Statistical Thermodynamic Analysis of Peptide and Protein Insertion into Lipid Membranes

Avinoam Ben-Shaul, Nir Ben-Tal, and Barry Honig
Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032 USA

ABSTRACT A statistical thermodynamic approach is used to analyze the various contributions to the free energy change associated with the insertion of proteins and protein fragments into lipid bilayers. The partition coefficient that determines the equilibrium distribution of proteins between the membrane and the solution is expressed as the ratio between the partition functions of the protein in the two phases. It is shown that when all of the relevant degrees of freedom (i.e., those that change their character upon insertion into the membrane) can be treated classically, the partition coefficient is fully determined by the ratio of the configurational integrals and thus does not involve any mass-dependent factors, a conclusion that is also valid for related processes such as protein adsorption on a membrane surface or substrate binding to proteins. The partition coefficient, and hence the transfer free energy, depend only on the potential energy of the protein in the membrane. Expressing this potential as a sum of a "static" term, corresponding to the equilibrium (minimal free energy) configuration of the protein in the membrane, and a "dynamical" term representing fluctuations around the equilibrium configuration, we show that the static term contains the "solvation" and "lipid perturbation" contributions to the transfer free energy. The dynamical term is responsible for the "immobilization" free energy, reflecting the loss of translational and rotational entropy of the protein upon incorporation into the membrane. Based on a recent molecular theory of lipid-protein interactions, the lipid perturbation and immobilization contributions are then expressed in terms of the elastic deformation free energy resulting from the perturbation of the lipid environment by the foreign (protein) inclusion. The model is formulated for cylindrically shaped proteins, and numerical estimates are given for the insertion of an α-helical peptide into a lipid bilayer. The immobilization free energy is shown to be considerably smaller than in previous estimates of this quantity, and the origin of the difference is discussed in detail.

INTRODUCTION

The free energy change associated with the transfer of a protein or protein fragment from an aqueous solution into a lipid membrane involves several contributions of different origin. The standard transfer free energy, ΔG°, is often written as a sum of a solvation free energy, $\Delta G^{\circ}_{\mathrm{solv}}$, a lipid perturbation free energy, $\Delta G^{\circ}_{\mathrm{lip}}$, and a term, $\Delta G^{\circ}_{\mathrm{imm}}$, which results from immobilization of the protein in the membrane environment (Engelman and Steitz, 1981; Jähnig, 1983; Jacobs and White, 1989; Ben-Tal et al., 1996). In this paper we develop a consistent statistical thermodynamic description of the transfer process that provides a rigorous definition of these individual free energy contributions. Our treatment allows as to arrive at a number of new results and shows how each of the free energy terms is related to specific contributions to the potential of mean force for the transfer of the protein from solution into the membrane.

The magnitudes of the various contributions to ΔG° depend, of course, on the detailed molecular structure of the protein and the lipid membrane. Nevertheless, some general insights into the mechanisms governing protein insertion into membranes can be gained by considering simple model systems. One class of systems that has been studied in some detail, both theoretically (Jähnig, 1983; Engelman et al., 1986; Milik and Skolnick, 1993; Ben-Tal et al., 1996) and experimentally (Leto and Holloway, 1979; Vogel, 1981; Moll and Thompson, 1994), is the incorporation of partially hydrophobic α-helical peptides into lipid bilayers. The "solvation" free energy, $\Delta G^{\circ}_{\mathrm{solv}}$, corresponding to the free energy change upon transferring the peptide from water to an oil-like medium, has been estimated by Jähnig (1983) and more recently by Ben-Tal et al. (1996). Estimates of $\Delta G^{\circ}_{\mathrm{lip}}$ and $\Delta G^{\circ}_{\mathrm{imm}}$ have also been made (Jähnig, 1983).

In this paper we present new models for $\Delta G^{\circ}_{\mathrm{lip}}$ and $\Delta G^{\circ}_{\mathrm{imm}}$. These two terms are found to be closely related; both of them arise from elastic deformations of the lipid bilayer. We estimate $\Delta G^{\circ}_{\mathrm{lip}}$ using a recent molecular-level theory of lipid-protein interaction (Fattal and Ben-Shaul, 1993; Ben-Shaul, 1995). Using this model we then express $\Delta G^{\circ}_{\mathrm{imm}}$ as the free energy cost associated with fluctuations (oscillations) of the protein axis along, and with respect to, the membrane normal. One of our central results is that the translational immobilization free energy depends (logarithmically) on the ratio between 1) the amplitude of the hindered protein motion normal to the membrane plane and 2) the membrane hydrophobic thickness. We also show that $\Delta G^{\circ}_{\mathrm{imm}}$ is independent (as it should be) of the relative amounts of lipid and water in the system.

Experimentally, ΔG° is determined by measuring the partition coefficient expressing the relative concentrations

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Address reprint requests to Dr. Avinoam Ben-Shaul, Department of Physical Chemistry and the Fritz Haber Research Center, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. Fax: 972-2-6513742, email:abs@batato.fh.huji.ac.il.

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of protein in the membrane and the aqueous solution. In the dilute solution limit (i.e., neglecting protein-protein interactions) this quantity, K, can be expressed as a ratio between the partition functions of the protein in the lipid and aqueous phases. Similar expressions apply to related "partitioning" phenomena such as ligand binding to substrates or protein binding to the surface of bilayers. The partition functions involve mass-dependent factors which, because of the change in character of certain degrees of freedom upon binding (e.g., translations become vibrations), appear to predict mass-dependent binding constants (Janin and Chothia, 1978; Dwyer and Bloomfield, 1981). One of our goals in this paper is to show that when all relevant degrees of freedom, i.e., those that change character in a binding (or insertion) process, can be treated classically, all of the mass-dependent factors cancel out identically. However, our main goal is to provide a general and physically meaningful analysis of the factors contributing to the free energy of protein insertion into membranes. To test the model, we consider one special case in some detail: the insertion of an α-helical peptide into a lipid membrane. We compare our numerical estimates with available experimental results and previous theoretical calculations.

THE PARTITION COEFFICIENT

Consider a lipid bilayer of total area A and a hydrophobic core of thickness d_L. The membrane is embedded in an aqueous solution of total volume V. Suppose that N protein molecules are present in the system, and assume partition between the membrane and the solution, so that N_s of them remain free in solution and N_m are incorporated into the membrane. For concreteness let us assume that the protein has a cylindrical shape, with d_P and R_P denoting its length and radius, respectively. The protein will be considered as "incorporated" if its center of mass is (somewhere) within the hydrophobic core of the membrane. As a specific example, we consider the case $d_P = d_L$, namely, the protein length matches exactly the hydrophobic thickness of the unperturbed lipid bilayer. Furthermore, we assume that in its minimum free energy (equilibrium) configuration in the membrane, the protein axis is perpendicular to the membrane plane, with its center of mass located at the midplane between the two lipid leaflets (Fig. 1 *a*). Because we are mainly concerned with the insertion free energy, let us assume that the conformation of the protein in both phases (water and membrane) is the same. It is straightforward to extend the model to more general cases. For instance, if the protein conformation in water is different from that in the membrane, then the conformational free energy change can be added as an extra contribution to ΔG°. In addition, the model can easily be generalized to cases in which $d_P \neq d_L$, as well as to the case of protein adsorption onto the membrane surface. It can also be generalized to treat the insertion of proteins or peptides of noncylindrical shapes.

The equilibrium distribution of proteins between the membrane and the solution is determined, as usual, by the

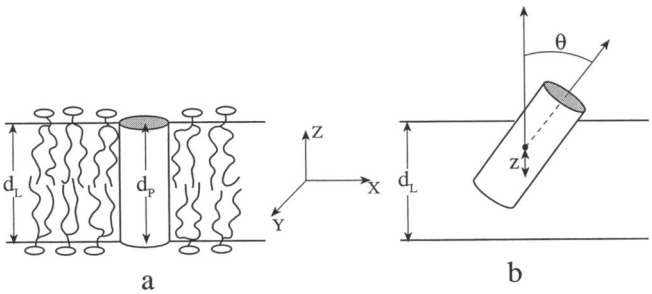

FIGURE 1 Schematic illustration of a cylindrical protein in a lipid bilayer. (*a*) Equilibrium configuration. (*b*) A nonequilibrium configuration; the protein center of mass is displaced a distance z from the bilayer midplane, and its long axis is oriented at angle θ with respect to the membrane normal.

equality of the protein chemical potentials in the two phases, $\mu_s = \mu_m$. In the limit of low protein concentration in solution we can write the ideal solution expression,

$$\mu_s = \mu_s^\circ + kT \ln \rho_s, \tag{1}$$

where $\rho_s = N_s/V$ is the three dimensional (3D) number density of proteins in the aqueous phase, k is Boltzmann's constant, and T is the absolute temperature. (We use μ to denote chemical potentials per molecule.) Similarly, when the 2D concentration of peptides in the membrane is low, we can write

$$\mu_m = \tilde{\mu}_m^\circ + kT \ln \sigma_s = \mu_m^\circ + kT \ln \rho_m, \tag{2}$$

where $\sigma_m = N_m/A$ is the 2D number density in the membrane. In the second equality of Eq. 2 we define $\rho_m = \sigma_m/d_L = N_m/V_m$ as the "effective" 3D number density of peptides in the membrane, with the corresponding standard chemical potential given by $\mu_m^\circ = \tilde{\mu}_m^\circ + kT \ln d_L$; $V_m = Ad_L$ is the total (hydrophobic) volume of the membrane. This change of variables is introduced for convenience only, so that the two standard chemical potentials, μ_s° and μ_m°, are expressed on the same (3D number density) scale.

When q_s and q_m are used to denote the partition functions of a single protein in the solution and the membrane, respectively, the standard chemical potentials can be expressed in the familiar forms (Hill, 1960)

$$\mu_s^\circ = -kT \ln(q_s/V) \tag{3}$$

$$\mu_m^\circ = -kT \ln(q_m/V_m). \tag{4}$$

From Eqs. 1–4 it follows that the partition coefficient, K, of the protein between the membrane and the solution is given by

$$K = \frac{\rho_m}{\rho_s} = \frac{q_m/V_m}{q_s/V} = \exp[-\Delta G^\circ/RT], \tag{5}$$

where $\Delta G^\circ/N_a = \mu_m^\circ - \mu_s^\circ$ denotes the standard free energy of transfer of the protein from solution into the membrane "on the number density scale," N_a is Avogadro's number, and $R = N_a k$ is the gas constant. The second equality in Eq.

5 defines the dependence of the partition coefficient on the number density (or, equivalently, on the molar concentration) scale. Both q_s and q_m are dimensionless. Because q_s/V is independent of V and q_m/A is independent of A (see below), the partition coefficient is only a function of T.

We have emphasized the obvious but important fact that K and hence ΔG^o refer to molar concentration scales, because quite often the partition coefficient is calculated on the mole fraction scale. Specifically, if the protein partitioning between membrane and solvent is expressed in terms of the mole fractions, $X_m/X_s \equiv K_x$, then the corresponding standard free energy change is $\Delta G_x^o = -RT \ln K_x = \Delta G^o - RT \ln(\nu_L/\nu_W)$, where ν_L and ν_W are the molar volumes of lipid and water, respectively.

PARTITION FUNCTIONS

Our assumption that the protein has the same conformation in both the solution and the membrane implies that it can be treated as a rigid body. (More specifically, we assume that the protein is cylindrical.) It is therefore characterized by six degrees of freedom. In the bulk solution three of these degrees of freedom correspond to the translational motion of the protein center of mass; the other three describe the external ("overall") rotations of the rigid body. These degrees of freedom can be safely treated classically. Let x, y, $z \equiv \vec{r}$ denote the center of mass coordinates and p_x, p_y, $p_z \equiv \vec{p}$ the conjugate translational momenta. The rotations can be specified in terms of three Euler angles, θ, η, $\phi = \vec{\Omega}$ and the three conjugate momenta p_θ, p_η, $p_\phi \equiv \vec{p}_\Omega$ (Mayer and Mayer, 1946). We shall use θ to denote the angle between the protein (i.e., the cylinder) long axis and the z axis of a cartesian system of coordinates attached to the protein center of mass ($0 \le \theta \le \pi$). The other two angles ($0 \le \phi \le 2\pi$ and $0 \le \eta \le 2\pi$) specify the angle between the projection of the protein axis and the xy plane and the angle of rotation around the long axis. As we shall see below, only the polar angle θ enters our expressions for K and ΔG^o.

When the protein is incorporated into the membrane, that is, when its center of mass is within the hydrophobic core, some of the degrees of freedom change their character. To specify the position and orientation of the protein in the membrane, we introduce a cartesian system of coordinates, x, y, z, the origin of which is located at (an arbitrary point on) the bilayer midplane, with the z axis perpendicular to this (xy) plane (Fig. 1). Because the membrane is isotropic in the xy plane, the protein center of mass can freely translate within the entire membrane area A. In other words, two translational degrees of freedom are essentially unrestricted. On the other hand, the motion along the z direction is obviously greatly restricted, as it leads either to protrusion of the protein into the aqueous region (left side of Fig. 2 *a*) or to a deformation of the lipid bilayer (left side of Fig. 2 *b*), both involving considerable free energy penalties (see below). Jähnig (1983) has treated this motion as a 1D translation restricted to a small range δz along the z axis. Below we shall model this motion as a vibrational mode (of amplitude $\sim\delta z$, depending on the lipid-protein interaction free energy).

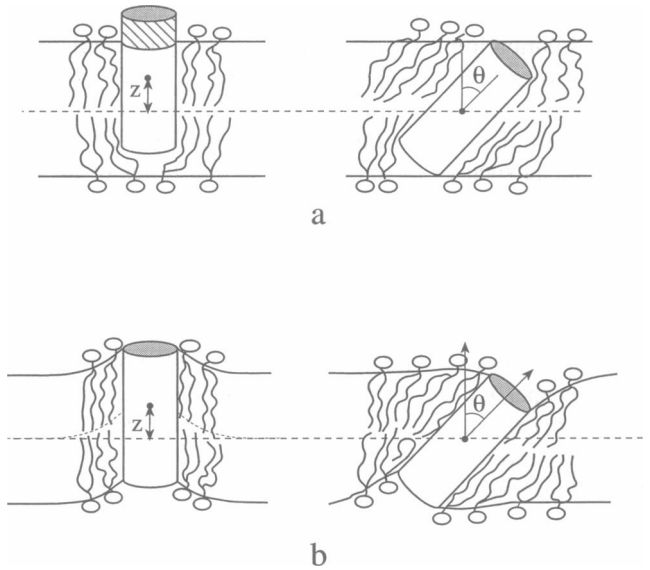

FIGURE 2 Possible models for calculating the energetic cost involved with nonequilibrium protein configurations in the membrane. (*a*) The membrane is assumed to be unaffected by changes in the protein configuration. Left: The protein protrudes beyond the membrane, exposing part of its hydrophobic surface to contact with water. Right: The protein tilts, and thus its two polar ends enter the hydrophobic core of the lipid membrane. (*b*) The elastic lipid chains distort in such a way as to avoid creating a hydrophobic mismatch (as in *a*). The free energy cost involved in these elastic deformations is lower than in model *a*.

Disregarding this difference, it is important to note that because δz is smaller than the membrane hydrophobic thickness, d_L, the protein center of mass is restricted to a volume $A\delta z \equiv V_m^f$, which is smaller than the total membrane volume $Ad_L = V_m$. This implies a considerable entropy loss, on the order of $k \ln(V_m^f/V_m) = k \ln(\delta z/d)$, as compared to free motion within the entire membrane volume. This entropy loss is the origin of the "immobilization" free energy mentioned in the introduction.

The rotational motions of the protein in the membrane are also severely hindered and thus also contribute to ΔG_{imm}^o. These effects are accounted for by the different potential energies $W_s(\vec{r},\vec{\Omega})$ and $W_m(\vec{r},\vec{\Omega})$ that denote the potential energies of the protein in the solution and the membrane, respectively. The Hamiltonians of the protein in the solution and the membrane are, respectively, $H_s = K_E + W_s$ and $H_m = K_E + W_m$, with K_E denoting the kinetic energy term, which is the same in both phases. The use of the Euler angles and the conjugate momenta to describe the motion of the protein implies that K_E depends not only on $\vec{p}$ and $\vec{p}_\Omega$ but also on $\vec{\Omega}$ (more precisely on θ and η) (Mayer and Mayer, 1946).

The classical partition function of the protein, treated as a rigid cylinder characterized by six degrees of freedom, is given by

$$q = \frac{1}{h^6} \int \cdots \int d\vec{p} d\vec{p}_\Omega d\vec{r} d\vec{\Omega} \exp\{-\beta[K_E(\vec{p}, \vec{p}_\Omega, \vec{\Omega}) + W(\vec{r}, \vec{\Omega})]\}, \quad (6)$$

with h denoting Planck's constant and $\beta = 1/kT$. Because the protein, both in the bulk solution and in the membrane, is surrounded by solvent molecules (water and lipids, respectively), the potential energy W is in fact a potential of mean force, which implicitly includes the averaging over the solvent degrees of freedom. Equation 6 applies to both media. In the solution $W = W_s$ defines $q = q_s$, and in the membrane $W = W_m$ defines $q = q_m$. The integration over the translational momenta in Eq. 6 is immediate, yielding the familiar result $(2\pi mkT)^{3/2}$ (see, for example, Hill, 1960), with m denoting the protein mass. The integration over the rotational momenta yields the factor $(2\pi kT)^{3/2}(ABC)^{1/2} \sin\theta$, with A, B, and C denoting the three principal moments of inertia (Mayer and Mayer, 1946). We can thus write

$$q = \Gamma \int \cdots \int d\vec{r} d\vec{\Omega} \sin\theta \exp[-\beta W(\vec{r}, \vec{\Omega})], \qquad (7)$$

with the factor Γ, hereafter referred to as "the momenta integral," defined by $\Gamma = (2\pi kT)^3(m^3ABC)^{1/2}/\hbar^6$. The momenta integral (or partition function) contains all of the mass-dependent factors in q. It should be stressed that the same factor, Γ, appears in both q_s and q_m and hence cancels out when we calculate the partition coefficient K. Consequently, neither K nor ΔG^o should contain any mass-dependent factors. The remaining integral in Eq. 7 is the configurational partition function of the protein. Because the aqueous solution is isotropic, it is clear that W_s is a constant that is independent of the center of mass position $\vec{r}$ or orientation $\vec{\Omega}$ of the protein. We shall set $W_s \equiv 0$, thus measuring the potential of mean force in the membrane, $W = W_m$, relative to its value in the solution.

The configurational integral corresponding to q_s yields

$$q_s = 8\pi^2 V \Gamma, \qquad (8)$$

with the factors V and $8\pi^2$ arising from the integrations over the coordinates and angles, respectively.

Consider now the partition function of the protein in the membrane, q_m. Because the membrane is isotropic in the xy plane, $W_m(\vec{r},\vec{\Omega})$ is independent of x and y. Hence the x, y integrations in Eq. 7 yield the membrane area A. Furthermore, because according to our model W_m depends only on the θ angle, the integrations over the angles ϕ and η yield the factor $4\pi^2$. Thus, noting that $W_m(\vec{r},\vec{\Omega}) = W_m(z,\theta)$, we obtain

$$q_m = 4\pi^2 A\Gamma \int_{-d_L/2}^{+d_L/2} dz \int_0^{\pi} \sin\theta \, d\theta \exp[-\beta W_m(z,\theta)] \equiv 8\pi^2 \Gamma V_m \tilde{q}_m, \qquad (9)$$

where $\tilde{q}_m$ is a reduced partition function ($\tilde{q}_m \equiv 1$ when $W_m \equiv 0$) defined by

$$\tilde{q}_m = \frac{1}{2d_L} \int_{-d_L/2}^{+d_L/2} dz \int_0^{\pi} \sin\theta \, d\theta \exp[-\beta W_m(z,\theta)]. \qquad (10)$$

Using our expressions for q_s and q_m, we can now rewrite Eq. 5 in the form

$$-RT \ln K = \Delta G^o = -RT \ln \tilde{q}_m. \qquad (11)$$

Our next step is to propose a model for $W_m(z,\theta)$ that is later used to derive an explicit expression for $\tilde{q}_m$, leading to explicit expressions for the various contributions to ΔG^o. Before doing so, however, we find it instructive to consider two limiting, albeit hypothetical, cases of interest. Suppose first that $W_m \equiv 0$, in which case the potential of mean force experienced by the protein in the membrane is not different from that in the solution. In other words, the membrane and the solution are simply two different regions of space. Then, the z integral in Eq. 10 yields the factor d_L, and the θ integration yields a factor 2, implying $\tilde{q}_m = 1$ and hence $K = 1$ or $\Delta G^o = 0$. Thus, the concentrations of the protein in the membrane and solution are the same. Of course, the numbers of protein molecules in the two phases will not be the same but, rather, partition according to the relative volumes of the two phases, that is, $N_m/N_s = V_m/V = Ad_L/V$.

As a second limiting case let us suppose that once the protein is inserted into the membrane the motion of its center of mass normal to the membrane midplane is confined to a small range, $\delta z \ll d_L$. Furthermore, its long axis rotations are confined to two small solid angle regions defined by $\delta\theta \ll \pi$, one around $\theta = 0$ and the other around $\theta = \pi$. Ignoring any other contribution to W_m, this implies that $W_m \equiv 0$ wherever $z \leq \delta z$ and $\theta \leq \delta\theta$ [or $\pi \geq \theta \geq (\pi - \delta\theta)$], whereas for all other values of z and θ we have $W_m = \infty$. This "step function" potential is equivalent to the model employed by Jähnig (1983) to calculate the "immobilization" term in ΔG^o. Using this model in Eqs. 10–11 we obtain

$$-\Delta G^o_{imm}/RT \approx \ln\left(\frac{\delta_z}{d_L}\right) + 2\ln\left(\frac{\delta\theta}{\sqrt{2}}\right). \qquad (12)$$

In the last equality we have approximated the result for the θ integral $[1 - \cos(\delta\theta)]$ by $(\delta\theta)^2/2$, (where θ is measured in radians). The first term on the right-hand side of Eq. 12 is due to the loss of translational entropy, whereas the second term results from the loss of rotational entropy of the protein in the membrane, as compared to the solution. In the next section we present a molecular model in which δz and $\delta\theta$ represent the amplitudes of normal oscillations of the protein center of mass relative to the bilayer midplane, and of the protein axis relative to the membrane normal, respectively.

THE TRANSFER FREE ENERGY

In its equilibrium configuration in the membrane, the protein axis is parallel to the z axis (i.e., $\theta = 0$), and its center of mass is at the bilayer midplane (i.e., $z = 0$) (see Fig. 1). Assuming that the fluctuations around the equilibrium po-

sition are small, we expand $W_m(z,\theta)$ to second order in z and θ or, more conveniently, in z and sin θ, so that

$$W_m(z, \theta) = W_m(0, 0) + \frac{1}{2}\lambda z^2 + \frac{1}{2}\omega \sin^2\theta, \qquad (13)$$

with λ and ω representing the second derivatives of W_m with respect to z and sin θ, respectively. Note that, by symmetry, the expansion does not include a "mixed" term (proportional to z sin θ). This follows from the fact that, for any given θ, $W_m(z,\theta) = W_m(-z,\theta)$. Note also that in writing Eq. 13, we regard $W_m(z,\theta)$ as a continuous function of z and θ. This assumption is not valid for the "protrusion model" described in Fig. 2 *a*, according to which W_m varies linearly with the exposed hydrophobic surface area and hence with z, or more precisely with $|z|$. In the next section we argue that the elastic deformation model of Fig. 2 *b* accounts more appropriately for the protein fluctuations around its equilibrium position in the membrane. For this model, W_m is a continuous function of z and θ and Eq. 13 is valid.

The constant (or "static") term in Eq. 13 is the free energy change associated with the transfer of the protein from the solution into its equilibrium position in the membrane. The other two terms account for the free energy cost associated with fluctuations around the equilibrium state. These are the terms that will give rise to the immobilization free energy ΔG^o_{imm}, as will be shown below.

The static term, $W_m(0,0)$, is a sum of several contributions. For the sake of comparison with previous analyses, we express this term as a sum of two contributions,

$$W_m(0, 0) = W_{solv} + W_{lip}. \qquad (14)$$

In the first term, W_{solv}, we include the difference in the electrostatic energy of the peptide between the aqueous phase and the lipid environment. It also includes the difference in the van der Waals and the hydrophobic free energies upon the transfer from water into the lipid environment. In other words, W_{solv} is the transfer free energy of the protein from water into a bulk liquid alkane, which consists of hydrocarbon chains of the kind composing the lipid hydrophobic tails. The second term accounts for the free energy cost associated with the reduced conformational freedom of the lipid chains around the incorporated protein. In many respects it is analogous to the hydrophobic free energy, because its origin is the indirect free energy cost involved in the reorganization of solvent molecules (in this case the lipids) around a solute.

Using Eqs. 10, 11, and 13 we can now write

$$\Delta G^o = \Delta G^o_{solv} + \Delta G^o_{lip} + \Delta G^o_{imm}, \qquad (15)$$

where $\Delta G^o_{solv} = N_a W_{solv}$, $\Delta G^o_{lip} = N_a W_{lip}$, and

$$\Delta G^o_{imm} = -RT \ln \tilde{q}_{imm}. \qquad (16)$$

Here

$$\begin{aligned}\tilde{q}_{imm} &= \tilde{q}_{imm,trans}\tilde{q}_{imm,rot} \\ &= \frac{1}{d_L}\int_{-dL/2}^{+d_L/2} dz \exp[-(\beta\lambda/2)z^2] \\ &\quad \times \frac{1}{2}\int_0^{\pi} d\theta \sin\theta \exp[-(\beta\omega/2)\sin^2\theta] \qquad (17) \\ &\approx \left(\frac{2\pi kT}{\lambda d_L^2}\right)^{1/2}\left(\frac{kT}{\omega}\right) \\ &\equiv \left(\frac{\delta z}{d_L}\right)\left(\frac{\delta\theta}{\sqrt{2}}\right)^2.\end{aligned}$$

The first equality in the last equation, according to which $\tilde{q}_{imm}$ is a product of vibrational (restricted translational) and librational (restricted rotational) partition functions, follows from the fact that the potential energy $W_m(z,\theta)$ is separable into z- and θ- dependent terms. The third equality in Eq. 17 is obtained using the assumption that the amplitudes of the vibrational and librational motions are small. More explicitly, the first factor is obtained when we replace the integration limits on z from $\pm d_L/2$ to $\pm\infty$. This is justified when $\beta\lambda d_L^2 \gg 1$. Similarly, if we assume $\beta\omega \gg 1$, then the main contributions to the θ integral arise from $\theta \approx 0$ and $\theta \approx \pi$. (The θ integral is twice the integral from $\theta = 0 \rightarrow \pi/2$. Because the major contribution to the integral arises from small θ, it can be evaluated by replacing sin θ by θ.) Noting that the probabilities of z and θ fluctuations are governed by the Boltzmann factors $\exp(-\beta\lambda z^2/2)$ and $\exp(-\beta\omega \sin^2\theta)$, one easily finds that the amplitudes of these fluctuations are $\Delta z \equiv \sqrt{\langle z^2\rangle} = \sqrt{kT/\lambda}$ and $\Delta\theta \equiv \sqrt{\langle \sin^2\theta\rangle} = \sqrt{2(kT/\omega)}$. This confirms that the assumptions $\beta\lambda d_L^2 \gg 1$ (implying $(\Delta z/d_L)^2 \ll 1$) and $\beta\omega \gg 1$ (implying $\Delta\theta \ll 1$) are indeed equivalent to assuming small z and θ fluctuations. In the next section we present a molecular model confirming that these assumptions are reasonable.

The last equality in Eq. 17 is obtained when we define

$$\delta z = (2\pi kT/\lambda)^{1/2} = \sqrt{2\pi}\Delta z \qquad (18)$$

$$\delta\theta = (2kT/\omega)^{1/2} = (\sqrt{2}\Delta\theta)^{1/2}, \qquad (19)$$

thus providing a correspondence between the "square well" result of Eq. 12 and Eqs. 16 and 17.

PROTEIN-MEMBRANE INTERACTION

In this section we consider in more detail the separate contributions to ΔG^o, with particular emphasis on the lipid perturbation and immobilization terms. Numerical estimates will be given for the insertion of an α-helical (20-mer polyalanine) peptide into a dimyristoyl phosphatidylcholine (DMPC) bilayer. The peptide length and the hydrophobic

thickness of the membrane are very similar, $d_P = d_L \approx 30$ Å. The radius of the helix cross section is $R_P \approx 5$ Å.

The solvation free energy can be expressed as a sum of electrostatic and "nonpolar" contributions, $\Delta G^o_{solv} = \Delta G^o_{el} + \Delta G^o_{np}$. The first term represents the different electrostatic free energies of the protein in water and in the membrane. The nonpolar term accounts for the different van der Waals energies in the two environments, as well as for the water structure effects known as the hydrophobic interaction. The lipid environment is regarded here as a bulk liquid alkane. The numerical values of the two terms in the solvation free energy depend, of course, on the detailed molecular structure of the protein and the membrane.

Recently both terms were calculated for the insertion of a 25-mer polyalanine α-helix into a lipid membrane (Ben-Tal et al., 1996). ΔG^o_{el} was calculated, using a continuum solvent model, to be ~25 kcal/mol. ΔG^o_{np} was calculated, by multiplying the water-accessible surface area of the helix by a surface tension coefficient derived from experimental free energies of transfer of alkanes from water to liquid alkanes, to be ~ -36 kcal/mol. Thus $\Delta G^o_{solv} \approx -11$ kcal/mol. This negative contribution to ΔG^o is the driving force for the peptide insertion into the membrane, inasmuch as the other two terms, ΔG^o_{lip} and ΔG^o_{imm}, are both positive.

The hydrophobic tails of the lipid molecules comprising the membrane are, on the average, stretched out along the membrane normal. The extent of chain stretching is often measured in terms of the "orientational order parameter," S, of the lipid tail. In the solid (or "gel") phase of the membrane, the lipid chains are fully stretched along the membrane normal, nearly all of them in their "all-*trans*" conformation. The order parameter in this state is nearly a maximum, $S \approx 0.8$ (Jähnig, 1983). In the fluid (or "liquid crystalline") phase of the membrane, which is of greater biological interest and on which we focus here, the order parameter is considerably smaller, typically $S \approx 0.2$ (Jähnig, 1983). The lower order parameter indicates that the lipid chains, although partially stretched, possess considerable conformational freedom, and hence, their entropy is higher than in the solid phase. The insertion of a rigid inclusion, such as a hydrophobic protein into a fluid membrane, stiffens the lipid chains in its immediate vicinity, thus lowering the conformational entropy of the system. This lipid-protein interaction, the origin of which is the lower elasticity (chain conformational freedom) of the membrane, provides a positive contribution $\Delta G^o_{lip} > 0$ to the free energy of transfer. In general, although not always (Fattal and Ben-Shaul, 1993), ΔG^o_{lip} is a minimum when the length of the protein matches exactly the hydrophobic thickness of the membrane, i.e., when $d_L = d_P$ (Fig. 1 *a*). Positive ($d_P > d_L$) or negative ($d_P < d_L$) hydrophobic mismatch results in an additional free energy penalty, associated with excess stretching or compression of the lipid molecules around the protein, to achieve perfect hydrophobic matching (see Fig. 3). This perfect matching ensures that no hydrophobic region of the protein protrudes beyond the membrane hydrophobic core, a process generally involving a higher free energy penalty.

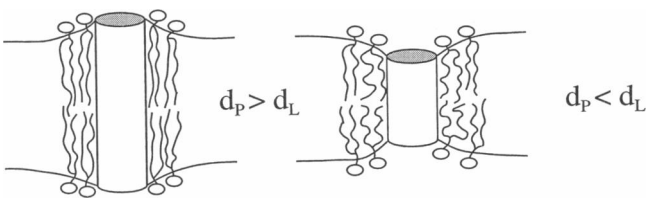

FIGURE 3 A schematic illustration of the lipid-protein interaction model used to estimate the energy of the elastic deformations described in Fig. 2 *b*. In the case of positive hydrophobic mismatch (*left*) the lipid chains stretch out to avoid exposure of the protein hydrophobic region to water. When the mismatch is negative (*right*), the chains are compressed, thus avoiding direct contact between the lipid tails and water.

We shall employ these notions below to formulate a simple model for calculating ΔG^o_{imm}.

In his estimate of ΔG^o_{lip}, Jähnig assumed that the orientational order of the lipids surrounding the incorporated protein is characterized by an order parameter S, the value of which is intermediate between those corresponding to the fluid and solid states of the lipid membrane. Then, based on a Landau-type theory, which relates the change in the order parameter to the entropy change in the fluid-solid transition, he derived the numerical estimate $\Delta G^o_{lip} \approx 2$ kcal/mol for the incorporation of an α-helix (with $d_P = d_L$) into a lipid bilayer. A more direct estimate of ΔG^o_{lip} can be obtained based on a recent molecular-level model of lipid-protein interaction (Fattal and Ben-Shaul, 1993). In this approach one calculates in detail the change in the chain conformational and headgroup interaction free energies as a function of d_P and d_L. Explicit numerical results were only reported for a cylindrical protein of cross-sectional radius $R_P \to \infty$, that is, $R_P \gg R_L$, where R_L is the average distance between lipid headgroups. (In other words, the protein presents a flat rigid wall to the lipid chains in its vicinity.) In principle, the same kind of calculation can be carried out for any R_P value. The numerical results obtained for the large ($R_P \gg R_L$) protein case suggest that, to a good approximation, the lipid-protein interaction free energy can be expressed in the form

$$\Delta G^o_{lip} = \Delta G^o_{lip,0} + \frac{1}{2} N_a \kappa (d_P - d_L)^2. \qquad (20)$$

The first term here represents the lipid perturbation free energy for the case of zero hydrophobic mismatch. The second term accounts for the additional free energy cost in cases of finite mismatch.

The concept of the hydrophobic mismatch plays an important role in various models of lipid-protein interactions in membranes. A representation similar to Eq. 20 was first suggested by Mouritsen and Bloom (1984) in their "mattress model" of lipid-protein interaction. However, their calculation of the restoring force κ is based on a different model than the one adopted in the present paper. The numerical values corresponding to the lipid perturbation energy in the case of a flat protein wall (R_P

$\gg R_L$) and a lipid bilayer in which the average cross-sectional area per chain (at the hydrocarbon-water interface) is ~34 Å^2 (implying $R_L \approx 3.3$ Å) are $\Delta G^o_{lip} \approx L \times 0.22$ kcal/molÅ and $N_a\kappa \approx L \times 0.09$ kcal/molÅ^3, where L is the length of the protein (cross-sectional) circumference. For a smaller, curved inclusion such as an α-helix with a radius of $R_P \approx 5$ Å, the perturbation free energy should be considerably smaller. If, as a crude curvature correction, we multiply the above value of $\Delta G^o_{lip,0}$ by the factor $(1 - R_L/R_P)$, we obtain for the helix insertion process $\Delta G^o_{lip,0} = 2\pi R_P(1 - R_L/R_P) \times 0.22 \approx 2.3$ kcal/mol. This value is very close to the numerical estimate obtained by Jähnig (1983) based on thermodynamic data (~2 kcal/mol). Using a similar curvature correction, we obtain $N_a\kappa \approx 0.07$ kcal/molÅ^2.

Consider now our expression (Eq. 16) for the immobilization free energy. The elastic constants λ and ω, appearing in Eq. 17, measure the free energy penalties corresponding to fluctuations of the protein around its equilibrium position. One can imagine two limiting models of these fluctuations. In one case, the lipid membrane remains unchanged, whereas the protein protrudes into the aqueous solution or fully enters the lipid membrane (Fig. 2 *a*). The free energy cost of such fluctuations can be estimated from the surface free energy associated with the hydrophobic protein area exposed to water, or the excess energy involved in introducing the generally polar protein termini into the hydrophobic core. Alternatively, as the protein moves up or down relative to the membrane midplane (z fluctuations) or tilts away (at some angle θ) from the bilayer normal, the lipid chains deform in such a way as to avoid the exposure of hydrophobic protein regions to water and to avoid penetration of the charged or polar protein ends into the membrane, as illustrated in Fig. 2 *b*. From our estimates of ΔG^o_{solv} and ΔG^o_{lip} it follows that the later scenario involves a lower free energy cost.

As the protein moves "up" ($z > 0$) or "down" ($z < 0$) with respect to the membrane midplane (Fig. 2 *b*), the two hydrocarbon-water interfaces of the bilayer deform in a fashion similar to that of the interfacial deformation corresponding to positive or negative lipid-protein hydrophobic mismatch (Fig. 3). More explicitly, one interface deforms as if $d_P > d_L$ (Fig. 3, *left*), and the other as if $d_P < d_L$ (Fig. 3, *right*). These deformations involve chain tilting (with respect to the membrane normal), splaying, as well as monolayer bending and stretching. The elastic energy cost associated with a fluctuation such as that in Fig. 2 *b* (*left*) is probably smaller than that corresponding to the "hydrophobic mismatch" model described in Fig. 3. Nevertheless, this model can be used to derive an estimate, or more precisely an upper bound, for the energetic cost of protein fluctuations. Thus, assuming that the upper monolayer in Fig. 2 *b* (*left*) deforms as in the case $d_P > d_L$, and the lower as in the case $d_P < d_L$, we find that $\lambda = \kappa$. Based on similar (approximate) considerations, it can be shown that $\omega \approx \kappa d_L^2$. Using Eq. 17 leads to the result

$$\tilde{q}_{imm} = \sqrt{2\pi}\left(\frac{kT}{\kappa d_L^2}\right)^{3/2}. \quad (21)$$

We conclude this section with a numerical estimate of the immobilization free energy of the α-helical peptide in a lipid bilayer. Using $d_L = 30$ Å and $N_a\kappa = 0.07$ kcal/molÅ^2, as estimated above from the lipid-protein interaction calculation, we obtain $\Delta G^o_{imm} = -RT \ln \tilde{q}_{imm} \approx 3.7$ kcal/mol. Because our estimate for κ is an upper bound, our estimate for the immobilization energy is an upper bound as well. The separate contributions representing the loss of translational and rotational entropies can be estimated by using Eq. 17 or, equivalently, by using Eq. 12 with $\delta z = \sqrt{2\pi}\,\Delta z = (2\pi kT/\kappa)^{1/2} \approx 7.3$ Å ($\Delta z = 2.9$ Å) and $\delta\theta = (\sqrt{2}\Delta\theta)^{1/2} = (2kT/\kappa d_L^2)^{1/2} \approx 7.9°$ ($\Delta\theta \approx 0.8°$). This yields $\Delta G^o_{imm} = \Delta G^o_{imm,trans} + \Delta G^o_{imm,rot} \approx 0.9 + 2.8 \approx 3.7$ kcal/mol.

Combining our estimates for the separate contributions to the transfer free energy, we obtain $\Delta G^o = \Delta G^o_{solv} + \Delta G^o_{lip} + \Delta G^o_{imm} \approx -11 + 2.3 + 3.7 = -5$ kcal/mol (Ben-Tal et al., 1996). Transforming to the standard free energy on the mole fraction scale (see Introduction), we obtain $\Delta G^o_x = \Delta G^o - RT \ln(\nu_L/\nu_W) \approx -7$ kcal/mol, where we have used $\nu_L = 1000$ Å^3, $\nu_W = 30$ Å^3. This value is close (perhaps fortuitously) to the value measured by Moll and Thompson (1994), $\Delta G^o_x = -5.5$ kcal/mol, for the binding of $(Ala)_{20}$-G-BPTI to large unilamellar vesicles of both DMPC and 1:1 DMPC:DPPC.

DISCUSSION

Our goal in this paper was to determine the origin of the various contributions to the free energy of inserting a protein into a lipid membrane. Starting with the basic statistical thermodynamic expressions governing protein partitioning between the two environments, we have shown that all of the contributions can be related to the terms appearing in the potential of mean force of the protein in the membrane. Our interpretation of the "solvation" and "lipid-perturbation" contributions to the transfer free energy is not conceptually different from those given in previous studies of the protein insertion problem. On the other hand, there are two issues that we have treated quite differently from previous theoretical studies and which are of general significance. First, we have shown that the partition coefficient and consequently the transfer free energy of the protein depend only on the configurational integrals appearing in the protein partition functions. When all degrees of freedom of the protein, regardless of their nature (e.g., translations versus vibrations, or rotations versus liberations) are treated classically, all of the mass-dependent factors (which enter through the momenta integrals) cancel out identically. Similar conclusions apply to related problems, such as protein and peptide adsorption on the membrane surface, and to ligand binding to substrate (Finkelstein and Janin, 1989),

provided, of course, that all of those degrees of freedom that change their character in the transition from the free to the bound state can still be treated classically.

The second point concerns the "immobilization" free energy. In the previous section, using Eq. 16 and our estimates of the vibrational amplitude $\delta z = 7.3$ Å ($\Delta z = 2.9$ Å) and rotational amplitude $\delta\theta = 7.9^\circ$ ($\Delta\theta = 0.8^\circ$), we found, for the helix insertion process, that $\Delta G^\circ_{imm} \approx 3.7$ kcal/mol, of which ~0.9 kcal/mol is due to the loss of translational entropy and the rest to lost rotational freedom. Jähnig (1983), using $\delta z = 1$ Å and $\delta\theta = 1^\circ$, obtained $\Delta G^\circ_{imm} = \Delta G^\circ_{imm,trans} + \Delta G^\circ_{imm,rot} = 8 + 2 \times 4 = 16$ kcal/mol. The large difference between the two estimates is due in part to the different values used for δz and $\delta\theta$ [had we used $\delta z = 1$ Å and $\delta\theta = 1^\circ$ in Eq. 16 (with $d_L = 30$ Å), the result would have been $\Delta G^\circ_{imm} = \Delta G^\circ_{imm,trans} + \Delta G^\circ_{imm,rot} = 2.0 + 2 \times 5.3 = 12.6$ kcal/mol] and in part to an inconsistent definition of the standard states of the helix in the aqueous phase and in the lipid bilayer, as discussed by Ben-Tal et al. (1996).

Finally, we note that the theoretical model presented here, which was applied only for the case of a single α-helix, is valid for all membrane proteins and, in addition, can be readily extended to proteins that are adsorbed on the surface of the membrane. A host of biological processes, for example, viral infection and signal transduction, involve interactions between proteins and lipids and between proteins or protein fragments in lipid bilayers (Shai, 1995). The theoretical development presented in this work provides a basis for a detailed study of the molecular events that underlie these processes.

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REFERENCES

Ben-Shaul, A. 1995. Molecular theory of chain packing, elasticity and lipid-protein interaction in lipid membranes. *In* Handbook of Biological Physics: Structure and Dynamics of Membranes, Vol. 1. R. Lipowsky and E. Sackmann, editors. Elsevier Science, Amsterdam. 359–401.

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

Dwyer, J. D., and V. A. Bloomfield. 1981. Binding of multivalent ligands to mobile receptors in membranes. *Biopolymers.* 20:2323–2336.

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

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

Fattal, D. R., and A. Ben-Shaul. 1993. A molecular model for lipid-protein interaction in membranes: the role of hydrophobic mismatch. *Biophys. J.* 65:1795–1809.

Finkelstein, A. V., and J. Janin. 1989. The price of lost freedom: entropy of bimolecular complex formation. *Protein Eng.* 3:1–3.

Hill, T. L. 1960. An Introduction to Statistical Thermodynamics. Addison-Wesley, Reading, MA.

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

Jähnig, F. 1983. Thermodynamics and kinetics of protein incorporation into membranes. *Proc. Natl. Acad. Sci. USA.* 80:3691–3695.

Janin, J., and C. Chothia. 1978. Role of hydrophobicity in the binding of coenzymes. *Biochemistry.* 17:2943–2948.

Leto, T. L., and P. W. Holloway. 1979. Mechanism of cytochrome b_5 binding to phosphatidylcholine vesicles. *J. Biol. Chem.* 254:5015–5019.

Mayer, J. E., and M. G. Mayer. 1946. Statistical Mechanics. Wiley, New York. 191–194.

Milik, M., and J. Skolnick. 1993. Insertion of peptide chains into lipid membranes: an off-lattice Monte Carlo dynamics model. *Proteins.* 15: 10–25.

Moll, T. S., and T. E. Thompson. 1994. Semisynthetic proteins: model systems for the study of the insertion of hydrophobic peptides into preformed lipid bilayers. *Biochemistry.* 33:15469–15482.

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

Shai, Y. 1995. Molecular recognition between membrane-spaning polypeptides. *Trends Biochem. Sci.* 20:460–464.

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