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Peptides in lipid bilayers: structural and thermodynamic basis for partitioning and folding

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The lipid bilayer is commonly, but incorrectly, viewed as a slab of bulk hydrocarbon liquid bounded by thin polar regions. Recent experimental and theoretical advances reveal the true complexities of the bilayer and its interactions with peptides. These are reviewed in the context of five fundamental questions that must be addressed in order to arrive at a structural and thermodynamic framework that is useful for studies of a broad array of biological processes.

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Introduction

Interactions between membranes and polypeptides are central to the insertion and folding of membrane proteins, the breaching of membrane barriers by toxins, the action of antibiotic peptides, and the interaction of hormones with membrane receptors. Although the interactions among these processes undoubtedly vary in many details, they all must involve, at an early stage, partitioning between water and a lipid bilayer. Unfortunately, this problem is very much like the protein folding problem, in that the general principles are understood, but the details necessary for quantitative predictions remain elusive.

The purpose of this review is to discuss progress being made on five fundamental questions which we think must be answered in order to arrive at a quantitative structural and thermodynamic framework for studies of the various biological processes. Many additional questions can be raised concerning such things as membrane lipid composition, bilayer phase behaviour, and the secondary structure propensities of oligopeptides but, in our view, such questions cannot be attacked effectively without answers to the five questions posed. We therefore focus narrowly in this review on papers of the last three or four years that are immediately relevant to the five questions below, or which provide novel approaches to their study.

What is the structure of fluid bilayers with respect to partitioning?

The thermal disorder associated with fluid bilayers precludes the possibility of obtaining atomic-resolu-

tion crystallographic images as can be done for crystallized membrane lipids [1,2^{••}]. For this reason, the 'structure' of a fluid bilayer is defined operationally as the time-averaged spatial distributions of the principal structural groups (such as carbonyls and phosphates) projected onto an axis normal to the bilayer plane [3^{••}]. Such structural images can be obtained from oriented multilamellar arrays of phospholipid bilayers by means of X-ray and neutron diffraction measurements using lipids labelled at specific sites with heavy metals [4–6] or deuterium [7–9]. Weiner, White and colleagues [3^{••},6,9,10[•],11,12^{••}] have developed a joint refinement procedure that allows one to combine X-ray and neutron data to arrive at fully-resolved images of fluid bilayers.

The first joint-refinement image obtained from dioleoylphosphocholine (DOPC) bilayers, shown in Fig. 1, provides a realistic and informative view of a fluid bilayer [12^{••}]. Time-averaged transbilayer distributions of the principal structural groups shown are equivalent to probability densities from which the probability of finding a particular structural group at a specific location can be determined. Areas under the peaks are equal to the number of structural groups (e.g. 28 methylenes) per lipid molecule.

Two aspects of these images require emphasis. First, the widths and positions of the peaks can be determined with high precision (0.02–0.5 Å). Second, the distributions are fully resolved images and are therefore accurate representations of the true thermal motion of the molecules [3]. This motion is a fundamental feature of fluid bilayers that must affect peptide-bilayer interactions.

From the perspective of peptide partitioning, the structure shown in Fig. 1 reveals the true complexity of the

Abbreviations

DOPC—dioleoylphosphocholine; FHCVF—Flory–Huggins-corrected volume-fraction units; FTIR—Fourier transform infra red; HCR—hydrocarbon core regions; IFR—interfacial regions; MF—mole-fraction.

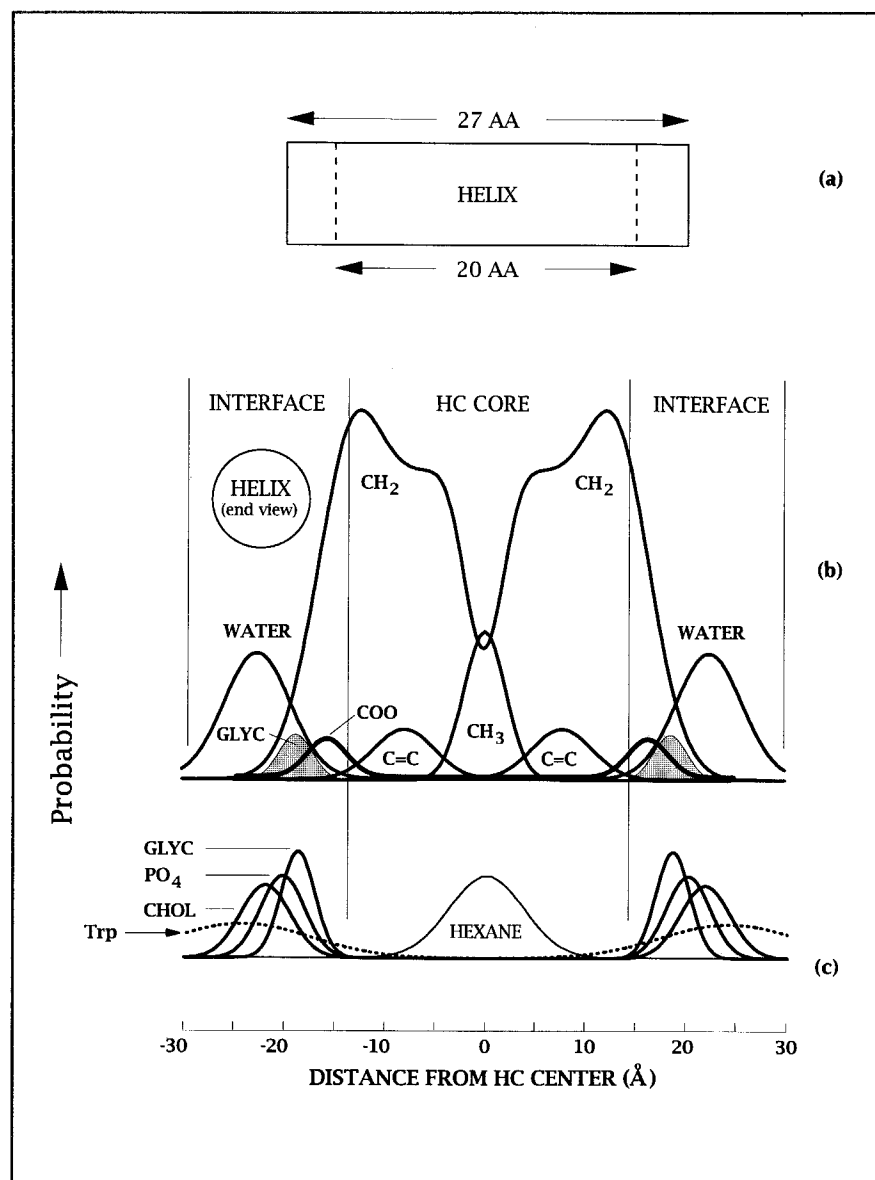


Fig. 1. The structure of a fluid DOPC bilayer determined by the joint refinement of X-ray and neutron diffraction data [12••]. The 'structure' consists of the time-averaged distribution of the principal structural groups of the lipid projected onto an axis normal to the bilayer plane. The distributions are constructed from Gaussian distributions whose areas equal the number of structural groups represented by the Gaussians; the distributions therefore represent the probability of finding a structural group at a particular location. (a) A representation of the length of a 27 amino acid transbilayer helix. (b) The distributions of the methyl (CH₃), methylene (CH₂), double bonds (C=C), carbonyls (COO), glycerol (GLYC), and water. The interfaces of the bilayer are defined as the regions occupied by water. Notice that an α -helix that is parallel to the bilayer can be comfortably accommodated in the interfaces. (c) The distributions of the glycerol, choline (CHOL), and phosphate (PO₄) groups. Also shown are the distributions of hexane [14] and the tryptophan of Ala-Trp-Ala-O-*tert*-butyl [22••] partitioned into the bilayer. The figure is that of White [74] modified to include the Trp distribution.

bilayer as a 'non-polar' phase. The image has been divided into interfacial (IFR) and hydrocarbon core (HCR) regions based upon the distribution of water. The HCR and the combined IFR each have a width of about 30 Å, so that the IFR account for 50% of the total thermal thickness of the bilayer. This refutes the widely held idea that the bilayer may be treated as a thin hydrocarbon slab separating two aqueous phases. Further, the structure of the IFR shows that they are a complex mixture of water and phosphocholine, glycerol, carbonyl and methylene groups. Even the HCR is not chemically uniform because of the varying content of methyl and ethylene groups and the anisotropy of the acyl chain motions [13]. HCR heterogeneity and anisotropy is demonstrated by the transbilayer distribution of dissolved hexane ([14]; Fig. 1c). A final important point concerns the thermal thickness of an interface relative to the dimensions of an α -helix (diameter $\approx$ 10 Å). A helix running parallel to the bilayer plane can

be comfortably accommodated within the 15 Å thickness of an interface.

The bilayer image just described is for a single lipid species at low hydration. In the future, bilayers formed from a variety of lipids over a broad range of hydrations must be examined. Our reliance on direct structural methods may eventually be reduced by the use of molecular dynamics and related simulations [15–20]. At the moment, however, simulations suffer from three problems. First, simulations of bilayers are computationally extremely demanding because of the large numbers of lipid and water molecules required to achieve meaningful results. Second, simulations presently extended over only a few tens, or hundreds, of picoseconds whereas lipids in bilayers execute motions over timescales that vary from nanoseconds for *gauche-trans* isomerizations to microseconds for whole-body motions. Third, none of the results of

bilayer simulations have been confirmed by rigorous comparisons to experiments such as those of Weiner and White [12••]. Until this is done, there can be no assurance that a particular protocol is satisfactory.

Where do peptides reside when partitioned into bilayers and what effects do they have on bilayer structure?

Even the most hydrophobic polypeptides cannot penetrate deeply into bilayers unless the peptide C=O and NH groups are hydrogen bonded [21,22••]. Thus, one generally expects peptides interacting with bilayers to be found near the bilayer surface, unless the hydrogen bonds can be satisfied internally as with an α -helix. Direct structural measurements both at the core and surface of bilayers have been made. Jacobs and White [22••,23,24] examined the interactions with bilayers of the tripeptides Ala-X-Ala-*O-tert*-butyl (where X = Gly, Ala, Leu, Phe, or Trp) which are too small to have secondary structure in solution. Neutron diffraction revealed that the tryptophan peptide in DOPC bilayers was confined to the interface regions, with the deuterated tryptophan spending much more time in the vicinity of the headgroups and water than in the hydrocarbon core ([22••]; Fig. 1c).

Brown and Huestis [25••] showed by means of NMR that the *tert*-butyl group of the Phe peptide contacts the hydrocarbon region, while the amino terminus contacts the aqueous phase with the Phe residue in the vicinity of the carbonyl groups. Furthermore, the partitioning was found to impose structure on the peptide in the form of preferred association between the Phe2 and Ala3 residues.

Direct X-ray diffraction measurements demonstrate that the model α -helical peptides Lys-Lys-Gly-Leu_n-Lys-Lys-Ala-amide (where $n = 16$ or 24) [26] span the bilayer [27•]. He *et al.* [28] used in-plane diffraction methods to show that gramicidin channels are randomly distributed in the plane of oriented bilayers. Several non-diffraction methods that show exceptional promise as tools for probing the structure of peptides in bilayers include oriented circular dichroism [29•,30], attenuated total reflection infra red spectroscopy [31•,32], quenching of peptide Trp fluorescence by lipid-bound quenchers [33•,34•], two- and three-dimensional NMR [35•], and so-called site-directed spin labelling [36•]. The recent determination of the structure of gramicidin A in oriented lipid multilayers by means of solid-state NMR is a particularly important accomplishment [37••].

Peptides in bilayers invariably affect the lipids. Zhang *et al.* [38] used Fourier transform infra red (FTIR) spectroscopy and differential scanning calorimetry to show that the Lys-Lys-Gly-Leu_n-Lys-Lys-Ala-amide peptides affect lipid acyl chains in their vicinity in a way that depends upon the acyl chain structure. Ala-X-Ala-*O-tert*-bu peptides affect the phase behaviour and acyl chain

motions of bilayers according to the size of the X residue [23,24]. The Trp peptide causes the thickness of DOPC bilayers to decrease, the acyl chain disorder to increase, and the phosphocholine headgroup to re-orient slightly [22••]. Partition coefficient measurements show that partitioning increases in proportion to accessible surface area of the X residue and is driven solely by entropy, which suggests that the hydrophobic effect is an important contributor to the association [22••]. However, NMR measurements demonstrate additional specific electrostatic interactions between the amino terminus and the lipid phosphate [25••]. Together, these measurements indicate that the complex interfaces of bilayers provide a favorable thermodynamic environment for peptides and thereby emphasize that the bilayer cannot, and should not, be treated as a simple hydrocarbon slab.

What system of units is appropriate for calculating partition coefficients and therefore the thermodynamics of partitioning?

The assumption has been made for many years that simple mole-fraction (MF) partition coefficient units should be used for calculating the thermodynamic parameters of partitioning processes [39]. However, two important papers [40••,41••] suggest that Flory-Huggins-corrected volume-fraction units (FHCVF) are more appropriate [42]:

$$\Delta G_{w \rightarrow b} = -RT \ln(K_V) + RT V_s (1/V_b - 1/V_w) \quad (1)$$

Eqn. 1 describes the free energy of transfer from water to bilayer where K_V is the volume-fraction partition coefficient and V_s , V_b , and V_w are the partial molar volumes of solute, lipid molecule, and water, respectively. The critical issue in partitioning is how one accounts for the simple entropy of mixing (cratic entropy), that is unrelated to entropy changes specific to the solute-solvent molecular interactions. MF units assume, implicitly, that solute and solvent molecules have the same molecular volumes, so that the entropy is due to simple combinatorial statistics. However, for solute molecules that are large compared to solvent molecules, the total volume of the solution is larger than expected on a MF basis, so that the total volume available per solvent molecule is increased. This causes the entropy to be higher than that calculated on a MF basis. The upshot of such considerations is that the Flory-Huggins correction term (right hand side of Eqn. 1) [41••] 'favors the dissolution of a large solute in a small solvent'. This means that the free energy cost of transferring a solute from water (the smallest known solvent) to any other phase consisting of larger solvent molecules is much higher than expected on a MF basis.

De Young and Dill [40••] found that the partition coefficients for benzene between water and *n*-alkanes were independent of alkane length only if FHCVF units were

used. The consequence of this is that the so-called solvation parameter C_s in the equation:

$$\Delta G_t = C_s \cdot A_a \quad (2)$$

used for calculating the free energy of transfer ΔG_t of a solute with an accessible surface area A_a from a non-polar phase to water is drastically altered. Based upon MF units, the usual value of C_s is $25 \text{ cal mol}^{-1} \text{ \AA}^{-2}$, whereas the FHCVF coefficients yield $43 \text{ cal mol}^{-1} \text{ \AA}^{-2}$. The correct value of C_s is extremely important in considerations of the absolute free energy changes associated with hydrophobic partitioning into bilayers, and with protein folding. At present, the question of the general use of FHCVF units is highly controversial for two reasons [44•]. First, theory cannot be directly tested in bulk-phase partitioning experiments because entropy, and hence free energy, cannot be measured directly; they can only be determined by calculation. Second, most partitioning experiments involve relatively small solute molecules for which the Flory-Huggins correction is not extremely large. This is shown in Fig. 2a, in which the change $\Delta\Delta G$ of the calculated water to non-polar phase ΔG relative to the MF value has been calculated for different solute and non-polar solvent molecular volumes. Notice that $\Delta\Delta G$ for small molecular volumes is relatively small, whereas for large molecular volumes it can be immense.

The appropriateness of FHCVF units could be more readily examined if a sufficiently large range of solvent and solute molecular volumes could be studied. Partitioning of polypeptides of different sizes into non-polar phases is an attractive approach, but is not feasible for bulk phases because of the energetic costs of transferring backbone carbonyl and amide groups. However, such experiments are feasible for bilayer phases because non-polar residues can satisfy hydrophobic interactions at the interface while the polar backbone residues maintain contact with the aqueous phase [22•]. Because the hydrophobic effect is the main driving force for partitioning onto neutral lipid bilayers, ΔG_t is expected to be a linear function of a peptide's A_a (Eqn. 2). We were therefore surprised when we analysed peptide MF partitioning data available in the literature ([22•, 45–58]; W Wimley and S White, unpublished data) for oligopeptides of various sizes and hydrophobicities. As can be seen in Fig. 2b, the ΔG_t for larger peptides saturates at about -8 kcal mol^{-1} and is largely independent of A_a . Re-analysis of the data using FHCVF units, however, yields values of ΔG_t that increase linearly with A_a . Under those circumstances, ΔG_t becomes extraordinarily large for large peptides, because of their large molecular volumes (Fig. 2a) which, of course, goes hand in hand with large accessible surface areas.

The two curves of Fig. 2b represent two very different testable hypotheses. The MF curve suggests that a fixed amount of accessible surface area is buried for peptides that are longer than about 10 amino acids. Some situations in which this might occur include 'tethering' of extended peptides to the interface by 10 or so of the amino acids, binding of partially folded peptides, or

micellization of the peptides. The FHCVF curve, on the other hand, suggests that an amount of accessible area is buried that is proportional to peptide length. These two hypotheses can be distinguished through measurements of the heat capacity change (ΔC_p) of partitioning because ΔC_p is proportional to the amount of accessible surface area that is buried [59•, 60, 61]. The first hypothesis seems unlikely to us because it would require that the partial folding or micellization of peptides be identical for peptides that vary widely in their proportions of charged and non-polar residues. If the second hypothesis is indeed correct, the data of Fig. 2b provide strong circumstantial evidence favoring the use of FHCVF units. Further, bilayer partitioning experiments would become extremely useful for understanding the fundamentals of protein folding in two ways. First, the bilayer interface is an ideal 'phase' for examining the hydrophobic partitioning of large oligopeptides. Second, because solutes at the interface can simultaneously satisfy polar and non-polar interactions, the hydrophobic effect contributions to folding can, in principle, be separated from polar contributions such as hydrogen bonding.

How can the relative contributions to partitioning of the hydrophobic effect, bilayer behaviour, and electrostatic interactions be established?

Lipid bilayers, like folded proteins, sit in a free energy minimum that results from small differences of very large energy and entropy terms. Thus, any perturbation of the bilayer may cause significant changes in the free energy of the system compared to the free energy changes expected on the basis of partitioning into bulk non-polar phases. The question thus arises as to how 'bilayer effect' free energy changes can be separated from the ones due solely to the hydrophobic effect. It has been known for years that the partitioning of hydrophobic and amphiphilic molecules into bilayers is often accompanied by large enthalpy changes not seen in bulk-phase partitioning [62]. We have observed, for example, that the partitioning of *N*-methylindole into cyclohexane gives $\Delta G = -7.2 \text{ kcal mol}^{-1}$, $\Delta H = +1.1 \text{ kcal mol}^{-1}$, and $\Delta S = 27.7 \text{ cal mol}^{-1} \text{ K}^{-1}$, whereas partitioning into bilayers yields values of $-8.1 \text{ kcal mol}^{-1}$, $-3.4 \text{ kcal mol}^{-1}$ and $15.5 \text{ cal mol}^{-1} \text{ K}^{-1}$, respectively [63, 59•]. The large ΔH that is unexpected from hydrophobic effect considerations has given rise to the concept of 'non-classical' hydrophobic effect, [62] and the possibility that partitioning into bilayers in some cases may be driven solely by thermodynamic changes in the bilayer [62, 64•].

Confusion over the nature of the hydrophobic effect in bilayers arises in part from considering the consequences of the hydrophobic effect solely in terms

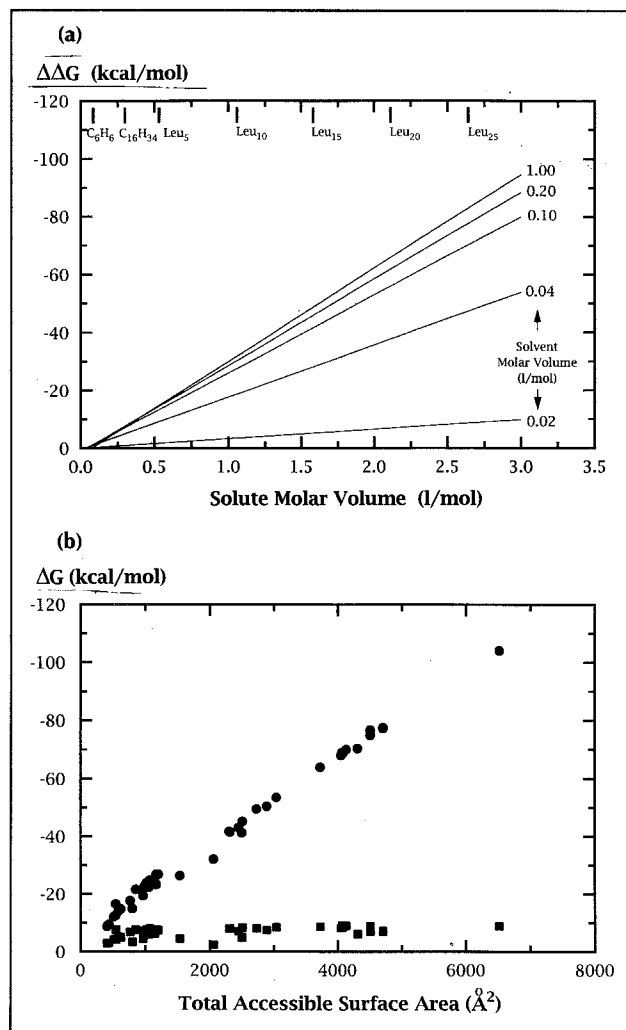


Fig. 2. Effects of the use of Flory-Huggins-corrected volume-fraction partition coefficients (FHCVF) for the calculation of the free energy of transfer ΔG of a solute from water to a non-polar phase ('solvent'). The figures compare results of FHCVF with those obtained using mole-fraction partition coefficients (MF). (a) $\Delta\Delta G$ is the change in the calculated ΔG (Eqn. 1) using FHCVF units relative to MF units. The calculations assume a fixed mole-fraction concentration of the solute in the water and non-polar solvent phase. Marked at the top of the figure are the molecular volumes of benzene (C_6H_6) and hexadecane ($C_{16}H_{34}$) and poly-leucine peptides (Leu_n) with $n = 5, 10, 15, 20$ and 25 . (b) Comparison of values of ΔG for a wide variety of peptides from water to neutral lipid bilayers [22, 45–58] calculated using MF units (solid squares) and FHCVF units (solid circles). Partitioning is expected to be driven by the total accessible non-polar surface area of the peptides (Eqn. 2). Such a relation is seen only if FHCVF units are used.

of changes in enthalpy and entropy. In reality, the best measure of the hydrophobic effect is the heat capacity change associated with partitioning [60,61]. We therefore examined the temperature dependence of partitioning of a series of indole compounds into cyclohexane and bilayer phases and used the heat capacity changes to separate the hydrophobic-effect contributions from bilayer contributions [61•]. This basic approach should be useful for a wide range of solute and lipid types. However, in our experiments we used electrically neutral solutes and bilayers. Electro-

static interactions may not be easy to separate from bilayer effects although at least two laboratories are making good progress in that area [64•,65•–70•].

How does partitioning of peptides affect the formation of secondary structure?

Kaiser and Kezdy [71] showed many years ago that the binding of peptides that had little or no secondary structure in free solution to lipid bilayers, introduced secondary structure. In free solution, the entropy reduction associated with secondary structure formation causes the unfolded state to be favoured. However, theory shows that if a peptide chain associates with an interface, the probability of secondary structure formation increases drastically because the interface restricts the degrees of freedom of the peptide [72•,73•]. The unfavourable reduction in conformational entropy that accompanies binding at the interface is compensated for by the free energy decrease that drives partitioning [22•]. This simple idea suggests the possibility of quantitative rules for the design of peptides with specified partition coefficients and secondary structure propensities.

Engleman and Steitz [21] proposed that transbilayer helices were inserted as so-called helical hairpins, but did not address the question of how the hairpins were formed. Jacobs and White [22•] amended the hypothesis by proposing that hydrophobic and amphiphilic residues of extended protein chains bind at the interface and thereby induce the formation of helical hairpins on the bilayer surface. As noted earlier, the interface regions are thick enough to accommodate an α -helix that is parallel to the bilayer surface, [12•,74] and partitioning induces structure even in very small peptides [25]. Off-lattice Monte-Carlo simulations performed by Milik and Skolnick [75•,76•] indicate that this binding-folding-insertion model is feasible.

Conclusions

The fluid bilayer is a complex phase that cannot be treated thermodynamically, or structurally, as a thin slab of alkyl liquid into which peptides partition according to the simple rules observed for bulk phases. The equilibrium bilayer structure results from the complex interplay of large forces that can be perturbed by the partitioning of peptides. As a result, the global free energy change associated with partitioning involves 'bilayer effects' as well as hydrophobic and electrostatic interactions. The interfacial regions, described by Wiener and White [12•] as regions of "tumultuous chemical heterogeneity", are important regions for the binding of peptides and the induction of secondary structure. They are likely to play an important role in the folding and insertion of membrane proteins not only because they

are the first sites of contact, but also because of their special physical properties. Experimental and theoretical advances of the past several years encourage us to believe that the development of a structural and thermodynamic framework for the quantitative prediction of peptide binding and secondary structure formation is a realistic possibility.

Acknowledgements

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