68
diphytanoylphosphatidylcholine, 90
directed random walk, 16
discocyte, 63
docosahexaenoic acid, 18
domain formation, 39, 44, 200
domain structure, 58
dominant negative suppression, 113
doxorubicin, 205
drug delivery, 204
drug encapsulation, 208
dynamic order, 10

E-cadherin, 142
echinocyte, 63
effective concentration, 123
elastic energy, 165
elastic membrane deformations, 78
electrophysiological measurements, 80
electrostatic binding energy, 124
electrostatic interactions, 123, 137
endocytosis, 23, 38, 39, 172, 195
endoplasmic reticulum, 23
excised inside-out patches, 189
eximer-to-monomer fluorescence emission, 51
exocytosis, 195

FcεRI, 150
flexural rigidity, 34
flickering, 34
fluctuating barriers, 23
fluctuation-dissipation theorem, 196
fluctuations
    normal to the membrane plane, 45
fluid mosaic model, 7
fluid-fluid immiscibility, 51
fluorescence energy transfer, 51
fluorescence recovery after photobleaching (FRAP), 22, 129
flux-ratio, 184, 185
free volume distribution, 178
fusion, 23
fusion pore, 161, 164, 165
fusion proteins, 162

gangliosides, 150
gauche conformations, 25, 26
gaussian curvature, 63
gel-fluid phase coexistence, 44, 52, 54
gel-fluid phase transition, 74
general anesthesia, 69
Gibbs surface excess, 125
glucose transporter, 156
glycophorin, 85
glycophorin dimers, 87
glycoprotein diffusion, 38
glycosphingolipids, 171
glycosyl-phosphatidylinositol anchors, 150
Golgi complex, 23
GPI-coupled hemagglutinin, 163
GPI-linked proteins, 151
gramicidin, 28, 67, 68, 77–83, 97

helical hairpins, 85
helix packing, 118
helix stability in bilayers, 85
helix tilt, 120
helix-coil transition, 86
helix-helix interaction, 86, 87
helix-lipid interaction, 87
hemagglutinin, 162
hemifusion, 161
hemifusion diaphragm, 162, 163
hexagonal phase HII, 177
HIV-1 Gag, 125
host defense peptides, 89
hydrocarbon core, 105
hydrogen bonds, 86
hydrophobic binding energy, 124
hydrophobic effect, 86, 106
    non-classical, 105
hydrophobic interactions, 74, 79, 81, 82, 123

hydrophobic length, 96, 131
hydrophobic matching, 28, 52, 53, 91, 100, 121, 130–132
hydrophobic partitioning, 124
hydrophobic slippage, 82
hydrophobic thickness, 131
hydrophobic/hydrophilic boundary, 81

inactivation kinetics, 110
indole ring, 105
infrared spectroscopy, 51
insulin, 157
integral membrane proteins, 17, 57, 78
  self-association, 135
integrins, 38
intercompartmental hop diffusion, 142
interfacial hydrophobicity scale, 103
interfacial solvation, 105
inverse hexagonal phase, 198
inverted non-lamellar phases, 177–179

kinks ($g^{\pm}tg^{\mp}$), 25

lag period, 44
lanosterol, 73
large unilamellar vesicle, 203
large-scale behavior, 195
laser trap, 22
laser tweezers, 140, 142
lateral associations
  transient, 22
lateral diffusion, 22, 58, 131, 142
lateral heterogeneity, 49
lateral pressures, 70
lateral stress profile, 29
leaky epithelia, 182
length-scales, 49, 58
line tension, 36
lipid acyl chain configurations, 71
lipid acyl chain length, 117, 131
lipid acyl chain mismatch, 44, 200
lipid acyl chain order, 199
lipid assymmetry, 169
lipid bilayer
  DPPC-cholesterol, 70–72
  DPPC-lanesterol, 74
  hydrophobic thickness, 120–121, 198
  material properties, 78
lipid bilayer compositional asymmetry, 58
lipid bilayer stability/instability, 83, 198
lipid distribution
  lateral heterogeneity, 130
lipid domains, 21
lipid exchange, 120
lipid fluidity, 118
lipid metabolites, 43
lipid organization, 44
lipid packing and lipid synthesis, 178
lipid phase coexistence, 59
lipid phase equilibria, 198
lipid phase preference and cell growth, 178
lipid phase transition, 29, 118
lipid scrambling, 172
lipid second messengers, 178
lipid selectivity, 52
lipid structural diversity, 130
lipid-cholesterol interactions, 71
lipid-lipid interactions, 27
lipid-protein interactions, 27, 74, 119, 135, 179
  cooperative, 50
  selectivity, 27
lipid-protein interface, 27, 52
lipid-synthesizing enzymes, 197–201
lipid-water interface, 68
lipids
  bilayer-forming, 200
  non-bilayer prone, 199, 207
local anaesthetics, 27
lysophospholipids, 45

magainin, 89, 91
magnetotaxis, 16
MARCKS, 125
melittin, 28, 67, 103
membrane adhesion, 29
membrane bending
  lipid translocation, 172
membrane bleb formation, 39, 40
membrane defects, 96
membrane deformation energy, 45
membrane domains, 21–23, 129, 139, 149, 150
  adhesion induced, 35
  definition, 21, 130
  detergent resistant, 149
  transient, 23
membrane dynamics, 39

membrane evolution, 72
membrane fluidity, 10, 17
membrane fusion, 161–166
  linkage model, 164
  spontaneous curvature, 165
  stunted, 166
membrane interface, 125
membrane lattice, 44
membrane lipid composition, 178
membrane mechanical stress, 45
membrane model
  formulation, 67–75
  history, 7–10
  principles, 7–11, 25–29, 67–75
membrane protein assembly, 110, 114
membrane protein conformation, 78, 199
membrane protein folding, 85
membrane proteins, 142
  basic, 27
  cooperative assembly, 143
  macroscopic diffusion coefficient, 140
  microscopic diffusion coefficient, 140
  oligomeric, 109–114
  polarized distribution, 139
  polytopic, 28, 109–114
membrane rafts, 39
membrane rigidity, 147
membrane skeletal network, 141
membrane skeleton, 22, 142, 143
membrane structure
  dynamic, 58
membrane surface, 26
membrane tension, 35, 39
membrane thickness, 39
membrane thinning, 91
membrane vesicles, 23
membrane-cytoskeleton linkage, 146
membrane-membrane interactions, 29
membrane-skeleton fence model, 141, 142
membrane-spanning domain, 78
membranes
  as fluid mosaics, 37
  tension free, 33
membranes and signal transduction, 150
metarhodopsin I, 18
metarhodopsin II, 18
microfilament cytoskeleton, 150
minimal models, 67–75
mismatch interactions, 74
model
  membrane, 11, 68, 69, 203, 204, 207
molecular dynamics, 67
molecular shape, 198
molten-globule, 26
Monte Carlo simulation, 52, 137
myelin basic protein, 26
myristoyl-electrostatic switch, 125
myristoylated peptides, 124

Na^+,K^+-ATPase, 28, 155–159, 184
  conformational modifications, 158
Na^+-recirculation, 181, 182, 187
nano-scale heterogeneity, 49–54
near-field scanning optical microscopy, 22
negative curvature, 18, 38, 178
neutron scattering, 91
nicotinic acetylcholine receptor, 53
non-annular sites, 119
non-equilibrium phenomena, 11, 195
non-equilibrium rearrangements, 52
non-equilibrium steady state, 50
non-ideal mixing, 59
non-lamellar lipid phases, 29
non-random organization, 10
numerical simulations vs analytic results, 74–75

off-lattice model, 71
Optimization of Physics Hypothesis, 15
oriented circular dichroism, 90

peptide adsorption, 93
peptide bonds, 103
peptide orientation, 90
peptide pores, 91
peptide-induced phase transition, 99
peptide-lipid interactions, 99
peptide-membrane interactions, 91
percolation properties, 52
permeability barrier, 57
Pf1 coat proteins, 67
phase coexistence, 44, 45
phase diagram, 45, 69
  DPPC-cholesterol, 70–72
  DPPC-lanosterol, 73
phase equilibria, 52

lamellar-to-nonlamellar, 200
phase separation, 45, 50, 68
lateral, 45
phase transition, 27
phosphatidylethanolamine, 177
phosphatidylethanolamine-PEG, 205
phosphatidylserine, 45, 53
phosphoinositides, 169
phospholipase A_2, 43–46, 52
phospholipase C, 53, 178
phospholipid bilayer
liquid crystalline, 118
phospholipid binding constants, 120
phospholipid exchange proteins, 171
phospholipid flippases, 170
phospholipid headgroup, 118
phospholipid molecules, 26
phospholipids
annular, 137
Poisson-Boltzmann equation, 124
polar head group, 125
poly-unsaturated fatty acyl chains, 18
polyethylene glycol, 205
porins, 28, 86
potassium channels, 109–114
protein aggregation, 44, 130, 132
protein anchoring, 28
protein array formation, 135–138
protein conformational change, 11, 79, 82
protein inclusions, 74
protein kinase C, 43–46, 53
protein-lipid interactions, 130, 132
protein-protein association, 59
protein-protein interactions, 113, 130, 132
proteins
membrane association, 26
segregation of, 23
selective retention of, 23
pumps, 195

radius of curvature, 35
receptor clusters, 150
receptor-mediated signaling, 151
receptors, 80
red blood cell membrane, 139–143
red blood cell membrane composition, 146
red blood cell membrane mechanical stability, 147
red blood cell shape, 63
red blood cells, 61
rhodopsin, 18
rotational diffusion, 58
rotational isomerism, 75

secondary structure formation, 104
self-assembly, 68
self-consistent field, 69
semi-local scale behavior, 196
sensing lipid bilayer properties, 199
shape instability, 64
shape transitions, 61
shear elasticity, 34, 38, 63
side chain partitioning
free energy of, 105
signaling, 10
Singer-Nicolson model, 7
single particle tracking, 130, 140
small-scale behavior, 195
small-scale structure, 49, 53
smectic liquid crystals, 33
solute-coupled water transport, 182
spectrin, 40, 141, 146
spectrin-based membrane skeleton, 147
sphingomyelin, 150, 169
splay, 92
spontaneous curvature, 29, 35, 62, 74, 75, 92, 173, 199
spring constant, 79
Src-family tyrosine kinases, 150
sterols, 72
strain-free membrane, 17
structural coupling between proteins and lipids, 95
structural organization
dynamic, 49
small-scale, 52
static, 49
surface charge density, 52, 198
surface tension, 173
surface undulations, 173

thermal disorder, 103
thermal shape fluctuations, 62
thermal thickness of the bilayer/solution interface, 103
time-scales, 49, 58

transbilayer helical organization, 87
transferrin receptors, 140, 142
transient pores, 93
transmembrane lipid traffic, 171
transport capacity, 155
triggering, 10
tryptophan, 28, 96, 97, 105
tryptophan-anchored peptides, 99
turnover number, 157

undulation forces, 33
unit membrane model, 7

vesicle budding, 33
vesicle shape, 61–64
    mechanical, 62
vincristine, 205

WALP peptides, 97
wetting, 75

Title. – Titles should be kept as short as possible and with an emphasis on words useful for indexing and information retrieval.

Abstract, Summary. – An abstract in English is compulsory. It should count 10-15 lines, outline main features, stress novel information and conclusions, and end with the author's name, title, and institutional and/or private postal address. – Papers in Danish may be provided with a summary in another language by agreement between author and Editor.

Typescript. – Page 1 should contain title, author's name and the name of the Academy. Page 2: Abstract, author's name and address. Page 3: Table of contents if necessary. Captions should be supplied on separate sheets. Footnotes should be avoided if at all possible; if indispensable, they, too, should be typed on separate sheets. Consult a *recent* issue of the series for general layout.

Typewrite with double space throughout and leave a 4 cm margin *right.* Indicate desired position of illustrations and tables with pencil in margin *and repeat it in the galley proof.*

Use three or fewer grades of heading unless more are indispensable. Avoid long headings. Indicate clearly the hierarchy of headings.

Figures. – Please submit two copies of each graph, map, photograph, etc., all marked with the author's name. Whenever possible all figures will be placed within the text; the nature of the illustrations will govern the Editor's choice of paper quality.

All figures, also line drawings, must be submitted as glossy, photographic prints suitable for direct reproduction. Prints fitting the indicated printed area are preferred, but the final size is the responsibility of the Editor. The scale should be indicated in the caption or, preferably, on the illustration itself.

Fold-out figures and tables should be avoided. Use distinct (but not dominant) capital letters for the items in composite figures. For transfer lettering use simple, semi-bold typefaces. The size of the smallest letters should not be less than 1.5 mm. Intricate tables are often more easily reproduced from line-drawings or from technically perfect original computer or type processor output.

References. – In general, the Editor expects all references to be formally consistent and in accordance with accepted practice within the particular field of research. Bibliographical references should preferably be given as, e.g., Shergold 1975, 16, the latter figure indicating the page number unless misunderstandable.

Correspondence

Manuscripts should be sent to the Editor, Det Kongelige Danske Videnskabernes Selskab, H. C. Andersens Boulevard 35, DK-1553 Copenhagen V, Denmark (tlf. +45 33 11 32 40). Questions concerning subscription to the series should be directed to the publishers.

Publisher

Munksgaard Export and Subscription Service
Nørre Søgade 35, DK-1370 Copenhagen K, Denmark

Editor: Poul Lindegård Hjorth

Biologiske Skrifter

Biol. Skr. Dan. Vid. Selsk.

Priser excl. moms/Prices abroad in Danish Crowns

Vol.

25 Six Papers in the Biological Sciences, being Part Two of *Sixteen Research Reports* by the Niels Bohr Fellows of the Royal Danish Academy of Sciences and Letters, published on the Occasion of the Centenary of Niels Bohr. 1985 ..... 200.-
(*Sixteen Research Reports* Part One is identical with: Ten Papers in the Exact Sciences and Geology, *Matematisk-fysiske Meddelelser* 41. 1985, 400.-)

26 JENSEN, HANS ARNE: Seeds and other Diaspores in Soil Samples from Danish Town and Monastery Excavations, dated 700-1536 AD. 1986 ..... 200.-

27 NILSSON, JYTTE R.: The African Heterotrich Ciliate, *Stentor andreseni* sp.nov., and *S. amethystinus* Leidy. A Comparative Ultrastructural Study. 1986 ..... 100.-

28 WUNDERLIN, RICHARD; LARSEN, KAI; and LARSEN, SUPEE SAKSUWAN: Reorganization of the *Cercideae (Fabaceae: Caesalpinioideae)*. 1987 ..... 80.-

29 JENSEN, HANS ARNE: Macrofossils and their Contribution to the History of the Spermatophyte Flora in Southern Scandinavia from 13000 BP to 1536 AD. 1987 ..... 200.-

30 DYCK, JAN: Structure and Light Reflection of Green Feathers of Fruit Doves *(Ptilinopus spp.)* and an Imperial Pigeon *(Ducula concinna)*. 1987..... 100.-

31 FRIIS, ELSE MARIE; CRANE, PETER R.; PEDERSEN, KAJ RAUNSGAARD: Reproductive Structures of Cretaceous Platanaceae. 1988 ..... 100.-

32 WINGSTRAND, K. G.: Comparative Spermatology of the Crustacea Entomostraca 2. Subclass Ostracoda. 1988..... 250.-

33 MIKKELSEN, VALD. M.: The Commons of Rejnstrup, Denmark. 1989 ..... 50.-

34 ØLLGAARD, BENJAMIN: Index of the *Lycopodiaceae*. 1989..... 150.-

35 SRINIVASAN, VIJAYALAKSHMI; FRIIS, ELSE MARIE: Taxodiaceous conifers from the Upper Cretaceous of Sweden. 1989 ..... 100.-

36 FRIIS, ELSE MARIE: *Silvianthemum suecicum* gen. et sp. nov., a new saxifragalean flower from the Late Cretaceous of Sweden. 1990..... 70.-

37 MOESTRUP, ØJVIND; THOMSEN, HELGE A.: *Dictyocha speculum* (Silicoflagellata, Dictyochophyceae), studies on armoured and unarmoured stages. 1990..... 100.-

38 MIKKELSEN, VALD. M.: Borrelyngen on Bornholm, Denmark. 1990..... 100.-

39 JØRGENSEN, C. BARKER: Water Economy in the Life of a Terrestrial Anuran, the Toad *Bufo bufo*. 1991..... 70.-

40 HANSEN, MICHAEL: The Hydrophiloid Beetles. Phylogeny, Classification and a Revision of the Genera (Coleoptera, Hydrophiloidea). 1991 ..... 700.-

41 FRIIS, ELSE MARIE; PEDERSEN, KAJ RAUNSGAARD; CRANE, PETER R.: *Esqueiria* gen. nov., fossil flowers with combretaceous features from the Late Cretaceous of Portugal. 1992 ..... 90.-

42 HENNINGSEN, KNUD W.; BOYNTON, JOHN E.; WETTSTEIN, DITER VON: Mutants at *xantha* and *albina* Loci in Relation to Chloroplast Biogenesis in Barley (*Hordeum vulgare* L.). 1993..... 700.-

43 Brain and Mind. Symposium on the Occasion of the 250th Anniversary of The Royal Danish Academy of Sciences and Letters August 17-20, 1992. Ed. by RODNEY M. J. COTTERILL. 1994..... 400.-

44 MIKKELSEN, VALD. M.: Borrelyngen on Bornholm, Denmark. 2. 1994..... 100.-

45 COTTERILL, RODNEY M.J.: Autism, Intelligence and Consciousness. 1994..... 150.-

46 BARFOD, ANDERS S.; KVIST, LARS PETER: Comparative Ethnobotanical Studies of the Amerindian Groups in Coastal Ecuador. 1996 ..... 300.-

47 HANSEN, BERTEL; LARSEN, KAI; OLSEN, SVEN-ERIK SANDERMANN: Protologues in seed catalogues from Botanic Garden Copenhagen 1843-1875. 1997..... 90.-

48 HANSEN, MICHAEL: Phylogeny and classification of the staphyliniform beetle families (Coleoptera). 1997..... 600.-

49 In Search of a New Biomembrane Model, edited by Ole G. Mouritsen & Olaf Sparre Andersen 1998 200,-

Printed in Denmark by Special-Trykkeriet Viborg a-s. ISSN 0366-3612. ISBN 87-7304-292-7