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

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"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.

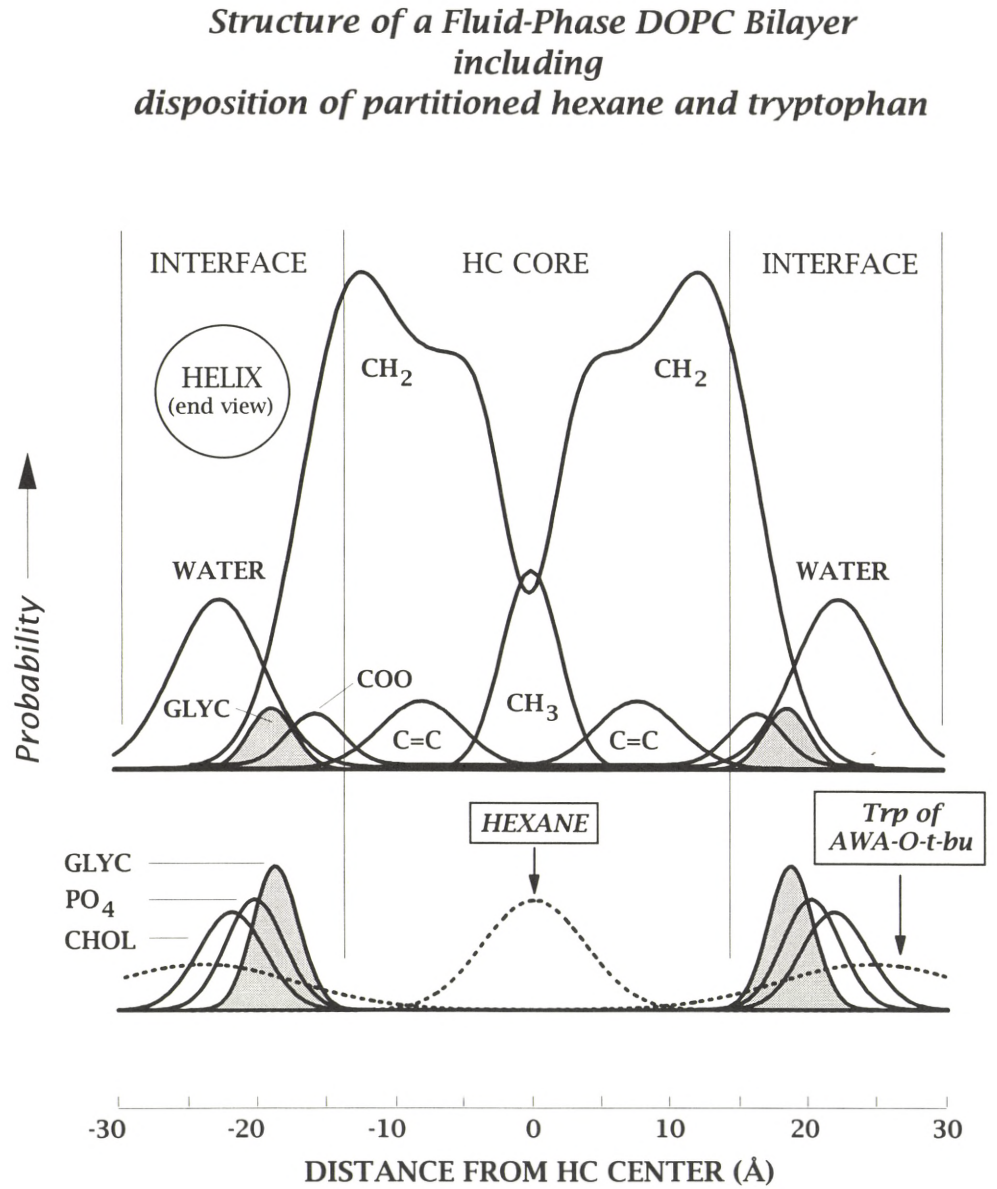

Figure 1. The transbilayer probability distribution functions for the principle structural groups of dioleoylphosphatidylcholine (DOPC) at 66% RH (5.4 waters/lipid) determined by x-ray and neutron diffraction (Wiener and White, 1992; White and Wimley, 1994; White and Wiener, 1996). The areas under the 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-

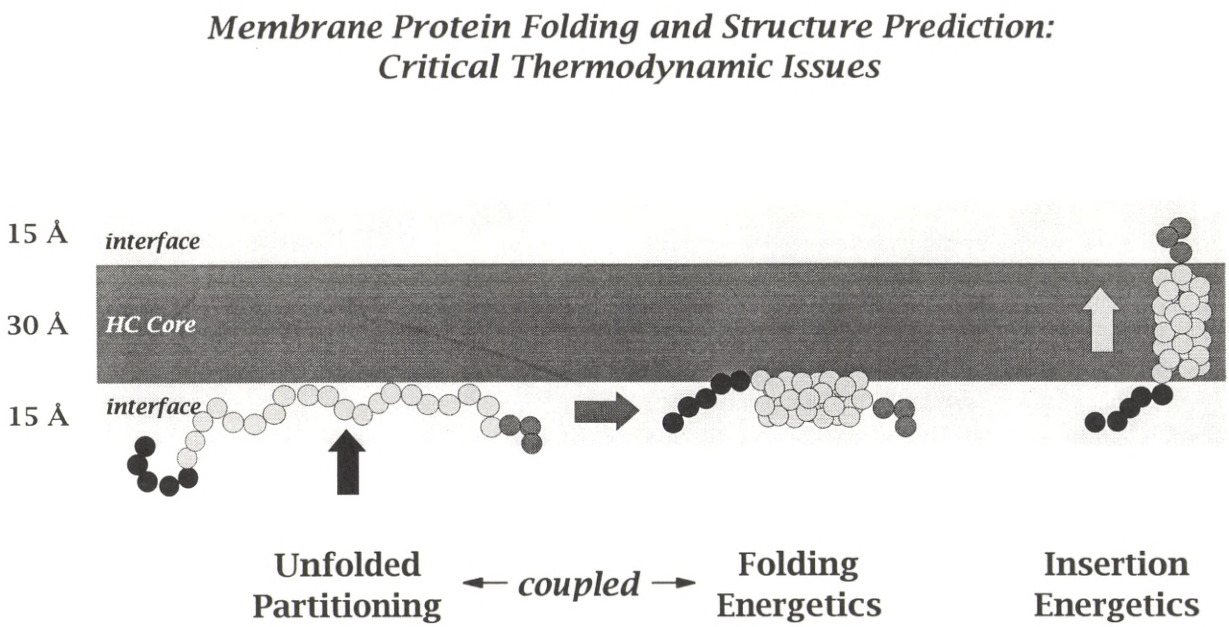

Figure 2. Summary of the critical thermodynamic issues of membrane protein folding and structure prediction. The figure emphasizes the three major classes of thermodynamic measurements that must be made in order to describe the stability of membrane proteins.

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

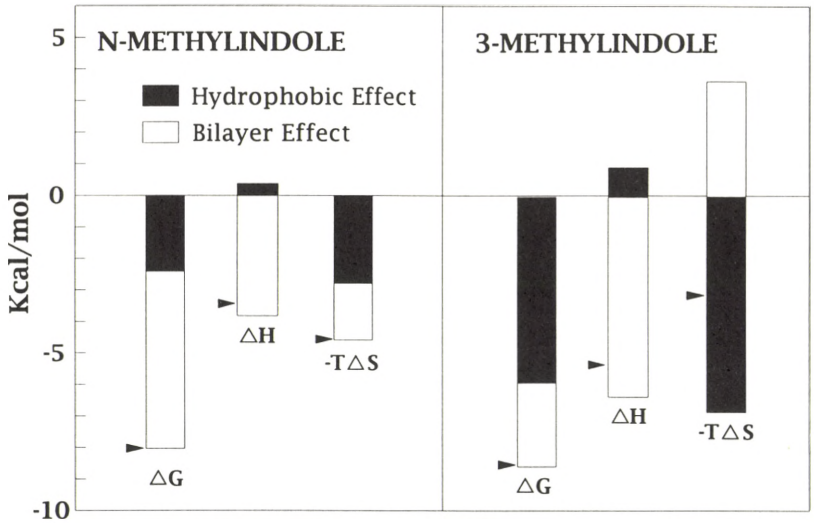

Figure 3. Relative contributions of the hydrophobic effect and so-called bilayer effects to the thermodynamics of the partitioning of *N*-methylindole and 3-methylindole into palmitoyloleoylphosphatidylcholine (POPC) bilayers. The net free energies (ΔG), enthalpies (ΔH), and the entropic (ΔS) contributions 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."

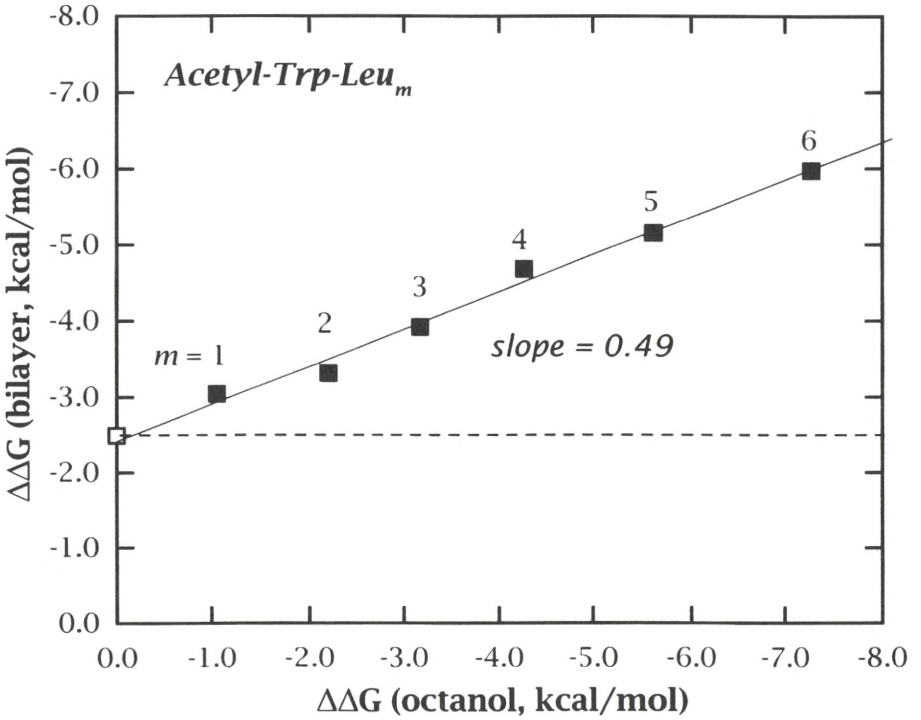

Figure 4. Comparison of the free energies of transfer of the peptides Ac-Trp-Leu$_m$ (m = 1 to 6) from water to POPC bilayer interfaces and from water to n-octanol. The slope of 0.49 indicates that the hydrophobic-contribution to partitioning into bilayers is apparently only half that expected for partitioning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

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