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

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*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.

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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 $\alpha$-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 $\beta$-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 $\alpha$-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 $\alpha$-helices; 2) the presence of $\beta$-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 $\beta$-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 $\beta$-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).

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