CHAPTER 8

Free Energy Determinants of Peptide Association with Lipid Bilayers

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In this chapter we describe different contributions to the free energy of peptide–membrane association and review computational methods for evaluating their magnitude at the mean-field level. The nature of interaction of different peptides with the bilayer, as reflected by the relative importance of each of the free energy contributions to peptide–membrane association, correlates strongly with the location of the peptide in the bilayer. Thus, we classify peptides into three different categories depending on their location in the membrane. The first category includes peptides that interact mainly with the hydrocarbon region of the membrane, the second includes peptides that interact predominantly with the polar headgroup region, and the third includes peptides that interact with both. We provide a review of recent studies of the energetics of peptide–membrane systems of each of the three classes at the mean-field level, emphasizing the relative importance of the free energy terms in each case, and highlighting pros and cons of the approach used. The emerging conclusion from this overview is that theoretical–computational treatment of peptides of the first two categories, that is, those that interact essentially either with the hydrocarbon region or with the polar headgroups only, is reasonably accurate. However, the theoretical treatment of peptides that interact with the whole bilayer structure (the hydrocarbon and the polar headgroup regions together) is substantially more difficult and current methodology is usually successful only if crucial, experimentally derived assumptions are made regarding the specific system at hand.

I. INTRODUCTION

A central problem in membrane biophysics is the fundamental understanding of the energetics of peptide–membrane systems. This has a bearing on understanding the interactions of signal peptides and signal anchors with lipid bilayers, the effects of antimicrobial peptides on cell membranes, the mechanism of voltage gating in ion channels, the nature of the immune response, and signal transduction, as well as on the prediction of structure and function in membrane proteins. A variety of powerful biochemical and biophysical (mainly spectroscopic) tools have been used for gathering information on peptide–membrane systems (reviewed by Cafiso, 1999; de Kroon *et al.*, 1993; White and Wimley, 1999). The ample experimental data thus collected, together with data from computer simulations and theoretical studies of peptide–membrane systems (reviewed by Forrest and Sansom, 2000; Sansom, 1998; White and Wimley, 1999), provide at least partial understanding of the energetics of such systems. In this chapter we discuss the various free energy contributions to peptide–membrane association and provide examples to illustrate their relative importance in different cases.

The lipid bilayer has a complex structure, including a central hydrocarbon region, which is hydrophobic in nature, and two flanking polar headgroup regions

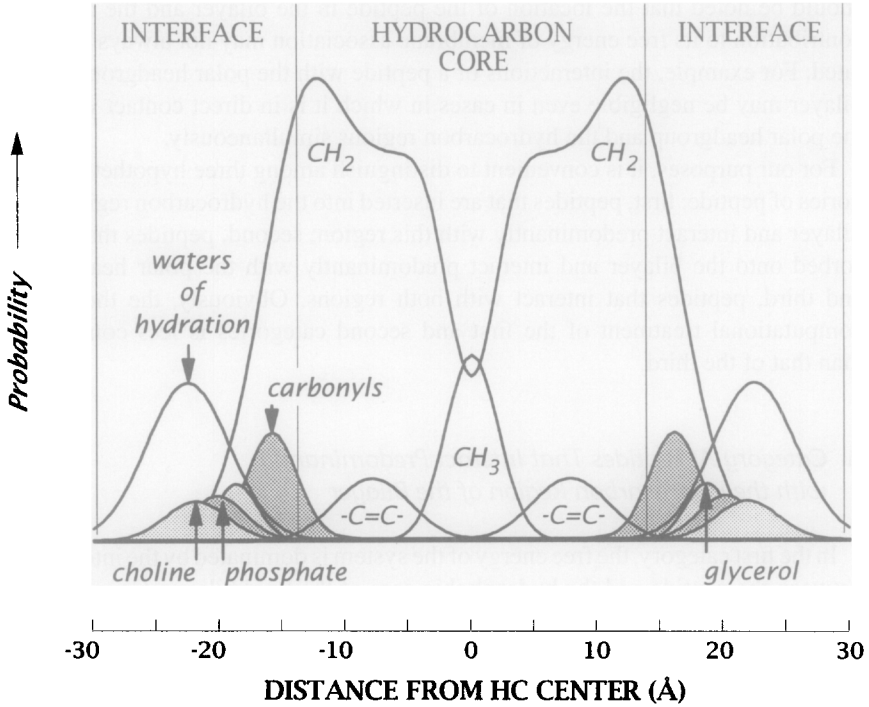


FIGURE 1 The structure of a fluid liquid crystalline dioleoylphosphocholine (DOPC) bilayer determined by the joint refinement of X-ray and neutron diffraction data (Wiener and White, 1992) (adapted from White and Wimley, 1999). The "structure" consists of the time-averaged distributions of the principal (quasi-molecular) structural groups of the lipid, projected onto an axis normal to the bilayer plane. The areas of the Gaussian distributions equal the number of structural groups represented by the Gaussians (one phosphate, two carbonyls, etc.); the distributions therefore represent the probability of finding a chemical group at a particular location. The interfaces of the bilayer, referred to in the text as the polar headgroup regions, are defined as the regions occupied by the headgroup's water of hydration. Although this structural image was obtained at low hydration (5.4 waters per lipid), more recent work demonstrates that the overall structure changes in relatively minor ways as the water content is increased (Hristova and White, 1998). Reprinted, with permission, from White and Wimley (1999). Copyright 1999 by Annual Reviews.

(Fig. 1). In most cases there is a strong correlation between the peptide's location in the membrane, as detected experimentally, and the dominant contributions to the free energy of its membrane association. For example, calculations suggest that the dominant contribution to the membrane association of alamethicin, a short transmembrane hydrophobic peptide (Barranger-Mathys and Cafiso, 1996; Huang and Wu, 1991; North *et al.*, 1995), comes from its interactions with the hydrocarbon region of the bilayer (Kessel *et al.*, 2000a). In this respect, it is useful to classify peptides according to their location in the membrane. However, it

should be noted that the location of the peptide in the bilayer and the dominant contribution to its free energy of membrane association may not always be correlated. For example, the interactions of a peptide with the polar headgroups of the bilayer may be negligible even in cases in which it is in direct contact with both the polar headgroup and the hydrocarbon regions simultaneously.

For our purposes, it is convenient to distinguish among three hypothetical categories of peptide: first, peptides that are inserted into the hydrocarbon region of the bilayer and interact predominantly with this region; second, peptides that are adsorbed onto the bilayer and interact predominantly with the polar headgroups; and third, peptides that interact with both regions. Obviously, the theoretical/computational treatment of the first and second categories is less complicated than that of the third.

A. Category A: Peptides That Interact Predominantly with the Hydrocarbon Region of the Bilayer

In the first category, the free energy of the system is dominated by the interactions between the peptide and the hydrophobic core of the lipid bilayer. Consequently, it is hardly affected by the chemical nature of the polar headgroup region of the bilayer. The association of peptides in this category with membranes is driven by the nonpolar contributions to the association free energy, commonly referred to as "hydrophobic interactions." In most cases these contributions are partially balanced by the electrostatic free energy penalty resulting from the transfer of polar groups, such as those in the peptide backbone, from the aqueous phase into the hydrocarbon region of the bilayer.

Biological systems that belong to this category include transmembrane pore-forming peptides, such as gramicidin (Wallace, 1990), alamethicin (Cafiso, 1994), and phospholamban (Arkin *et al.*, 1997; Herzyk and Hubbard, 1998). Certain membrane proteins (e.g., ion channels and receptors) are composed of α-helical transmembrane segments, which are synthesized separately and assemble inside the lipid bilayer to form the intact protein. The general behavior of these transmembrane segments and their interactions with the lipid bilayer are similar to those of membrane-associated peptides, and therefore can be included in this category. A well-known example is the acetylcholine receptor (AChR), which is composed of five subunits that assemble inside the lipid bilayer to form an ion-conducting pore (Hucho *et al.*, 1996). Glycophorin provides another example; it forms homodimers of known three-dimensional structure (MacKenzie *et al.*, 1997).

The transmembrane domain of many membrane proteins is composed of a bundle of hydrophobic α helices, which originate from the same polypeptide chain. Studies of these proteins, such as bacteriorhodopsin, show that at least some of the transmembrane α helices can separately refold into lipid bilayers when they have been disconnected from each other by the removal of the connecting loops.

Moreover, when these fragments are subsequently brought together they spontaneously yield a functional protein (Popot, 1993; Popot and Engelman, 1990; Popot *et al.*, 1987). Such transmembrane helices may also be included in category A. Studies suggest that it should be possible to predict the locations of at least some of the transmembrane helices in the sequence of membrane proteins based on energetic considerations (see Section III.A.4 below).

B. Category B: Peptides That Interact Predominantly with the Polar Headgroup Region of the Bilayer

In the second category, the free energy of the system is dominated by the interactions between the peptide and the polar headgroup of the lipid bilayer. In such cases, the peptides are generally adsorbed onto the surface of the lipid bilayer, without significant penetration into the polar headgroup region. Consequently, they hardly interact with the hydrocarbon region of the bilayer. For example, studies indicate that the neuronal anchoring protein, AKAP79, associates mainly electrostatically with lipid bilayers; it contains three regions enriched with basic residues that interact with the acidic lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) (Dell'Acqua *et al.*, 1998). In addition, many important peripheral proteins are membrane-anchored by a combination of a hydrocarbon chain, which interacts with the hydrophobic core of the bilayer, and a region of positively charged residues that interact (mainly) electrostatically with negatively charged lipids in the membrane (reviewed by McLaughlin and Aderem, 1995). Examples include cytochrome *c* (Heimburg and Marsh, 1995, 1996; Pinheiro, 1994; Pinheiro and Watts, 1994a,b), myelin basic protein (MacNaughtan *et al.*, 1985), phospholipases (Roberts, 1996), Src (Buser *et al.*, 1994; Resh, 1993, 1994; Sigal *et al.*, 1994), myristoylated alanine-rich C-kinase substrate (MARCKS) (Aderem, 1992; Blackshear, 1993; McLaughlin and Aderem, 1995), human immunodeficiency virus (HIV) matrix protein (C. P. Hill *et al.*, 1996; Massiah *et al.*, 1994; Zhou *et al.*, 1994), K-Ras (Cadwallader *et al.*, 1994; Hancock *et al.*, 1990), and human carbonic anhydrase IV (Stams *et al.*, 1996). In some of these cases it is possible to consider the contribution of the region of basic residues to the association free energy separately, and there is ample experimental and computational data suggesting that the region of basic residues interacts predominantly with the polar headgroup of the bilayer (Murray *et al.*, 1997, 1998).

C. Category C: Peptides That Interact with Both the Hydrocarbon and Polar Headgroup Regions of the Bilayer

In the third category, the peptide partially penetrates the lipid bilayer and interacts both with the polar headgroups and the hydrocarbon region of the bilayer.

Antibacterial peptides, such as magainin (Bechinger *et al.,* 1998), dermaseptin, and mammalian cecropin, belong to this category (Shai, 1999). These basic peptides are synthesized by a wide range of organisms as part of their defense system against invading bacteria (Bechinger, 1997; Saberwal and Nagaraj, 1994). The antibacterial action of such peptides is believed to be the result of their ability to induce structural disorder in the packing of lipid chains in the bacterial plasma membrane. According to the "carpet-like mechanism" suggested by Shai and co-workers (Pouny *et al.,* 1992; Shai, 1995), the antibacterial peptides are adsorbed, via their basic domain, to the negatively charged bacterial plasma membrane, each of them partially penetrating the hydrophobic core of the bilayer. The combined action of these peptides on the lipid bilayer eventually leads to its lysis and subsequently to the death of the bacterium.

Certain protein segments behave in a similar fashion. For example, the fusion peptide of influenza hemagglutinin mediates the fusion of internalized virions with the mature endosomal membrane (Carr and Kim, 1993, 1994), probably through structural rearrangements of the lipid bilayer. Several studies indicate that the fusion peptide penetrates the endosomal membrane, interacting with both the polar and nonpolar regions of the lipid bilayer (Durell *et al.,* 1997; Han *et al.,* 1999, 2001; Luneberg *et al.,* 1995; Macosko *et al.,* 1997).

D. Theoretical and Computational Studies

The structural complexity of peptide–membrane systems makes them a challenge for theoretical treatments. Thus, state-of-the-art computational methods usually involve *ad hoc* assumptions derived from experimental data on the particular system at hand. For example, in all-atom molecular dynamics (MD) simulations, the method of choice in theoretical studies of peptide–membrane systems, the choice of the initial peptide–membrane configuration is often based on available experimental data. Some MD simulations even involve the addition of new energy terms tailored for the specific system involved.

The theoretical methods that are currently used for the study of peptide–membrane systems differ from each other with respect to the level of detail used for representing the peptide and the bilayer. Straightforward MD simulations are based on atomic-detail models of both the peptide and the hydrated lipid bilayer (reviewed by Forrest and Sansom, 2000; Roux and Woolf, 1996; Sansom, 1998). MD simulations may provide insight into some aspects of peptide–membrane interactions (e.g., hydrogen bonding), but they do not usually provide energetic guidelines for processes such as peptide adsorption onto, and insertion into, lipid bilayers, primarily due to limitations on simulation time and size.

Continuum solvent models (e.g., Ben-Tal *et al.,* 1996a,b, 2000a; Berneche *et al.,* 1998; La Rocca *et al.,* 1999; May and Ben-Shaul, 1999; see also Section III)

are at the opposite extreme in terms of the level of detail used for describing the system, relying on implicit description of the aqueous phase and, in some cases, of the bilayer. Thus, simulations that are based on these models are more feasible for the study of biological events that occur on long time scales and for thermodynamic analysis.

Monte Carlo (MC) simulations usually involve an intermediate level of representation. Preliminary tests have demonstrated their ability to deal with peptide–membrane systems (reviewed by Skolnick and Milik, 1996). However, their accuracy depends strongly on the quality of the energy function, which is, as yet, of questionable quality (Pastor, 1994), although efforts are being made to generate new and better energy functions (e.g., Schlenkrich *et al.,* 1996).

Biggin and Sansom (1999) have recently reviewed various theoretical treatments of peptide–membrane and protein–membrane systems. They concentrated mainly on MD simulations and generally avoided thermodynamic aspects. This chapter is complementary to their review, in that it focuses mainly on the thermodynamic (and long-term kinetic) aspects and reviews recent attempts to estimate the major free energy components of peptide–membrane interactions at the mean-field level. We review recent continuum solvent models and Monte Carlo studies to provide a general framework for mean-field treatment of peptide–membrane interactions. This framework should hold for peptide–membrane systems of each of the three categories mentioned above, and it may be useful in the critical assessment of many simple model studies of peptide–membrane systems.

II. THEORY

The total free energy difference between a peptide in contact with the membrane and one in the aqueous phase (ΔG_{tot}) can be decomposed into components in several ways (Ben-Tal *et al.,* 1996a; Cafiso, 1999; Engelman and Steitz, 1981; Fattal and Ben-Shaul, 1993; Honig and Hubbell, 1984; Jacobs and White, 1989; Jähnig, 1983; Milik and Skolnick, 1993; White and Wimley, 1999). Figure 2 presents an option that is compatible with the mean-field approach used here. ΔG_{tot} is a sum of differences of (1) effects due to changes in the pK_a of titratable residues (ΔG_{pKa}), (2) peptide conformation (ΔG_{con}) and immobilization (ΔG_{imm}) effects, (3) electrostatic and nonpolar contributions to the solvation free energy (ΔG_{sol}), (4) lipid perturbation effects (ΔG_{lip}), (5) membrane deformation effects (ΔG_{def}), (6) effects resulting from interactions between the peptide and lipid headgroups (ΔG_{hg}), and (7) effects resulting from specific peptide–lipid interactions (ΔG_{sp}). Thus,

$$\Delta G_{tot} = \Delta G_{pKa} + \Delta G_{con} + \Delta G_{imm} + \Delta G_{sol} + \Delta G_{lip} + \Delta G_{def} + \Delta G_{hg} + \Delta G_{sp}. \quad (1)$$

e Water
Water
Water
f Water
Liquid alkane
Water
d Water
Water
Water
g Water
Liquid alkane
Water
c Water
b Water
a Water
k Water
Water
h Water
Water
i Water
Water
j Water
Water
ΔG_trans
ΔG_con
ΔG_pKa
ΔG_rot
−ΔG°_ele
ΔG_wl
ΔG_m
ΔG°_ele
ΔG_ap
ΔG_lef
ΔG_rig
ΔG_sp

FIGURE 2 A schematic diagram describing the various contributions to the total free energy of association of a peptide with the lipid bilayer. The association process is depicted as a sequence of different thermodynamic substates, designated a–k. The various contributions to the total free energy of peptide–membrane association characterize the passages between the different substates. The peptide is schematically depicted as a green curve (in its unfolded states a and b) or as two joined green helices (in its folded states c–k), and ΔG_{con} is the free energy due to conformational change. The black (in state a) and pink (in b–k) patches on the peptide represent two different protonation states of a titratable residue, and ΔG_{pKa} is the free energy associated with the change of protonation state. The central hydrophobic region of the peptide is colored blue, and the two horizontal lines represent the boundaries of the hydrocarbon region of the membrane. The free energy penalty of peptide immobilization (c–d) is denoted ΔG_{imm}. The solvation free energy (d–g) is denoted ΔG_{sol}. Here d–g, surrounded by light blue shading, describe a thermodynamic cycle based on the separation of ΔG_{sol} into electrostatic and nonpolar contributions. The hydrophobic core of the peptide is discharged in water (d–e; $-\Delta G^1_{elc}$). It is then transferred into a liquid alkane (e–f; ΔG_{np}) and recharged again in the liquid alkane (f–g; ΔG^2_{elc}). The unperturbed lipid chains are represented by the black wavy lines. Peptide-induced lipid perturbation effects (g–h) are denoted ΔG_{lip}; state h shows lipid chains in the vicinity of the peptide, which become more rigid due to their interactions with the peptide core; the ΔG_{lip} contributions reflect the associated entropic penalty. Membrane deformation effects (h–i) are denoted ΔG_{def}. The neutral and acidic headgroups are represented by gray and red spheres, respectively. Effects resulting from the interactions between the peptide and lipid headgroups, for example, positively charged residues of the peptide with negatively charged lipids (i–j), are denoted ΔG_{hg}. The yellow sphere represents a polar headgroup that interacts specifically with the peptide (the peptide segment that is involved in this interaction is colored yellow). Effects resulting from specific peptide–lipid interactions (j–k) are denoted ΔG_{sp}. (See color plate.)

A. Effects Due to pK_a Changes: ΔG_{pKa}

The transfer of a charged residue from water to a less polar medium, such as the lipid bilayer, involves a large electrostatic desolvation free energy penalty. This free energy penalty may be lowered significantly by protonation or deprotonation of the residue, which leads to its neutralization (e.g., Honig and Hubbell, 1984). Thus, peptide–membrane association may induce changes in the protonation state of titratable residues in the peptide (Fig. 2a to Fig. 2b). These changes may be a direct consequence of the peptide interactions with either the polar headgroup region (Esmann and Marsh, 1985; Horvath *et al.,* 1988) or the hydrocarbon region of the membrane (Honig and Hubbell, 1984). Alternatively, they may be associated with conformational changes that the lipid bilayer induces in the peptide (White and Wimley, 1999). Regardless of the cause of the change in the protonation state of an amino acid, the free energy change associated with it, ΔG_{pKa}, may be calculated as

$$\Delta G_{pKa} = -2.3kT(pK_a - pH), \tag{2}$$

where k is the Boltzmann constant, T is the absolute temperature, and K_a is the ionization equilibrium constant of the residue. For example, ΔG_{pKa} for the neutralization of aspartic or glutamic acid ($pK_a \approx 4$) at neutral pH is ~4 kcal/mol.

B. Peptide Conformation Effects: ΔG_{con}

The transfer of a peptide from a polar environment, such as water, to a less polar medium, such as the membrane, may involve conformational changes in the peptide, with the resulting free energy contribution ΔG_{con}. Indeed, lipid bilayers are known to induce formation of secondary structure elements (e.g., Deber and Goto, 1996; Engelman and Steitz, 1981; Popot, 1993; White and Wimley, 1999). The conformational changes usually take place to lower the free energy of transfer of unsatisfied hydrogen bonds from the aqueous phase into the bilayer. Experimental data (reviewed by Engelman *et al.,* 1986; Popot, 1993; Popot and Engelman, 1990; White and Wimley, 1999) and simulations (Milik and Skolnick, 1993, 1995; see also review by Biggin and Sansom, 1999) suggest that the sequence of events is probably such that the conformational changes in the peptide follow its association with the membrane. Nevertheless, in many cases it is computationally more convenient to account for ΔG_{con} in the aqueous phase (Fig. 2b to Fig. 2c). In some cases the structural changes are limited to a short amino acid stretch going, say, from random coil to α-helix conformation upon membrane insertion. Both experiments (Yang and Honig, 1995) and theory (Wojcik *et al.,* 1990) suggest that for stretches of up to about 15 residues, ΔG_{con} in the aqueous phase is small and sometimes even negligible. Thus, in such cases it is reasonable to assume that $\Delta G_{con} \approx 0$.

It may be difficult to get an estimate of ΔG_{con} associated with the folding of a membrane protein in the aqueous phase. In this respect, the approach of White and Wimley (1999), who experimentally derived an estimate for the reduction in free energy due to secondary structure formation in bilayers, may be a more feasible option for treating proteins.

C. *Peptide Immobilization Effects:* ΔG_{imm}

Any association process—the binding of a ligand to a protein, the dimerization of two proteins, the insertion of a peptide into a lipid bilayer (Fig. 2c to Fig. 2d), or the adsorption of a peptide onto a bilayer—involves an entropy loss, reflecting the conversion of free translational and rotational, degrees of freedom into bound motions. For example, in bulk solution, a ligand has three translational degrees of freedom of its center of mass and three rotational degrees of freedom. Thus, depending on its concentration (i.e., on the choice of the standard state), a certain volume V and all of the "orientational space," that is, $8\pi^2$, will be accessible to the ligand molecule when it is free in solution. Upon binding to a receptor, some, usually all, of these degrees of freedom become confined. Thus, only a portion V_b of the volume and a portion γ of orientational space are accessible for the ligand molecule. The immobilization free energy penalty ΔG_{imm} is a sum of contributions from the restrictions on translational, ΔG_{tr}, and rotational, ΔG_{rot}, degrees of freedom imposed on the ligand upon binding (Brady and Sharp, 1997; Erickson, 1989; Gilson *et al.*, 1997; T. L. Hill, 1985; Janin, 1996):

$$\Delta G_{imm} = \Delta G_{tr} + \Delta G_{rot} = -kT \ln(V_b/V) - kT \ln(\gamma/8\pi^2). \quad (3)$$

It is rather difficult to measure ΔG_{imm} directly, because it is usually not the only entropy-based contribution to association; for example, ΔG_{sol} and ΔG_{con} usually involve significant entropic components. Finkelstein and Janin (1989) derived a theoretical estimate of $\Delta G_{imm} \approx 15$ kcal/mol for protein dimerization by calculating V_b and γ from the root mean square fluctuations (*B*-factors) in protein crystals. Their estimate seems reasonable for tight binding and when all six external translational and rotational degrees of freedom are confined due to binding (Holtzer, 1994; Horton and Lewis, 1992; Murphy *et al.*, 1994; Novotny *et al.*, 1989; Page and Jencks, 1971; Searle and Williams, 1992; Searle *et al.*, 1992; Tidor and Karplus, 1994; Vajda *et al.*, 1994, Weng *et al.*, 1996).

The planar pseudo-symmetry of bilayers and the nature of peptide–membrane interactions mean that peptide–membrane associations differ from, for example, binding of a ligand to a receptor. Of the three center-of-mass translational degrees of freedom of a peptide in solution, two, associated with translation in the membrane plane (x–y), are retained. Similarly, one of the three rotational degrees of freedom of a peptide in solution—the one associated with peptide rotation around the

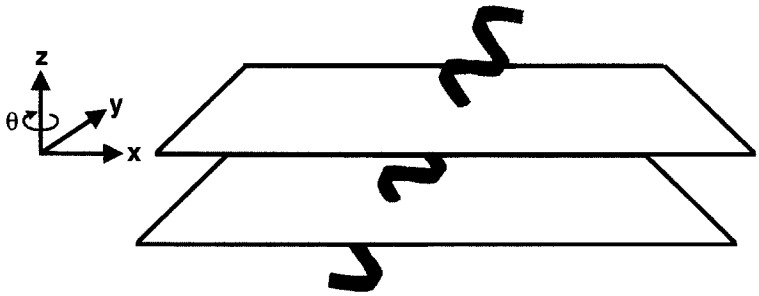


FIGURE 3 Peptide immobilization effects. Upon membrane association, the peptide may still freely translate as a rigid body in the membrane plane (x–y), but its motion along the membrane normal (z) becomes limited. Similarly, the peptide can freely rotate around the normal (θ) even when membrane-associated, but its two other rigid-body rotational motions become restricted.

membrane normal (θ)—does not markedly change its character upon association (Fig. 3). Therefore, because of the pseudo-symmetry of the membrane, usually only three degrees of freedom—one translation (in the z axis) and two rotations—are confined upon association.

The upper bound of the value of ΔG_{imm} for the insertion of a 25-mer polyalanine α helix into a lipid bilayer has been estimated to be ~3.7 kcal/mol (Ben-Shaul *et al.*, 1996; Ben-Tal *et al.*, 1996a). This estimate is much smaller than that obtained for binding processes, because to the fluidity of the bilayer enables it to tolerate many peptide configurations prohibited by a rigid receptor. Recently, an even smaller value of $\Delta G_{\text{imm}} \approx 1.3$ kcal/mol was estimated for the electrostatically driven adsorption of the positively charged peptide pentalysine onto membranes containing negatively charged lipids (Ben-Tal *et al.*, 2000b). Membrane fluidity was not taken into account in this study. The peptide resides about 3 Å from the van der Waals surface of the bilayer, and the small value of ΔG_{imm} in this case was attributed to the screening effect of water, which significantly reduces the Coulomb attraction between the peptide and the membrane.

The $\Delta G_{\text{imm}} \approx 1.3$ kcal/mol estimate for pentalysine is comparable to the data obtained by Peitzsch and McLaughlin's (1993) estimate based on the measured partitioning of fatty acids and acylated peptides between the aqueous phase and phospholipid vesicles; in both studies the same number of degrees of freedom is restricted. They reported that their measured association free energy is consistent with an empirical rule derived by Tanford (1991) from the partitioning of fatty acids into *n*-heptane, in which association entropy plays no part. They therefore concluded that ΔG_{imm} should be small, ~2 kcal/mol. A similar estimate can be derived from measurements of the association of model acylated proteins with lipid bilayers (Pool and Thompson, 1998).

D. Hydrophobicity, Hydropathy Plots, and the Solvation Free Energy: ΔG_{sol}

The water environment has significant effects on soluble proteins, and solvation free energy ΔG_{sol} has primary importance in stability and binding in globular proteins (Eisenberg and McLachlan, 1986; Honig and Nicholls, 1995; Honig *et al.*, 1993; Kellis *et al.*, 1988; Kyte and Doolittle, 1982; McCammon, 1998; Sheinerman *et al.*, 2000; Warshel, 1991; Yue and Dill, 1996). An estimate of ΔG_{sol} may be obtained from hydrophobicity scales; indeed, hydropathy plots have been proved valuable in the discrimination between residues that are solvent-exposed and residues that are buried in the core of soluble proteins (e.g., Eisenberg and McLachlan, 1986; Kyte and Doolittle, 1982).

Hydrophobicity scales are also commonly used in structure predictions of membrane proteins. An implicit assumption involved in the use of these scales is that a particular solvent mimics the membrane environment. For example, the widely used Kyte and Doolittle scale (Kyte and Doolittle, 1982) uses vacuum-to-water transfer free energies to assign a hydrophobicity value to each amino acid. A moving window is then applied to the sequence to determine which segments are most likely to appear within the bilayer. A different approach, taken by von Heijne (1981a,b) and Engelman *et al.* (1986), is based on defining free energy contributions of individual chemical groups to partitioning. Another approach is based on the Eisenberg and McLachlan (1986) and Efremov *et al.* (1999c) schemes, which assume that transfer free energies are proportional to a sum of atomic properties that are derived from a least squares fit to experimental transfer free energies.

All of these schemes share a common feature: Free energies of transfer are assumed to be proportional to some inherent property, whether that of an individual atom, a chemical group, or an entire amino acid side chain. Although they work fairly well in predicting the helical spanning regions of membrane proteins (Fasman and Gilbert, 1990; Jähnig, 1990; White and Wimley, 1999), they fail in complex cases, where residues are involved in interactions and cannot be accurately represented by simple numbers or categories (e.g., Rose, 1987). Clearly, a prediction based on the transfer free energies of individual groups into a bulk phase cannot account for specific interactions between groups that characterize heterogeneous systems (see discussions by White and his co-workers: Jacobs and White, 1989; White and Wimley, 1999). Thus, transfer free energies of individual amino or carbonyl groups cannot be used to predict the free energy of transfer of a molecule in which these groups are hydrogen-bonded to one another. Similarly, a group that partitions near an interface and may form specific interactions with lipid headgroups cannot be treated as if it were completely immersed in a bilayer. Moreover, groups that are buried near an interface can still interact quite strongly with water (Gilson *et al.*, 1985), but this

interaction clearly cannot be accounted for with numbers based on partitioning into a bulk phase.

Hydrophobicity scales are commonly used to approximate ΔG_{sol} (Fig. 2d to Fig. 2g) in simulations of peptide–membrane systems (Biggin *et al.,* 1997; Edholm and Jähnig, 1988; Efremov *et al.,* 1999a–c; Gersappe *et al.,* 1993; Milik and Skolnick, 1993, 1995; Seagraves and Reinhardt, 1995; Skolnick and Milik, 1996). Such simulations are based on a simplified description of the aqueous phase and the lipid bilayer as two structureless media connected to each other by a smooth boundary (e.g., La Rocca *et al.,* 1999; Skolnick and Milik, 1996). Numbers associated with partitioning between polar and nonpolar media are assigned to atoms, chemical groups, or whole residues (depending on the resolution of the simulation) as they cross the bilayer–water boundary. The outcome of this approach is encouraging, in that in all of the reported simulations, the calculations are consistent with available experimental data (e.g., regarding the most likely conformation and orientation of the peptide in the bilayer or its free energy of association with the bilayer). However, in view of the limited number of peptide–membrane systems studied using this approach, and particularly the small number of cases tested using a single variant of this approach, it is premature to evaluate its usefulness at this point.

ΔG_{sol} of Fig. 2d to Fig. 2g is the free energy of transfer of the hydrophobic core of the peptide from water into a bulk hydrocarbon phase. It accounts both for electrostatic contributions (ΔG_{elc}; Fig. 2d to Fig. 2e, and Fig. 2f to Fig. 2g) resulting from changes in the solvent dielectric constant and for van der Waals and solvent structure effects, which are grouped in the nonpolar term (ΔG_{np}; Fig. 2e to Fig. 2f); together they define the classic hydrophobic effect:

$$\Delta G_{sol} = \Delta G_{elc} + \Delta G_{np} = \Delta G^{1}_{elc} - \Delta G^{2}_{elc} + \Delta G_{np}. \quad (4)$$

The main contributions to ΔG_{sol} usually come from the interactions of the peptide with the hydrocarbon region of the bilayer (e.g., Fig. 2; the blue segment of the peptide). ΔG_{sol} usually involves an electrostatic penalty, such as that due to the partitioning of backbone hydrogen bonds into the hydrocarbon region of the membrane. However, for hydrophobic peptides this penalty is overcompensated for by the nonpolar contributions, resulting in net attraction between the peptide and the membrane.

Mean-field calculations show that for hydrophobic peptides ΔG_{sol} is usually the dominant contribution to ΔG_{tot} and its value for membrane-associated peptides of about 20 residues is typically -10 to -20 kcal/mol (Ben-Tal *et al.,* 1996a; Kessel *et al.,* 2000a; see also below).

Since it is known that peptide adsorbing to the polar headgroup region often induces secondary structure formation (White and Wimley, 1999, and references therein), it is obvious that interactions of peptides with the polar headgroups involve

ΔG_{sol} components. Headgroup contributions to ΔG_{sol} may be due to the removal of water molecules from a peptide segment, the headgroups, or both (Ben-Tal *et al.*, 1996b, 1997a). Because of the polar nature of the headgroups, it is anticipated that both the electrostatic desolvation penalty and the nonpolar contributions associated with partitioning into the headgroup region will be smaller in magnitude than those associated with partitioning into the hydrocarbon region. The headgroups' contributions to the solvation free energy may be included in the ΔG_{sol} or in the ΔG_{hg} term of Eq. (1).

1. Electrostatic Contributions to Solvation: ΔG_{elc}

The electrostatic contribution to the solvation free energy (ΔG_{elc}; Fig. 2d to Fig. 2e, and Fig. 2f to Fig. 2g) can be obtained from finite-difference (FD) solutions to the Poisson–Boltzmann (PB) equation (the FDPB method) (Honig and Nicholls, 1995; Honig *et al.*, 1993):

$$\nabla \cdot [\varepsilon(r)\nabla\phi(r)] - \varepsilon_r \kappa(r)^2 \sinh[\phi(r)] + e^2\rho^f(r)/(\varepsilon_0 kT) = 0. \tag{5}$$

Here $\varepsilon(r)$ is the spatially dependent dielectric constant, $\kappa(r)$ is the Debye–Hückel parameter, $\rho^f(r)$ is the distribution of fixed charges, $\phi(r)$ is the electrostatic potential, k is the Boltzmann constant, e is the electron charge, ε_r is the dielectric constant of the aqueous phase, ε_0 is the permittivity of free space, and T is the absolute temperature. The spatial distributions $\varepsilon(r)$, $\kappa(r)$, and $\rho^f(r)$ are obtained from the structure of the peptide and membrane and the equation is solved for $\phi(r)$. The electrostatic free energy, ΔG_{elc} is obtained, by integration, from the spatial distributions of $\rho^f(r)$ and $\phi(r)$. In the absence of salt ions [or equivalently when $\phi(r)$ is small and the Poisson–Boltzmann equation can be linearized], ΔG_{elc} is given by

$$\Delta G_{elc} = 1/2 \int dr\, \rho^f(r) \cdot \phi(r). \tag{6}$$

2. Nonpolar Contributions to Solvation: ΔG_{np}

By definition, the ΔG_{np} term in Eq. (4) includes all the contributions to the solvation free energy that are missing in the ΔG_{elc} term. Thus, ΔG_{np} (Fig. 2e to Fig. 2f) includes contributions of electrostatic origin, such as changes in the van der Waals (dispersion) force in the transfer from the aqueous phase to the membrane, as well as contributions from solvent structure effects, collectively referred to as the classic hydrophobic effect. ΔG_{np} can be derived from the partitioning of nonpolar molecules, such as alkanes, between water and nonpolar solvents. Normally there is a linear relation between the measured log partition coefficient (or equivalently the transfer free energy) and the alkane size. A common method to account for the ΔG_{np} contribution is to relate it to the water accessible surface area A of the

alkanes,

$$\Delta G_{np} = \gamma A + b, \tag{7}$$

where γ is a surface tension coefficient and b is an intercept.

A choice of alkane partitioning between water and liquid alkanes, giving $\gamma = 0.0278$ kcal/(mol Å^2) and $b = -1.71$ kcal/mol (Sitkoff *et al.,* 1996), is particularly suitable for calculations with peptides and small molecules that interact predominantly with the hydrocarbon region of the bilayer (Ben-Tal *et al.,* 1996a, 2000a; Kessel *et al.,* 2000a,b). Experimental data suggest that the partitioning of amino acids between the aqueous phase and the polar headgroup region of palmitoyloleoylphosphatidylcholine (POPC) bilayers can be approximated using $\gamma = 0.012$ kcal/(mol Å^2) (Wimley and White, 1996).

E. Lipid Perturbation Effects: ΔG_{lip}

On average, the hydrophobic tails of the lipid molecules in a membrane are oriented along the membrane normal. The extent of chain stretching is often measured in terms of the average orientational order parameter of the tail S, which lies between 0 and 1. In the solid (or "gel") phase of the membrane, the lipid chains are nearly all in an "all-*trans*" conformation, lying fully stretched along the membrane normal, with order parameter $S = 0.8$ (Jähnig, 1983). In the fluid (or "liquid crystalline") phase of the membrane, which is of greater biological interest, the order parameter is considerably smaller, typically $S = 0.2$ (Jähnig, 1983). This lower value indicates that the partially stretched lipid chains possess considerable conformational freedom. Their entropy is thus higher than in the solid phase. The insertion of a rigid inclusion, such as a hydrophobic peptide, into a fluid membrane may stiffen the lipid chains in its immediate vicinity, thus lowering the conformational entropy of the system (Fig. 2g to Fig. 2h). This peptide–lipid interaction results from the lower elasticity (chain conformational freedom) of the membrane, which gives rise to a positive contribution to ΔG_{tot}.

ΔG_{lip} is obviously entropic in origin and its magnitude is proportional to the contact area between the peptide and the lipid tails, just as ΔG_{np} is proportional to the water-accessible surface area. Thus, one should expect a negligible value of ΔG_{lip} for peptides that are adsorbed at the membrane–water interface and do not interact with the lipid chains directly (Ben-Tal *et al.,* 1996a). For the transmembrane insertion of a 25-mer polyalanine α helix into a model bilayer, ΔG_{lip} has been estimated to be 2.3 kcal/mol (Ben-Shaul *et al.,* 1996). Since the effective circumference of all transmembrane helices is roughly the same and since there is very little variation in the width of the hydrocarbon region of biological

membranes, this estimate may be viewed as typical for ΔG_{lip} associated with helix insertion into biological membranes.

Such treatment does not take into account the extra work that must be done against surface pressure when inserting a protein or a peptide into only one side of a bilayer. It has been suggested that this is the reson for the 100-fold change in the activity of phospholipase C upon changing the pressure of a monolayer (Boguslavsky *et al.*, 1994).

F. Membrane Deformation: ΔG_{def}

When the length of the hydrophobic core of the peptide exactly matches the hydrophobic thickness of the membrane, the free energy contribution associated with the effect of the peptide on the organization of the lipid chains is generally, although not always (Fattal and Ben-Shaul, 1993), minimal, and includes only ΔG_{lip}. Negative hydrophobic mismatch, which occurs when the width of the hydrocarbon region of the bilayer is larger than the peptide's hydrophobic core (Fig. 2h), results in an additional free energy penalty (ΔG_{def}). This is associated with excess compression of the lipid molecules around the protein to achieve perfect hydrophobic matching (Fig. 2i), following the "mattress model" (Mouritsen and Bloom, 1984). This perfect matching facilitates the removal of polar regions in the peptide from the hydrocarbon region of the membrane into the aqueous phase, which would otherwise involve a high free energy penalty contribution from ΔG_{el}. A molecular model of the lipid chains can be used to estimate the dependence of ΔG_{def} on the mismatch between a lipid bilayer and a flat hydrophobic inclusion (Fattal and Ben-Shaul, 1993) (Table I). ΔG_{def} is usually small; even a severe helix-induced membrane deformation of 5 Å results in a free energy penalty of less than 1 kcal/mol.

The inverse situation, positive mismatch, in which the width of the hydrocarbon region of the bilayer is smaller than the peptide's hydrophobic core, is also energetically unfavorable: It involves the exposure of hydrophobic regions in the peptide core to the polar aqueous phase or water–membrane interface. The lipid tails may be expected to compensate by expanding in response to positive mismatch and it is possible to derive the corresponding ΔG_{def} contributions from Fatal and Ben-Shaul's (1993) calculations (Table I). Alternatively, the peptide may adopt a tilted orientation in the membrane (Killian, 1998). In fact, most transmembrane helices in membrane proteins are somewhat tilted (Bowie, 1999; Killian, 1998; White and Wimley, 1999). Similarly, peptides like alamethicin and isolated protein segments like the M2 segment of the nicotinic acetylcholine receptor have been shown to adopt a tilted orientation inside lipid bilayers (Kessel *et al.*, 2000a; Opella *et al.*, 1999).

TABLE I

Dependence of the Membrane Deformation Free Energy ΔG_{def} on the Hydrophobic Mismatch between the Lipid Bilayer and a Transmembrane Peptide/Protein with a 30-Å-Long Hydrophobic Core[a]

Membrane width[b] (Å)	$\Delta G_{def}/l$[c] (kcal/mol Å)	ΔG_{def}(5 Å)[d] (kcal/mol)	ΔG_{def}(17 Å)[e] (kcal/mol)
40	0.33	3.57	28.76
38	0.22	2.34	18.80
36	0.13	1.36	10.96
34	0.06	0.65	5.22
32	0.02	0.20	1.58
30	0.00	0.00	0.00
28	0.01	0.08	0.64
26	0.04	0.41	3.33
24	0.09	1.01	8.13
22	0.18	1.87	15.03
20	0.28	2.99	24.04

[a] The dependence is based on the calculations of Fattal and Ben-Shaul (1993), in which the transmembrane protein/peptide was treated as a flat inclusion of infinite radius. In later studies, we applied curvature corrections of the form $2\pi R_P(1 - R_L/R_P)$, where $R_L = 3.3$ Å and R_P are the radii of the lipid chain and the helical peptide, respectively.

[b] The width of the hydrocarbon region of the lipid bilayer.

[c] The results of Fattal and Ben-Shaul (1993) for membrane deformation induced by a hydrophobic inclusion of infinite radius. l is the inclusion's circumference.

[d] ΔG_{def} associated with a hydrophobic inclusion of radius 5 Å, corresponding to a monomeric transmembrane helix.

[e] ΔG_{def} due to a hydrophobic inclusion of radius 17 Å, corresponding to a transmembrane hexameric helical bundle.

G. Polar Headgroup Effects: ΔG_{hg}

Peptide–membrane association may involve interactions with the lipid headgroups (Fig. 2i to Fig. 2j). The free energy contributions associated with these interactions are collectively referred to as ΔG_{hg}. The desolvation contributions to these interactions (e.g., Ben-Tal *et al.*, 1997a) may be considered as part of ΔG_{sol}. However, the polar headgroups may add unique contributions, such as the nonspecific attraction between acidic lipid heads and basic residues in the peptide (McLaughlin and Aderem, 1995; Murray *et al.*, 1997). Many peptides contain basic residues that interact electrostatically with acidic headgroups in lipid bilayers. Examples include signal sequences (von Heijne, 1998; Zheng and Gierasch, 1996) and antimicrobial peptides (Shai, 1999; White *et al.*, 1995).

Chapter 3 in this book focuses on the headgroups' contributions to peptide and protein association with lipid bilayers. Here, we briefly present a few examples, focusing on the contributions of basic residues to the free energy of membrane

association. Under physiological conditions (membranes containing 25–30 mol% of monovalent acidic lipids at a concentration of 150 m*M* monovalent salt ions), each positively charged residue (lysine or arginine) in the vicinity of the bilayer plane contributes about 1 kcal/mol to ΔG_{tot} (e.g., Ben-Tal *et al.,* 1996b; see Section III.B.3 below).

H. Specific Peptide-Lipid Interactions: ΔG_{sp}

Certain peptides or protein segments may interact specifically with membrane lipids (Fig. 2j to Fig. 2k). For example, the PH domain of PLC-δ_1 has been demonstrated to interact with high affinity and stereospecificity with both membrane phosphatidylinositol 4,5-bisphosphate and D-*myo*-inositol 1,4,5-trisphosphate (Lemmon *et al.,* 1995). Furthermore, the specific interaction between a protein and a certain lipid molecule may affect the activity of the protein, as in, for example, protein kinase C (Newton, 1993) and β-hydroxybutyrate (Cortese *et al.,* 1989). Specific peptide–lipid interactions, which may involve different parts of the lipid molecule, may be electrostatic or nonpolar in nature. However, for computational convenience they may be considered as a distinct free energy contribution, denoted ΔG_{sp}.

I. Peptide-Peptide Interactions and Lipid Demixing

Throughout this chapter we consider the interactions between a single peptide molecule and the lipid bilayer and do not take into account direct or membrane-mediated interactions between peptide molecules. Similarly, we do not take into account the ability of the peptide to attract certain kinds of lipids (e.g., acidic) into its vicinity. These contributions have been considered in previous studies (e.g., Denisov *et al.,* 1998; Heimburg and Marsh, 1995; Heimburg *et al.,* 1999; May *et al.,* 2000; Murray *et al.,* 2001).

III. MEAN-FIELD STUDIES OF PEPTIDE-MEMBRANE SYSTEMS

We present here a summary of recent studies of peptide–membrane systems of the three categories mentioned in Section I, that is, systems in which the peptide interacts with the hydrocarbon region only, the headgroup region only, or both. We selected simple model studies of several peptides that were carried out using different methodologies. We describe each of the methodologies in terms of the general framework of Fig. 2 and evaluate the relative importance of the free energy terms of Eq. (1) in each case.

A. Category A: Peptides That Interact Predominantly with the Hydrocarbon Region of the Bilayer

For peptides (and protein fragments) that interact predominantly with the hydrocarbon region of the bilayer, the free energy of the peptide–membrane system should be dominated by the solvation free energy term ΔG_{sol} and should hardly be affected by the chemical nature of the polar headgroups of the bilayer. We review three sets of studies: (1) Monte Carlo (MC) simulations, incorporating a hydrophobicity scale, of two bacteriophage coat proteins, *Pf1* and *fd*, each of which contains a short transmembrane segment of about 20 residues, which dominates the energetics of the system (Milik and Skolnick, 1993, 1995); they therefore fall into category A, even though they may appear to interact with the entire lipid bilayer; (2) MD simulations, using a "hydrophobicity potential" to represent the membrane, on the pore-forming antimicrobial peptide alamethicin, which is typical for this category; (3) continuum solvent model studies, on a model α-helical peptide, $(Ala)_{25}$, and on alamethicin.

1. Pf1 and fd Coat Proteins

Pf1 and *fd* are coat proteins which belong to two classes of filamentous bacteriophages (Clark and Gray, 1989; Nakashima *et al.*, 1975). These proteins are relatively short and contain two linked α-helical fragments. One of the segments is hydrophobic and the other is amphipathic. The sequence of *Pf1* is

GV**I***D***T**[**SAV***E***SAIT**]*D*GQG*D*[M<u>K</u>**AIGGYIVGALVILAVAGLIYSM**]L<u>RK</u>**A**,

where strictly hydrophobic (A, I, L, P, and V) and aromatic (F, Y, and W) residues are in bold black, positively charged residues (K and R) are underlined, and negatively charged residues (E and D) are in italic. The brackets indicate sequences that assume helical conformation (Roux and Woolf, 1996). The sequence of *fd* is

A*EGDD***P**[A<u>K</u>**AAF***D***SLQASAT***E*][**YIGYAWAMVVVIVGATIGI**]
<u>K</u>**LF**<u>**KK**</u>**FTS**<u>K</u>**AS**,

where the left and right brackets indicate sequences that may form amphipathic and hydrophobic α helices, respectively (McDonnell *et al.*, 1993). When membrane-associated, the hydrophobic helix of each protein assumes a transmembrane orientation, whereas the amphipathic helix of each becomes surface-oriented (Shon *et al.*, 1991).

Milik and Skolnick used MC simulations to study the membrane association of these proteins (Milik and Skolnick, 1993, 1995). They used a simplified model of the proteins and the lipid bilayer and an approximated form of some of the free

energy terms in Eq. (1) and Fig. 2. The proteins were described as chains of spheres with centers at the C_α positions. The ΔG_{sol} contributions of the side chains were derived from Roseman's octanol/water hydrophobicity scale (Roseman, 1988a). Different ΔG_{sol} contributions of the backbone were used depending on the secondary structure, that is, α-helix versus extended conformations. The ΔG_{hg}, ΔG_{lip}, and ΔG_{imm} contributions were estimated from experimental data on the partitioning of very short peptides into dioleylphosphatidylcholine (DOPC) lipid vesicles (Jacobs and White, 1989). The ΔG_{con} component was taken into account by the addition of an empirical energy term that was set to maintain the helical structure of the proteins. These free energy terms were incorporated into MC simulations to search the energetically favored peptide conformations and protein–membrane configurations.

The simulations indicated that the transfer of the C-terminal transmembrane fragments of *Pf1* and *fd* from the aqueous solution into the lipid bilayer proceeds through the following steps. First, the sequence is adsorbed onto the surface of the lipid bilayer in a random-coil conformation. Then, the fragment assumes a distorted-coil conformation. Finally, it is inserted into the bilayer while assuming an approximate α-helical conformation.

Milik and Skolnick's (1993, 1995) simulations were heavily based on water–octanol partitioning data, assuming that the amphiphilic medium provided by octanol is a good approximation of the hydrocarbon region of the lipid bilayer. In fact, octanol mimics the water–bilayer interface well, but it is significantly more polar than the hydrocarbon region of the bilayer (Thorgeirsson *et al.*, 1996; White and Wimley, 1999; Wimley and White, 1996; Yu *et al.*, 1994). The results of the simulations were in accord with the available experimental data, in spite of the crude representation of the membrane (and the peptide). However, this may be attributed in part to the nature of the peptides chosen for the study: Because they are predominantly helical, they fit well with the enhancement of α-helix conformations in the model. In addition, they consist of a highly hydrophobic (transmembrane) segment flanked by regions of polar and charged residues and are therefore bound to assume transmembrane orientations.

A more accurate treatment of protein–membrane interactions would probably involve the combination of two different hydrophobicity scales, one for residue partitioning into the interface and the other for residue partitioning into the hydrocarbon core (Efremov *et al.*, 1999a; White and Wimley, 1999). The incorporation of both scales (which have only recently become available) into Milik and Skolnick's energy function might have provided more realistic simulations.

2. Polyalanine α Helices

As a first stage in determining the energetics of membrane protein stability, Ben-Tal *et al.* (1996a) and Ben-Shaul *et al.* (1996) studied polyalanine α helices

to determine factors that are common to the insertion of all peptides and proteins, such as those due to the peptide backbone. They used continuum solvent model calculations and the thermodynamic cycle of Fig. 2 to estimate ΔG_{tot} of Eq. (1). ΔG_{elc} was obtained from finite-difference solutions to the Poisson–Boltzmann equation (the FDPB method) (Honig and Nicholls, 1995; Honig *et al.*, 1993). The $(Ala)_{25}$ α helix was represented in atomic detail, with atomic radii and partial charges defined at the coordinates of each nucleus. The charges and radii were taken from PARSE, a parameter set that was derived to reproduce vacuum-to-water (Sitkoff *et al.*, 1994) and liquid alkane-to-water (Sitkoff *et al.*, 1996) solvation free energies of small organic molecules. The lipid bilayer was described implicitly, as a low-dielectric slab. ΔG_{np} was calculated from the change in the water-accessible surface area of $(Ala)_{25}$ upon membrane association, using Eq. (7). The peptide free energy contributions due to peptide immobilization and lipid perturbation were calculated from a statistical thermodynamic model of the lipid tails (Ben-Shaul *et al.*, 1996; Fattal and Ben-Shaul, 1993).

Two configurations of a membrane-associated 25-mer polyalanine helix were found to be lower in free energy than the isolated helix in the aqueous phase. The first corresponded to a transmembrane orientation, in which the helix termini protruded from either side of the bilayer. The second minimum was for a surface orientation, in which the helix was adsorbed onto the surface of the bilayer with its principal axis parallel to the membrane plane. This orientation is similar to that found in the crystal structures of the amphipathic α helices in the membrane-exposed faces of the peripheral membrane proteins Cox1 and Cox2 (Kurumbail *et al.*, 1996; Picot *et al.*, 1994) and in the interfacial helix in the structure of rhodopsin (Palczewski *et al.*, 2000).

Previous attempts to estimate the free energy of association of polyalanine α helices with bilayers (e.g., Engelman *et al.*, 1986) produced values of ~ -30 kcal/mol, which are much more negative than the values of ~ -5 kcal/mol obtained from measurements on related systems (e.g., Moll and Thompson, 1994; White and Wimley, 1999, and references therein). The main reason for the discrepancy between the calculated and measured values was the neglect of the electrostatic penalty associated with the transfer of the helix backbone from the aqueous phase into the bilayer. This penalty was taken into account by Ben-Tal *et al.* (1996a) and the calculated values of the free energy in both minima, -4 kcal/mol for the transmembrane orientation (Ben-Tal *et al.*, 1996a) and -3 kcal/mol for the horizontal orientation (N. Ben-Tal, unpublished results), corresponded well with the measured values.

3. The Contribution of the Backbone Hydrogen Bond

As mentioned in Section II.D, many attempts have been made to develop polarity (hydrophobicity) scales that indicate the relative tendencies of the amino acids to be inserted into lipid bilayers. The scales are based on the implicit assumption

that each side chain has a fixed free energy contribution regardless of its neighbors and that, by adding the contribution of the backbone, it is possible to estimate the total free energy of transfer of the amino acid. However, this assumption is misleading, because transfer free energies are context-dependent. For example, the free energies of transfer of the hydrogen-bonded (N—H· · ·O=C) and nonbonded (N—H, O=C) groups between two solvents depends quite sensitively on their environment. The water-to-liquid alkane free energies of transfer of these groups were calculated as 3.8 and 7.8 kcal/mol, respectively, when they were part of either an *N*-methylacetamide (NMA) dimer or monomer (Ben-Tal *et al.*, 1997b). Much smaller values, 2.1 and 6.4 kcal/mol, respectively, were calculated when these groups were part of a polyalanine α helix (Ben-Tal *et al.*, 1996a). The reason for the difference is that NMA and the helix form differently shaped cavities in the solvent. Specifically, the amide groups in an α helix are surrounded by a low-dielectric region even in the aqueous phase, such that their free energies of transfer into a liquid alkane are smaller than those of NMA. The same logic dictates that the transfer free energies of amino acid chains that are part of an α helix would be different from those of the isolated amino acid. The situation is compounded by the possibility of side chain–side chain and side chain–backbone hydrogen-bonding interactions. It may be possible, however, to take these effects into account in devising context-dependent hydrophobicity scales, such as the one presented in Section III.A.4 below.

4. Computationally Derived Hydrophobicity Scale

Using the same methodology as presented in Sections III.A.2 and III.A.3, Ben-Tal (1997, and unpublished data) developed a hydrophobicity scale based on the free energies of transfer of the amino acids from aqueous phase into lipid bilayers. Unlike other hydrophobicity scales (e.g., Kyte and Doolittle, 1982), which assume that the free energies of transfer are proportional to some inherent property (of an individual atom, a chemical group, or an amino acid), this scale was derived using the theoretical model presented above (Section III.A.2), and it accounts for the amino acids being located at the center of an α helix. The scale is given in Table II and is compared to three other scales in Fig. 4. The scales are generally similar, all having the hydrophobic and charged amino acids at the two extremes. Ben-Tal's scale (filled circles in Fig. 4) is the least hydrophobic of the four. This is mainly because, unlike other scales, it includes the free energy penalty of inserting the helix backbone into the bilayer.

The most significant difference between Ben-Tal's scale and the others is the energy penalty of inserting an arginine (R) residue into the bilayer; it is much larger in Ben-Tal's scale than in the others. It should also be noted that the energy penalty of inserting arginine into the bilayer significantly exceeds that of lysine (K) in Ben-Tal's scale, both residues being taken in their deprotonated (neutral) form to avoid the penalty of inserting a charge into the hydrocarbon region of the

TABLE II

Hydrophobicity Scale Representing Free Energies of Transfer of Each of the 20 Amino Acids from Water into the Center of the Hydrocarbon Region of a Model Lipid Bilayer[a]

Amino acid	ΔG_{tot} (kcal/mol)
I	−2.6
L	−2.6
F	−1.5
V	−1.2
A	−0.2
G	0.0
C	+0.4
S	+0.8
T	+1.1
M	+1.3
W	+1.3
P	+2.8
Y	+4.3
Q	+5.4
H	+6.8
K	+7.4
N	+7.7
E	+9.5
D	+11.5
R	+19.8

[a]The scale was computationally derived, as described in Section III.A.4. The amino acid residues are presented using a single-letter code.

bilayer. The source of the difference between the values of arginine and lysine in Ben-Tal's scale is the different charge distributions between these two residues, guanidinium versus amine. However, it may well be that the difference was overexpressed in the PARSE set of partial charges used in the calculations (Sitkoff *et al.*, 1996). Further theoretical and experimental work is needed to clarify this and other issues regarding the relative hydrophobicity of the amino acids in the scale. Nevertheless, preliminary tests have shown that the scale is significantly more potent in detecting transmembrane helices in the sequence of membrane than other hydrophobicity scales (H. Ben-Ami, T. Seifer, B. Honig and N. Ben-Tal, manuscript in preparation).

5. Alamethicin

Alamethicin is a 20-amino-acid antibiotic peptide produced by the fungus *Trichoderma viride,* which self-assembles to form ion channels in lipid bilayers.

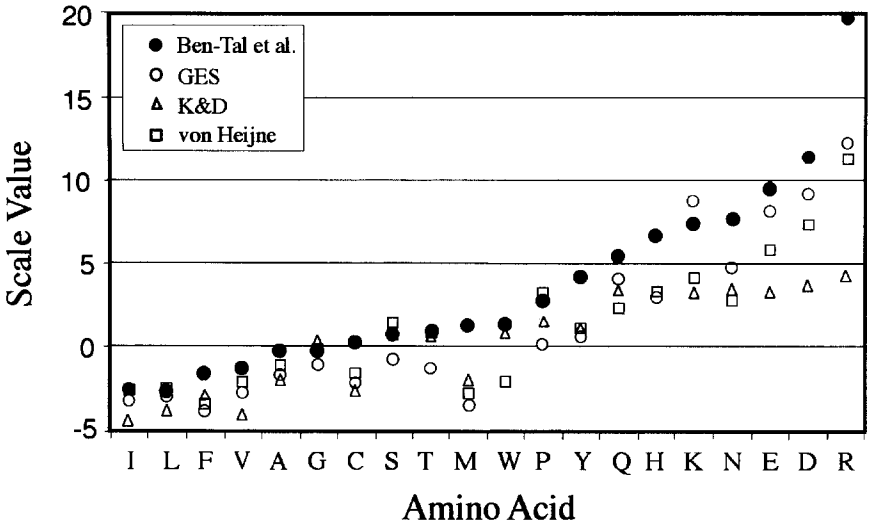
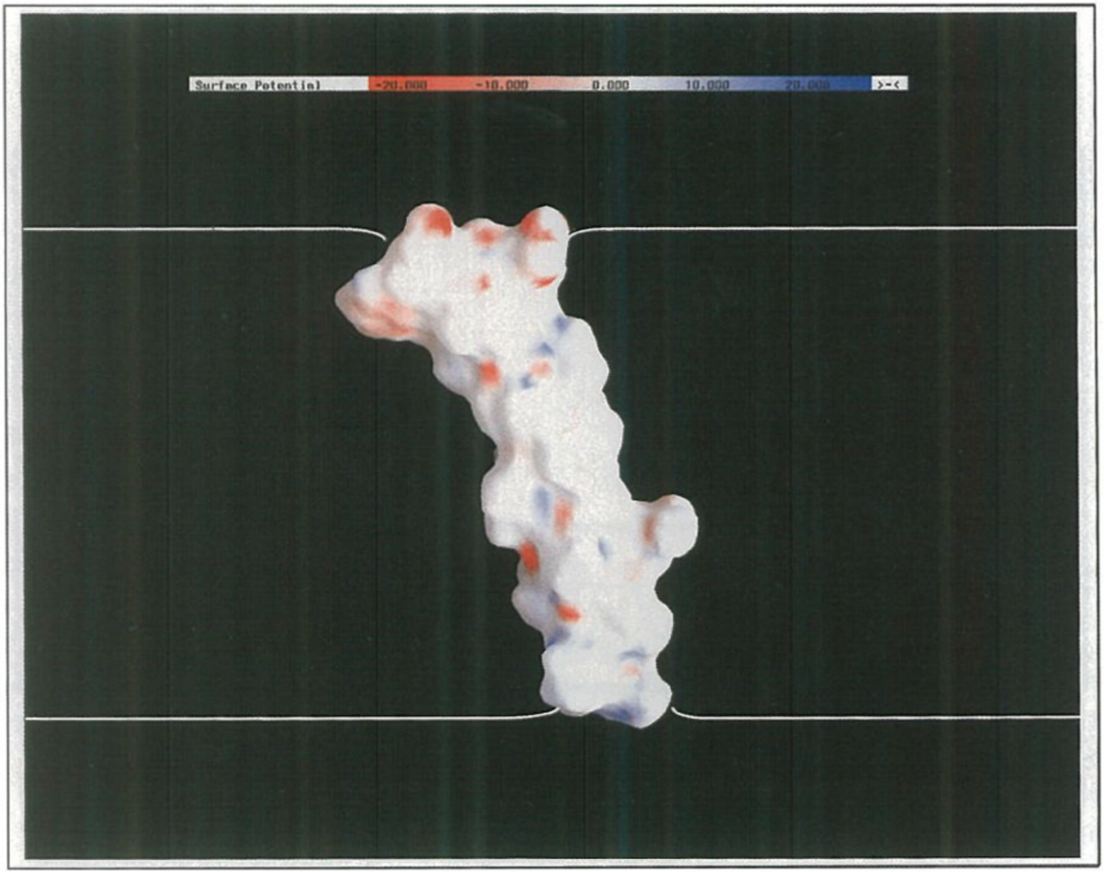


FIGURE 4 Comparison of four different hydrophobicity scales of the amino acid residues. Values for the scales, on the vertical axis, are as follows: free energies in kcal/mol for Ben-Tal *et al.*'s (unpublished data) scale (filled circles), for the Goldman–Engelman–Steitz (GES; Engelman *et al.*, 1986) scale (open circles), and for the von Heijne (1981a,b) scale (open squares), and scale index for the Kyte–Doolittle (Kyte and Doolittle, 1982) scale (open triangles).

The sequence of alamethicin is Ac-**UPUAUAQUVUGLUPVUUQQF**-OH, where Ac is acetyl, U is α-amino isobutyric acid, and F-OH is phenylalaninol, and, again, the hydrophobic and aromatic residues are in bold. The peptide is predominantly α-helical in solution (Banerjee and Chan, 1983; Esposito *et al.*, 1987; Fox and Richards, 1982; Yee and O'Neil, 1992), and the helical structure of its N-terminal segment is maintained in dimyristoylphosphatidylcholine (DMPC) (North *et al.*, 1995). The small size of alamethicin makes it an attractive model for the investigation of peptide–membrane interactions, and indeed, it has been studied intensively both theoretically and experimentally (see reviews by Cafiso, 1994, Sansom, 1998).

Sansom and his co-workers (Biggin *et al.*, 1997) studied alamethicin using a simplified representation of the membrane as a "hydrophobic potential," adapted from Milik and Skolnick's (1993, 1995) Monte Carlo simulations of peptide–membrane systems, described above (Section III.A.1). In this study, alamethicin structures were generated by simulated annealing, and a restrained molecular dynamics simulation, in the presence of a transbilayer voltage, was carried out. The interactions between alamethicin and the lipid bilayer were represented by a hydrophobicity index assigned to the side chains of the amino acid residues, and a constant value of 4.1 kcal/mol was assigned to each potential hydrogen-bonding group exposed to the bilayer. The authors stated that the main limitation of this


Surface Potential
-20.000
-10.000
0.000
10.000
20.000

study was the coarse-grained treatment of the interactions between alamethicin and the lipid bilayer, namely, the use of a residue-based hydrophobicity index, which obscures the atomic detail of these interactions.

Free energy calculations have very recently been carried out within the framework of the mean-field approach of Eq. (1) and Fig. 2 to study alamethicin–membrane interactions in the limit of low peptide concentration (Kessel *et al.*, 2000a,b). The main results are described below.

a. ΔG_{tot} and the Most Likely Alamethicin–Bilayer Configuration. For the alamethicin–membrane system, the total free energy of Eq. (1) can be approximated as $\Delta G_{\text{tot}} \approx \Delta G_{\text{sol}} + \Delta G_{\text{imm}} + \Delta G_{\text{lip}} + \Delta G_{\text{def}}$ (Kessel *et al.*, 2000a). (Note that $\Delta G_{\text{p}K\text{a}} = 0$, because the alamethicin isoform used here does not contain titratable residues). Recent studies suggest that alamethicin interacts mainly with the hydrocarbon region of the bilayer and that its conformation does not change significantly upon association with the membrane (Barranger-Mathys and Cafiso, 1996; Jayasinghe *et al.*, 1998; Kessel *et al.*, 2000a,b; J. R. Lewis and Cafiso, 1999). Thus, the ΔG_{con}, ΔG_{hg}, and ΔG_{sp} terms are approximately zero (discussed in Kessel *et al.*, 2000a). The ΔG_{sol} term of Eq. (4) was calculated with the same method used for the polyalanine α-helix calculations described in Section III.A.2. The contributions of the ΔG_{imm}, ΔG_{lip}, and ΔG_{def} terms were estimated based on a statistical thermodynamic model of the lipid bilayer (Ben-Shaul *et al.*, 1996; Fattal and Ben-Shaul, 1993). The values were given in Sections II.C, II.E, and II.F.

Again, two configurations of a membrane-associated alamethicin were found to be lower in free energy than isolated alamethicin in the aqueous phase: transmembrane and surface configurations. The most stable of these, associated with the most negative ΔG_{tot} value of Kessel *et al.*'s (2000a) calculations, was the transmembrane orientation (Fig. 5), in agreement with experimental measurements (Barranger-Mathys and Cafiso, 1996; Huang and Wu, 1991; North *et al.*,

FIGURE 5 Schematic representation of the orientation of alamethicin in the 2-Å deformed bilayer. Alamethicin is depicted with the electrostatic potential ϕ color-coded on its molecular surface. This was calculated using DelPhi (Nicholls and Honig, 1991) and is displayed on the molecular surface using GRASP (Nicholls *et al.*, 1991). Negative potentials ($0 > \phi > -20$ *kT/e*) are red, positive potentials ($0 < \phi < 20$ *kT/e*) are blue, and neutral potentials are white. Calculations have been carried out for the conformation found in the crystal structure (Protein Data Bank, accession number 1AMT). The peptide is shown with its N-terminus pointing up and its more polar C-terminus pointing down. The two white lines represent the boundaries of the hydrocarbon region of the lipid bilayer. The polar termini of alamethicin are outside the hydrocarbon region of the deformed lipid bilayer, and the hydrophobic core of the peptide is immersed inside this region. (See color plate.)

1995). The ΔG_{tot} value associated with this configuration was −5.5 kcal/mol, again in agreement with the value derived from nuclear magnetic resonance (NMR) measurements (J. R. Lewis and Cafiso, 1999).

The hydrophobic region of alamethicin is about 3 Å shorter than the native width of the hydrocarbon region of the lipid bilayer (Fig. 5). One of the interesting results of Kessel *et al.*'s (2000a) calculations is that the transmembrane insertion of alamethicin into a lipid bilayer of native hydrocarbon region of 30 Å is likely to involve an approximately 2-Å deformation of the bilayer, to match the hydrophobic length of the peptide (Fig. 5). This is in agreement with experimental data (He *et al.*, 1996; J. R. Lewis and Cafiso, 1999; Wu *et al.*, 1995). A 2-Å deformation of the lipid bilayer may be insignificant, considering the dynamic nature of the bilayer. However, recent calculations with other hydrophobic peptides, such as the M2δ segment of the acetylcholine receptor (A. Kessel and N. Ben-Tal, unpublished data) and gramicidin (S. Bransburg-Zabary, A. Kessel, M. Gutman and N. Ben-Tal, unpublished data), indicate that the transmembrane insertion of such peptides may lead to membrane deformation of as much as 10 Å. These preliminary results suggest that peptide-induced membrane deformation is a general characteristic of such systems.

The hydrophobic mismatch between alamethicin and the lipid bilayer, which is responsible for the membrane deformation upon alamethicin insertion, may explain the assembly of the intact channel. The mismatch results mainly from the limitation of the length of the peptide's central hydrophobic region by the C-terminal polar Gln18 side chain. The conservation of this polar residue throughout evolution suggests an evolutionary advantage for this mismatch. The formation of the ion channel results from aggregation of the alamethicin monomers, which reduces the extent of peptide–bilayer interactions. Therefore the deformation of the membrane should also be decreased. Thus, a peptide such as alamethicin is likely to oligomerize and form ion channels to reduce its unfavorable interactions with the lipid bilayer. This hypothesis is supported by experimental studies of alamethicin (Keller *et al.*, 1993; J. R. Lewis and Cafiso, 1999). The general role of the hydrophobic mismatch in protein oligomerization is also demonstrated by a study of bacteriorhodopsin (B. A. Lewis and Engelman, 1983a).

b. Flip-Flop across the Lipid Bilayer. Alamethicin can reverse its orientation in the bilayer, that is, undergo a "flip-flop" motion. The flip-flop of alamethicin in POPC vesicles has been studied by NMR spectroscopy (Jayasinghe *et al.*, 1998), and the results suggest that it involves an energy barrier, presumably due to the free energy of transfer of the peptide termini across the bilayer. Recent mean-field calculations, following the framework of Eq. (1) and Fig. 2, have suggested a path for the flip-flop motion (Kessel *et al.*, 2000b).

The obvious path, which involves the rotation of the peptide around its center of mass, is characterized by a very high free energy barrier (~30 kcal/mol), resulting from burying the two termini in the bilayer simultaneously (Kessel *et al.*, 2000a). A more plausible option is a sequential rotation of the peptide around one terminus at a time (Fig. 6); This flip-flop path involves two free energy barriers, each associated with inserting one of the peptide termini from the aqueous phase into the bilayer. The flip-flop rate was estimated by calculating the ΔG_{tot} values associated with the configurations above and below each of these two free energy barriers (Kessel *et al.*, 2000b). The ΔG_{tot} values and the barrier heights are given in Fig. 6. The flip-flop rate derived from these calculations, $\sim 10^{-7}$/s, is close to the measured value of 1.7×10^{-6}/s (Jayasinghe *et al.*, 1998). Selective charge neutralization of polar groups in alamethicin confirms that the main free energy barrier of the flip-flop process results from the water-to-lipid bilayer transfer of the C-terminus.

6. Conclusions

The studies presented in Sections III.A.1–III.A.5 used three different mean-field approaches to treat membrane systems in which the energetics is dominated by the interactions of a transmembrane hydrophobic span with the hydrocarbon region of the lipid bilayer.

The common advantage of these approaches is that their simplicity makes it possible to use them to describe both thermodynamic and long-term kinetic aspects of peptide–membrane systems. The common disadvantage of these approaches is the lack of atomic description of the lipid bilayer. This is especially important in the description of the interactions between the peptide and the chemically diverse polar headgroup region of the bilayer (Fig. 1). Although these interactions are insignificant in the systems presented above, this may not always be the case (e.g., see peptides of category C, Section III.C below).

Although the three approaches have many qualities in common, they do differ in some respects. The continuum solvent model of Section III.A.2–III.A.4 is based on atomic description of the peptide and a slab representation of the lipid bilayer. Its limitations have been discussed at length (Ben-Tal *et al.*, 1996a, 2000a). The major shortcomings are the model's complete neglect of the polar headgroup region and the fact that, at least in its current implementation, the model requires a known high-resolution structure of the peptide. The model of Milik and Skolnick (1993, 1995) is based on an approximate description of both the protein and the bilayer, and the model used by Sansom and his co-workers (Biggin *et al.*, 1997) relies on a residue-based hydrophobicity index, which obscures much of the atomic detail of the interactions between the peptide and the bilayer. However, although these two models do not provide an accurate estimate of ΔG_{sol}, both provide a means with which to consider conformational changes of

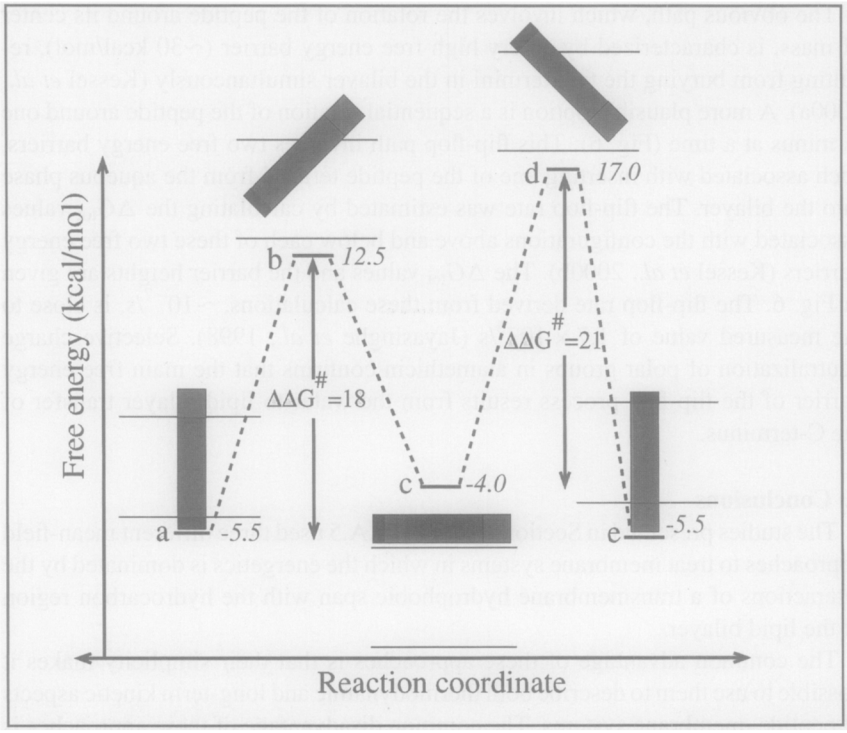


FIGURE 6 A schematic representation of the two suggested paths for alamethicin flip-flop. Each of the two paths involves the following configurations, each of which is obtained from the previous one by rotation: (a) The initial (or final) transmembrane orientation of the peptide in the bilayer, with the C-terminus (medium gray) facing upward and the N-terminus (black) downward. (b) Insertion of the N-terminus of the peptide into the lipid bilayer, while the C-terminus remains at the water–bilayer interface. The long axis of the peptide is tilted approximately 45° with respect to the bilayer normal. The path from configuration a to b involves rotation around the C-terminus residue. (c) Peptide adsorption on the surface of the lipid bilayer. The path from configuration b to c involves further rotation around the C-terminus residue. (d) Insertion of the C-terminus of the peptide into the lipid bilayer, while the N-terminus remains at the water–bilayer interface. This orientation is the reciprocal of the orientation described in b. The path from configuration c to d involves rotation around the N-terminus residue. (e) The final (or initial) transmembrane orientation of the peptide in the bilayer, with the C-terminus facing downward and the N-terminus upward. This transmembrane orientation is the reciprocal of orientation a. The path from configuration d to e involves further rotation around the N-terminus residue. The two paths differ from one another in the order of occurrence of the configurations a → e versus e → a. Alamethicin is schematically depicted as a rectangle. The central hydrophobic region of the peptide is dark gray, the C-terminus of the peptide is medium gray, and the N-terminus is black. The borders of the hydrophobic core of the lipid bilayer are marked by the two horizontal lines. The calculated free energy values of the alamethicin–membrane system in each configuration are given in italic. The values of the free energy barriers of the most probable path, from the left-hand side to the right-hand side, are given in roman.

the peptide upon membrane insertion. The approach of Sansom and co-workers also considers transbilayer voltage effects, which are important in many biological systems.

B. Category B: Peptides That Interact Predominantly with the Polar Headgroup Region of the Bilayer

For peptides (and protein fragments) that interact mainly with the polar headgroups of bilayer lipids, the free energy of the peptide–membrane system should be dominated, in essence, by the ΔG_{hg} and ΔG_{sp} contributions and should hardly be affected by the hydrocarbon region of the bilayer.

Short basic peptides, such as pentalysine, which interact electrostatically with acidic lipids in the membrane, are typical examples for this category. Because the exact chemical nature of the lipid does not matter, provided that it is negatively charged (as opposed to zwitterionic) and that it can electrostatically attract the positively charged peptides, the ΔG_{sp} contributions should be negligible. For example, pentalysine associates with a negatively charged membrane made up of phosphatidylserine (PS) or phosphatidylglycerol (PG) with essentially the same ΔG_{tot} (Kim *et al.*, 1991; Mosior and McLaughlin, 1992). A brief summary of some studies on the interaction of such peptides with the bilayer follows. A more detailed description can be found in Chapter 3.

1. Short Basic Peptides

Many important peripheral proteins contain clusters of basic residues that interact electrostatically with acidic lipids in the membrane. Tri-, penta-, and heptalysine have been studied to determine factors that are common to the membrane association of all of these peripheral proteins (Ben-Tal *et al.*, 1996b; Murray *et al.*, 1999). The electrostatic free energy of interaction of the basic peptides with phospholipid bilayers, that is, the electrostatic contribution to ΔG_{hg}, was calculated by applying the (nonlinear) Poisson–Boltzmann equation [Eq. (5)] to atomic models of the peptides and bilayers in aqueous solution. The electrostatic free energy of interaction, which arises from both the long-range Coulombic attraction between the positively charged peptide and the negatively charged lipid bilayer and the short-range Born repulsion, had a minimum at a distance of ~3 Å (i.e., one layer of water) between the van der Waals surfaces of the peptide and the lipid bilayer. The molar association constant K, calculated as the Boltzmann average over many peptide–membrane configurations, was in accord with the measured values: K was typically about 10-fold smaller than the experimental value (i.e., a difference of about 1.5 kcal/mol in the free energy of association). The predicted dependence of K (or the association free energies) on the ionic strength of the solution, on the mole percent of acidic lipids in the membrane, and on

the number of basic residues in the peptide agreed very well with experimental measurements.

Furthermore, the same model has been applied to calculate the electrostatic free energy of membrane association of the positively charged toxin charybdotoxin (CTX) and analogues of known NMR structure (Ben-Tal *et al.*, 1997a) and of a basic peptide corresponding to residues 2–19 of Src (Ben-Tal *et al.*, 1997a; Murray *et al.*, 1998). Again, the calculated and measured binding free energies agreed and the calculations provided a molecular interpretation of the experimental data.

The limitations of the model have been discussed in detail elsewhere (Ben-Tal *et al.*, 1996b, 1997a). The main weakness of the model is apparently the static representation used for the polar headgroups. As a result, any peptide-induced structural modifications, including small adjustments in the location of the polar headgroups that are in direct contact with the peptide, are not taken into account.

2. Neuromodulin

Neuromodulin, a calmodulin substrate, is thought to play an important role in the calcium/phospholipid second-messenger signaling system (Liu and Storm, 1990). It is believed to be attached to the cell membrane by the two palmitoyl groups that are covalently attached to cysteine residues near its N-terminus (Houbre *et al.*, 1991; Skene and Virag, 1989). The calmodulin-binding domain of human neuromodulin (residues 39–55) contains a cluster of basic residues; when neuromodulin is free, rather than bound, these residues may interact with acidic lipids in the bilayer.

The peptide KIQASFRGHITRKKLKG, which corresponds to residues 37–53 of bovine neuromodulin (Skene, 1989), contains two arginine, four lysine, and no negatively charged residues. McLaughlin and his co-workers (Kim *et al.*, 1994) have shown that the membrane affinity of this peptide increases as a function of the mole percent of acidic lipid. Upon serine phosphorylation by protein kinase C, the net charge of the peptide decreases from +6 to +2, and the measurements show that the membrane affinity of the phosphorylated peptide is significantly lower than that of the nonphosphorylated peptide.

Ben-Tal (1997, and unpublished data) used the continuum solvent model as described in the studies of short basic peptides (Section III.B.1) to calculate the binding of the neuromodulin peptide to lipid bilayers. A model of the peptide (in extended conformation) was constructed using InsightII (MSI, San Diego, CA), and its interaction free energy with atomic models of lipid bilayers containing different mole percent of acidic lipids at 100 m*M* salt concentration was calculated for different peptide–membrane configurations. Again, the most negative value of the interaction free energy was obtained when the van der Waals surfaces of the

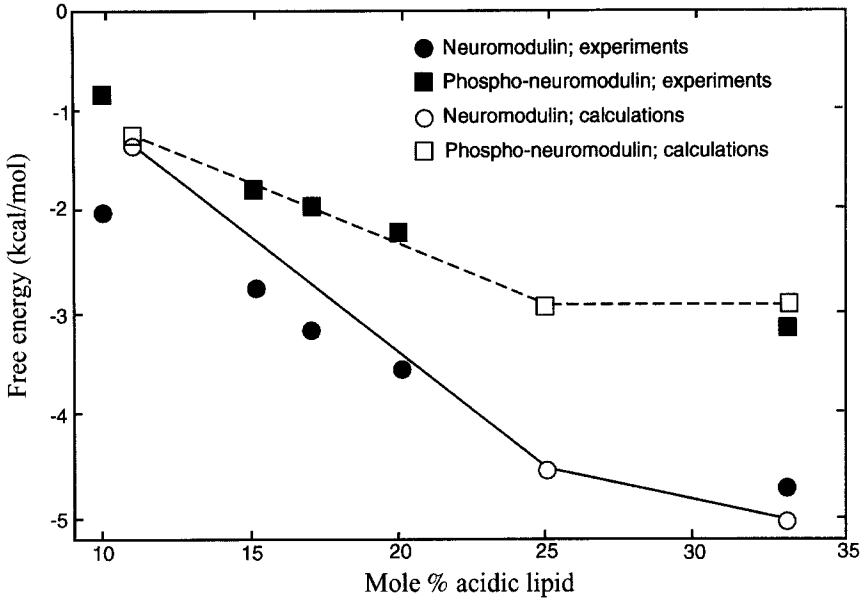


FIGURE 7 Neuromodulin and phospho-neuromodulin association with lipid bilayers: the experimentally determined standard Gibbs free energies of association of neuromodulin (39–55) (*circles*) and phospho-neuromodulin (39–55) (*squares*) to PC:PG unilamellar vesicles in 100 m*M* KCl (Kim *et al.*, 1994). The open circles and open squares represent the theoretical estimates for the association free energies of these peptides, respectively.

peptide and the bilayer were about 3 Å from each other. The association free energy ΔG_{tot} was approximated, and the value of the free energy at this minimum and the results are presented in Fig. 7 along with the measured values of McLaughlin and his co-workers (Kim *et al.*, 1994).

Overall, the calculated values of the association free energy were in good agreement with measured values. The calculations reproduced the dependence of the association free energy on the mole percent of acidic lipids in the membrane both for the phosphorylated and the nonphosphorylated peptides. There were deviations between the calculated and measured values at low concentration of acidic lipid (12 mol%); the calculated value was approximately 0.7 kcal/mol higher (i.e., less negative) than the measured value. This is presumably because of the oversimplified model used (discussed in Ben-Tal *et al.*, 1996b). The model does not take into account lipid motions and it may well be that at low acidic lipid concentrations, peptide adsorption onto the bilayer induces an accumulation of acidic lipids at the expense of zwitterionic lipids near the peptide (e.g., May *et al.*, 2000).

The model involves several arbitrary assumptions. First, the structure of the peptide is unknown. Extended conformation was used as an approximation of a random coil. Preliminary results showed that the membrane affinity of the peptide is significantly lower in an α-helical conformation. This is because the α-helix conformation prevents at least some of the basic residues from being in close proximity to the membrane plane. The main advantage of the extended form over other possible conformations is that it enables all the basic residues to be in the vicinity of the membrane plane, and thus it is likely to provide the maximal ΔG_{hg} contribution to the association free energy.

Second, the model is based on the assumption that the peptide does not penetrate the membrane. There is experimental evidence that this is the case for the tri-, penta-, and heptalysine peptides (Ben-Tal *et al.*, 1996b), and the very hydrophilic nature of the neuromodulin (39–55) peptide suggests that it behaves similarly. However, there are no experimental data in support of this assumption. In view of these shortcomings in the calculations, the agreement between the calculations and experiments with respect to the membrane affinity of the neuromodulin (39–55) peptide may have been fortuitous.

3. Conclusions

Several generalizations can be made about the association of basic peptides with bilayers containing acidic lipids. First, continuum solvent model calculations suggest that the long-range Coulomb attraction between the oppositely charged peptide and membrane is overly compensated for by the short-range image charge and Born repulsion. The balance between these two effects leads to a minimum in the electrostatic free energy when the van der Waals surfaces of the peptide and the membrane are about 3 Å (i.e., one water layer) from each other. Experimental data suggest that basic peptides are adsorbed onto membrane surfaces in very close proximity to the headgroups and yet they do not penetrate the bilayer (e.g., Ben-Tal *et al.*, 1996b, 1997a). However, it is unclear whether there is a layer of water between the peptide and the headgroup region.

Second, continuum solvent models based on atomic models of the peptide and the lipid bilayer are often successful in reproducing experimental partitioning coefficients of peptide–membrane association in cases where the peptides do not penetrate the membrane. A theoretical treatment of peptides that penetrate the membrane is likely to require replacing the static model of the lipid bilayer with one that incorporates lipid motions.

Third, the calculations and measurements suggest a rule of thumb to approximately account for the contributions of basic residues to the association of peptides and proteins to membranes. At physiological conditions, that is, about 25–33 mol% acidic lipid at a monovalent salt concentration of about 150 m*M*,

each lysine or arginine residue in the vicinity of the acidic lipids contributes about 1 kcal/mol to the free energy of adsorption onto the cytoplasmic side of cellular membranes.

C. *Category C: Peptides That Interact with Both the Hydrocarbon and Polar Headgroup Regions of the Bilayer*

This category includes peptides and protein fragments that are in physical contact with the hydrocarbon and the polar headgroup regions simultaneously and whose interaction free energies with these two regions are of comparable magnitude. We will discuss two examples here: the N-terminal segment of the peripheral membrane protein Src, and the pore-forming domain of the insecticidal toxin δ-endotoxin produced by *Bacillus thuringiensis*.

1. The Src Peptide

The v-Src oncoprotein ($pp60^{v\text{-}src}$) and its normal cellular homologue, c-Src ($pp60^{c\text{-}src}$), are membrane-bound, nonreceptor tyrosine kinases (Resh, 1993). Their N-termini contain two motifs essential for membrane localization: a stretch of polar amino acids rich in positively charged residues and a myristate attached cotranslationally to the N-terminal glycine by *N*-myristoyltransferase (Murray *et al.*, 1997). Association of Src with the plasma membrane is inhibited by mutations that produce nontransforming phenotypes that either reduce the net positive charge in the N-terminal cluster of basic residues (Kaplan *et al.*, 1990; Sigal *et al.*, 1994) or prevent myristoylation (Buss *et al.*, 1986; Cross *et al.*, 1984; Kamps *et al.*, 1985).

Experimental data suggest that the cluster of basic residues at the N-terminus of Src interacts electrostatically with acidic lipids in the membrane, but does not penetrate the membrane (Murray *et al.*, 1997, and references therein), whereas the myristate partitions into the hydrocarbon region of the lipid bilayer (Peitzsch and McLaughlin, 1993). These key observations were later used by Murray *et al.* (1998) to experimentally and theoretically investigate the binding of myristoylated and nonmyristoylated peptides corresponding to residues 2–19 of avian c-Src, GSSKSKPK*D*PSQRRRS*LE*. They used the continuum solvent model presented in Section III.B and atomic models of the peptide and the bilayer to calculate the free energy of interaction of the peptides with lipid bilayers for different peptide–membrane configurations. They then used these free energy values to deduce the molar partition function of the peptides between the bilayer and the aqueous phase, carrying out statistical thermodynamic averaging to calculate the partition function of the free and membrane-adsorbed peptides.

Again, the results were in good agreement with the measured peptide partitioning onto phospholipid vesicles. Membrane association of the nonmyristoylated Src peptide increased as the mole percent of acidic lipid in the vesicles was increased, the ionic strength of the solution was decreased, or the net positive charge of the peptide was increased (due to phosphorylation). The theoretical model also correctly predicted the measured partitioning of the myristoylated peptide, myr-Src(2–19); for example, adding 33% acidic lipid to electrically neutral vesicles increased the partitioning of myr-Src(2–19) by a factor of 100. Phosphorylating either serine 12 or serine 17 decreased the partitioning of myr-Src(2–19) onto vesicles containing acidic lipid by a factor of 10.

The success of the theoretical model in this study was based on two facts. First, the coupling between the contributions of the myristate and the cluster of basic residues to membrane association is weak (Buser *et al.*, 1994; Murray *et al.*, 1997). Thus, the computational effort was dedicated to accurately calculating the electrostatic free energy of interaction of the cluster of positively charged residues with the negatively charged membrane. The contribution of the myristate was added at a later stage and the assumption was that it is, in essence, independent of changes in the membrane affinity of the cluster of positively charged residues, such as those due to peptide phosphorylation. Second, experimental data indicated that Src(2–19) is a random coil both in the aqueous phase and when membrane-associated, and that it does not penetrate the bilayer. Thus, approximate atomic models of the peptide (in extended form) and of a bilayer patch were built and the calculations involved a search in configurational space, considering the peptide and the bilayer as rigid bodies.

In summary, experimental data suggest that the theoretical treatment of the Src peptides requires explicitly taking into account only the peptide interactions with the polar headgroups. The experimentally derived contributions due to interactions of the myristate with the hydrocarbon region can be added separately.

2. The *Bacillus thuringiensis* δ-Endotoxin

δ-Endotoxins are highly potent insecticidal toxins produced by *Bacillus thuringiensis*. Research over the past three decades has attempted to elucidate the structure of the δ-endotoxins and its implications for their activity. The X-ray crystal structure of the *Bacillus thuringiensis* δ-endotoxin in its soluble form showed that the toxin comprises three domains (Gouaux, 1997; Li *et al.*, 1991). We will focus here on one of them: the pore-forming domain, which is an α-helix bundle consisting of seven helices.

The structure of the *Bacillus thuringiensis* δ-endotoxin in lipid bilayers was, until recently, unknown. In a combined experimental and theoretical effort, Gazit *et al.* (1998) elucidated the structure and organization of the pore-forming domain of δ-endotoxin in lipid bilayers. They used fluorescent and FTIR measurements

to determine the affinity and orientation of seven synthetic peptides in small unilamellar vesicles, corresponding to each of the seven helices of the pore-forming domain. In addition, they used resonance energy transfer measurements of all possible combinatorial pairs of membrane-bound helices to map the network of interactions between helices in their membrane-associated state. The results are consistent with a situation in which helix 1 does not associate with the lipid bilayer, helices 4 and 5 insert into the membrane antiparallel to each other as a helical hairpin structure, and helices 2, 3, 6, and 7 lie on the membrane surface, radiating outward from their point of entry into the bilayer like the spokes of a wheel.

Gazit *et al.*'s study also reported a series of Monte Carlo simulations to probe low-energy configurations of peptides corresponding to helices 2–7 in the lipid bilayer. The energy function used in the simulations included the X-PLOR force field (Brunger, 1992) to account for ΔG_{con} contributions to ΔG_{tot}. The ΔG_{sol} and ΔG_{hg} contributions to ΔG_{tot} were taken into account using the "hydrophobic potential" approach (Biggin and Sansom, 1996; La Rocca and Sansom, 1998) described in Section III.A.5. The results of the simulations showed that each of the six peptides tends to adopt a helical conformation and to associate with the membrane. The calculated value of ΔG_{tot} was usually larger in magnitude than the measured value, in some cases by a factor of two, but the correlation between the calculated and measured values was good. However, the situation was not as good regarding the most likely orientations of the peptides in the bilayer. For example, the calculations suggested that all the helices, including helices 4 and 5, which have been experimentally shown to be transmembrane (Gazit *et al.*, 1998), are surface-bound in oblique orientations. The authors suggested that the reason for the discrepancy between the theoretical results and the measurements may be that membrane insertion of these two helices involves the formation of homodimers.

In summary, Gazit *et al.*'s study provides yet another example of the fact that current methodology usually cannot fully describe peptide–membrane systems, unless specific assumptions about the system at hand are included.

3. Conclusions

We have reviewed two different methods that have been used to elucidate thermodynamic aspects of peptide–membrane interactions for peptides of category C, that is, peptides that interact both with the hydrocarbon and the headgroup regions of the lipid bilayer. The first method is based on continuum solvent model; the peptide is represented in atomic detail and the membrane is represented as an all-atom model in one of them (Murray *et al.*, 1998). The other method (Gazit *et al.*, 1998) is based on MD simulations, in which the peptide is described in atomic detail and the bilayer is described as a structureless medium.

The two methods were successful in the reproduction of some, but not all, of the experimental data on the systems at hand. In cases where the structure of the peptide and its orientation in the bilayer are accurately predicted, the value of ΔG_{tot} is unreasonably high in magnitude, and in cases where the value of ΔG_{tot} is reasonable, the orientation of the peptide in the membrane is in conflict with experimental values. The most successful study of the above was on the interactions of the Src(2–19) peptide with lipid bilayers. However, the success in this case may be attributed to the fact that ample experimental data on the structure of the peptide and the nature of its interactions with the bilayer were implemented in the theoretical model. Overall, our theoretical understanding of the thermodynamics of association of peptides of category C with lipid bilayers is far from being satisfactory and requires that experimental data be incorporated into the theoretical model in one way or another.

IV. OPEN QUESTIONS

We have described a theoretical framework to clarify the energetics of peptide–membrane association (Fig. 2) and demonstrated its utility in mean-field-based computational studies of peptides that interact with the hydrocarbon region of the membrane, the polar headgroup region, or both. In many cases, the theoretical treatment is successful in reproducing experimental data (e.g., regarding the most likely conformation and orientation of peptides in the membrane), thus providing molecular interpretation of the experimental data. Nevertheless, the failure of the mean-field model to deal with certain peptide–membrane systems implies that our theoretical understanding of the thermodynamics of such systems is incomplete. Indeed, many peptide–membrane systems that have been studied experimentally are too complicated for a theoretical treatment, and for many cases in which theoretical models were applied, the experimental data could not be fully explained. For example, in most of the approaches described here it is assumed that the structure of the membrane does not change upon peptide association and in some of the approaches the three-dimensional structure of the peptide is taken as a given, based on prior experimental knowledge.

A successful theoretical treatment of the energetics of peptide–membrane systems requires that several fundamental questions about peptide–membrane interactions be answered. For example, the free energy of transfer of the peptide backbone hydrogen bond from the aqueous phase into the hydrocarbon region of the membrane is not yet known; estimates range from 0.6 (Roseman, 1988b) to 2.2 kcal/mol (Ben-Tal *et al.*, 1996a). As discussed by White and Wimley (1999), this value, which is of central importance to our understanding of peptide–membrane interactions, has major implications for folding and stability in

membrane proteins. The free energy of transfer of the peptide backbone hydrogen bond from the aqueous phase into the polar headgroup region of the bilayer is also unknown (White and Wimley, 1999).

The contribution of the polar headgroup region to the energetics of peptide–membrane systems requires further clarification. For example, it is unclear why the aromatic residues Phe and Trp have high affinity to the water bilayer interface (e.g., Killian and von Heijne, 2000; Pilpel *et al.*, 1999; White and Wimley, 1999; Yau *et al.*, 1998). Most of the hydrophobicity scales indicate that these residues are highly hydrophobic, and yet they are much less commonly found at the hydrocarbon region of the bilayer than the aliphatic residues (Ala, Leu, Ile, and Val). The high affinity of the aromatic residues to the polar headgroup region has been variously attributed to amphipathic interactions of the imino group's hydrogen bonding, specific dipolar interactions, enhanced acyl-chain van der Waals interactions, and cation–π interactions (Yau *et al.*, 1998). Another possible explanation is that the rigidity of the aromatic residues leads to yet another entropy penalty due to their confinement effect on the lipid chains. This qualitative explanation needs to be numerically tested. Overall, estimates of ΔG_{lip} and ΔG_{def} have been derived from models in which simplified geometry was used to represent the peptide (e.g., Fattal and Ben-Shaul, 1993). The assumption is that peptides can be approximated as cylinders and that their effective diameter is approximately constant. Continuum solvent model calculations have shown that the exact geometry of the peptide significantly affects the electrostatic and nonpolar contributions to the solvation free energy (Honig and Nicholls, 1995). It may well be that ΔG_{lip} and ΔG_{def} are similarly affected. This issue awaits further studies using detailed statistical thermodynamic models at the atomic level.

The effect of the polar headgroups on the pK_a of the titratable amino acids is well documented experimentally (e.g., Section II.A), but awaits a theoretical explanation. For the most part this effect can probably be explained based on desolvation, but the extent of desolvation and the contributions of the different chemical groups need to be elucidated. Specific interactions between polar residues and the polar headgroups need to be studied.

The value of the dielectric constant in the polar headgroup region is unknown, and, given the chemical complexity of this region, it is not even clear if assigning a single (average) value to this region is meaningful (Fig. 1; White and Wimley, 1999). Peptide and protein association with lipid bilayers may involve association with the phosphate groups of the lipid polar heads. This, in turn, may lead to full or at least partial desolvation of these groups. The energetic cost of this process is unknown (Ben-Tal *et al.*, 1997a).

Answering these questions requires a combination of theoretical and experimental effort.

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