[4] Protein Folding in Membranes: Determining Energetics of Peptide–Bilayer Interactions

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Introduction

Although the problem of the folding of soluble proteins continues to resist solution, we at least have a strong understanding of the general thermodynamic principles[1,2] and have available a wealth of thermodynamic data.[3-5] The study of membrane protein folding and stability is much less advanced: Some general principles are emerging,[6-9] but the amount of thermodynamic data available remains quite limited. The energetics of the partitioning of peptides into membranes constitutes one especially important class of data. We will demonstrate how such data can be used for clarifying the folding of peptides and small proteins in membranes and then describe the principles and methods used for determining the energetics of the partitioning of peptides into bilayer membranes.

[1]K. A. Dill, *Biochemistry 29,* 7133 (1990).

[2]T. E. Creighton, "Protein Folding." W. H. Freeman and Company, New York, 1992.

[3]A. Chakrabartty and R. L. Baldwin, *Adv. Prot. Chem. 46,* 141 (1995).

[4]E. Freire, *Annu. Rev. Biophys. Biomol. Struct. 24,* 141 (1995).

[5]G. I. Makhatadze and P. L. Privalov, *Adv. Protein Chem. 47*, 307 (1995).

[6]J.-L. Popot, *Curr. Opinion Struct. Biol. 3,* 532 (1993).

[7]S. H. White and W. C. Wimley, *Curr. Opinion Struct. Biol. 4,* 79 (1994).

[8]S. H. White, "Membrane Protein Structure: Experimental Approaches." Oxford Univ. Press, New York, 1994.

[9]D. C. Rees, A. J. Chirino, K.-H. Kim, and H. Komiya, *in* "Membrane Protein Structure: Experimental Approaches" (S.H. White, ed.), p. 3. Oxford Univ. Press, New York, 1994.

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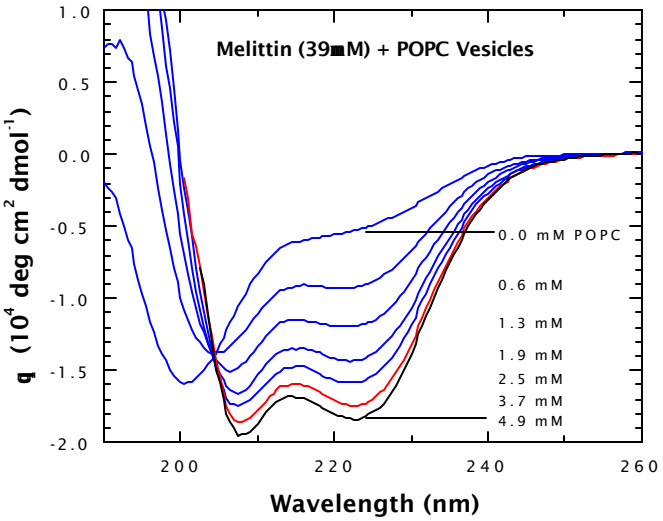

FIG. 1. Dependence of circular dichroism (CD) spectra of the peptide melittin on the concentration of large unilamellar vesicles formed from palmitoyloleoyl phosphocholine (POPC) (A. S. Ladokhin, unpublished). Spectra are plots of molar ellipticity Θ against wavelength in nanometers. The spectrum for 0 m*M* POPC is that of a largely disordered peptide with a residual α helicity of about 15%. The spectrum for 4.9 m*M* POPC, corresponding to nearly completely membrane-bound melittin, is about 60% helical. The isodichroic point indicates that there are only two populations of melittin: disordered melittin in free solution and largely helical melittin on the membrane. Data similar to these have been published by H. Vogel, *FEBS Lett. 134*, 37 (1981).

Coupling of Folding to Partitioning: Energetic Principles

The partitioning of peptides and small proteins into membranes is often accompanied by folding that results in the formation secondary structure. This process is referred to as partitioning-folding coupling.[10] The result of this coupling is that peptides bound to membranes often have a much higher secondary structure content than they do in membrane-free solutions. Although a detailed thermodynamic explanation for this phenomenon is lacking, the folded peptide must have a lower free energy in the membrane than the unfolded peptide. The bee venom peptide melittin provides a classical example of this effect,[11] as shown by the data in Fig. 1, but there are many other peptides that show the same type of behavior.[12-14] Melittin is monomeric at low concentrations in aqueous solution and exists in a disordered state with

[10]W. C. Wimley and S. H. White, *Nature Struct. Biol. 3,* 842 (1996).
[11]H. Vogel, *FEBS Lett. 134,* 37 (1981).
[12]V. K. Mishra, M. N. Palgunachari, J. P. Segrest, and G. M. Anantharamaiah, *J. Biol. Chem. 269,* 7185 (1994).
[13]V. K. Mishra and M. N. Palgunachari, *Biochemistry 35,* 11210 (1996).
[14]L. K. Tamm, *in* "Membrane Protein Structure: Experimental Approaches" (S. H. White, ed.), p. 283. Oxford Univ. Press, New York, 1994.

low α-helical content as judged by its circular dichroism (CD) spectrum. When bilayer vesicles are titrated into the solution, the fraction of melittin partitioned into the vesicles and the average melittin helicity increase concomitantly. The maximum helicity observed when binding is essentially complete corresponds to that of an almost completely helical peptide. Thus, the binding of melittin to membranes induces the formation of secondary structure. The presence of a very distinct isodichroic point in the CD spectra shown in Fig. 1 (see also Vogel[11]) is indicative of a two-state transition in which there are only two populated states: monomeric melittin in water with low helicity and membrane-bound melittin with high helicity.

The significance of this coupling of folding to partitioning is that there are two equilibria that contribute simultaneously to the observable process, as shown schematically in Fig. 2 where we have drawn a thermodynamic cycle for a peptide that binds to membranes and gains secondary structure there. To understand this partitioning-folding coupling, the thermodynamics of each state must be determined independently. The bound random-coil state, however, frequently cannot be observed directly. To circumvent this problem, we have established an experimental interfacial hydrophobicity scale based on the energetics of the membrane partitioning of small peptides (two to six residues) that

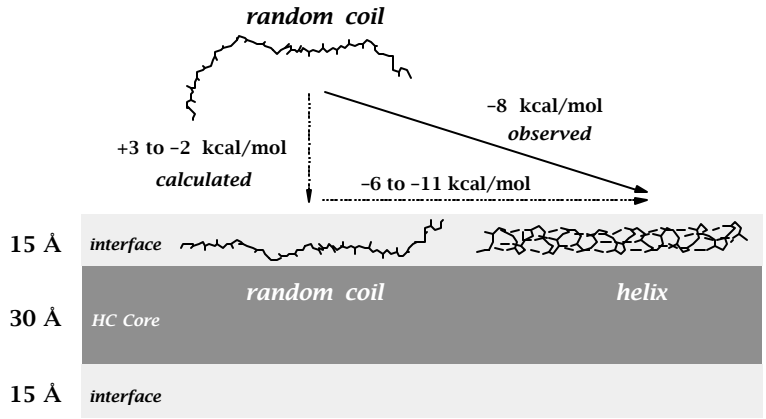

FIG. 2. The thermodynamic cycle for the partitioning of melittin between water and bilayers. The free energy of transfer of melittin from water, where it is disordered, to the bilayer interface, where it is helical, can be determined directly from experiments such as those shown in Fig. 1. The free energy of transfer of disordered melittin cannot be determined directly because disordered melittin on the membrane cannot be detected.[74] However, the free energy can be estimated using the interfacial hydrophobicity scale of Wimley and White, *Nature Struct. Biol. 3*, 842 (1996). The "low resolution" schematic of the bilayer is based upon neutron and X-ray diffraction measurements.[16]

are known to lack secondary structure in water and in membranes.[10] The thermodynamic scale obtained from these peptides thus constitutes an unfolded reference state for peptides in membranes. This scale is used in Fig. 2 to estimate the free energy of the partitioning of unfolded melittin so that the free energy of the folding of melittin can be estimated to be −6 to −11 kcal/mol. We have suggested that secondary structure formation on the membrane is promoted through reduction of the high cost of peptide-bond partitioning by hydrogen bond formation.[10] For melittin, the free energy of folding can be explained by a modest reduction of 0.2 to 0.4 kcal/mol in the partitioning of each peptide bond.

This simple example of the partitioning of melittin into membranes demonstrates the value of measurements of partitioning free energies in studies of the interactions of peptides and small proteins with lipid bilayers.

Partitioning, Binding (Not!), and Free Energy

One is accustomed in biochemistry and enzymology to thinking about associations among molecules in terms of chemical equilibria, as in the stoichiometric binding of small molecules to specific sites on macromolecules.[15] The free energy of the association ΔG is related to an association constant k_a by means of $k_a = \exp(-\Delta G/RT)$. Although this approach is quite appropriate for membrane systems when considering the association of ligands with membrane-embedded receptors, conceptual difficulties arise when it is applied to the association of proteins or peptides with the fabric of membranes, *i.e.* the lipid bilayer. For example, the preferential association of peptides (P) with lipid (L) vesicles is frequently treated in the literature as a simple chemical equilibrium between peptide and lipid molecules: $L + P \leftrightarrow LP$. Alternatively, the lipid bilayer vesicles that receive the peptide are treated as macromolecules with n discrete binding sites comprised of m lipid molecules: $P + mL \leftrightarrow P{\cdot}\, mL$. Two assumptions are implicit in either formulation: (1) the stoichiometry between peptide and lipid is fixed and (2) the association is a characteristic of individual or groups of lipid molecules. Such assumptions are suspect because of the fluid nature of membranes and because the hydrophobic and electrostatic interactions that drive most peptide–bilayer interactions arise from the collective properties and behavior of the lipids in the bilayer. That is, the interactions of peptides and proteins must be considered as interactions with a lipid assembly, the bilayer, rather than the individual molecules comprising it. Thus, chemical equilibria such as $P + L \leftrightarrow PL$ or $P + mL \leftrightarrow P{\cdot}\, mL$ are inappropriate descriptions of peptide–bilayer interactions.

[15] J. Wyman and S. J. Gill, "Binding and Linkage," University Science Books, Mill Valley, CA, 1990.

Biologically relevant lipid bilayers are in highly thermally disordered fluid states.[16] Because such bilayers have a specific molecular composition and their extent can be defined by geometric surfaces,[17] they constitute separable phases of matter dispersed in an aqueous phase. The association of peptides with bilayers should therefore be treated as partitioning between two immiscible fluid phases (water and bilayer) instead of as a chemical equilibrium between peptides and lipid binding sites. This partitioning approach is conceptually more accurate and avoids certain problems associated with defining thermodynamic standard states in binding-site formulations.[18] Although the terms peptide binding or bound peptide are used frequently, this is strictly for convenience: *Binding* should always be interpreted to mean *partitioning*.

The chemical potential (partial molar free energy) of a solute (peptide) in a phase (bilayer or water) is given by

$$m = m^0 + RT \ln a \tag{1}$$

where the composition of the phase is described by the solute activity a with $a^0 = 1$ being the activity in the standard state. Mole fraction, molar, volume fraction, and Flory-Huggins-corrected volume-fraction units[19,20] can be used for solute activities. The choice of composition units and standard state is being vigorously debated in the context of so-called Flory-Huggins corrections for differences in molecular volumes between solute and solvents.[19,21-27] Such corrections can have large effects on calculated free energies and their microscopic interpretation. The fundamental issue of the debate is how to extract so-called contact interaction energies from partitioning measurements in a way that correctly accounts for differences in the translational and configurational entropies of the two phases of the partitioning experiment. Chan and Dill[28] have given a lucid and detailed account of the issues involved that the reader is encouraged to consult.

[16]M. C. Wiener and S. H. White, *Biophys.J. 61*, 434 (1992).
[17]J. W. Gibbs, *in* "The Collected Works of J. Willard Gibbs" (Anonymous), p. 55. Longmans, Green, and Co., New York, 1931.
[18]J. Janin, *Proteins 24,* i (1996).
[19]L. R. De Young and K. A. Dill, *J. Phys. Chem. 94,* 801 (1990).
[20]K. A. Sharp, A. Nicholls, R. Friedman, and B. Honig, *Biochemistry 30,* 9686 (1991).
[21]D. Sitkoff, K. A. Sharp, and B. Honig, *Biophys. Chem. 51,* 397 (1994).
[22]D. Sitkoff, K. A. Sharp, and B. Honig, *J. Phys. Chem. 98,* 1978 (1994).
[23]B. Lee, *Biopolymers 31,* 993 (1991).
[24]A. Holtzer, *Biopolymers 35,* 595 (1995).
[25]S. K. Kumar, I. Szleifer, K. Sharp, P. J. Rossky, R. Friedman, and B. Honig, *J. Phys. Chem. 99,* 8382 (1995).
[26]H. S. Chan and K. A. Dill, *J. Chem. Phys. 101,* 7007 (1994).
[27]A. Ben-Shaul, N. Ben-Tal, and B. Honig, *Biophys. J. 71,* 130 (1996).
[28]H. S. Chan and K. A. Dill, *Annu. Rev. Biophys. Biomol. Struct. 26,* 425 (1997).

Until the debate is settled and until statistical mechanical models appropriate for bilayer partitioning are developed, we continue to follow the admonishment of Lewis and Randal[29] (p. 248) that, "for nearly every purpose the mole fraction furnishes the most advantageous method of measuring composition, and the employment of this measure in aqueous as well as in nonaqueous solutions is to be encouraged" and note that conversion between mole fraction and other units is straightforward.[20,30] Thus, as does Tanford,[31] we use mole fraction x as the measure of composition and take infinite dilution of the solute ($a/x \to 1$ as $x \to 0$) as the standard state.[29]

The chemical potentials [Eq. (1)] of a peptide in the bilayer (bil) and water phases must be equal at equilibrium so that $\mu_{bil} - \mu_{water} = 0$. The standard free energy of transfer from water to bilayer is therefore

$$\Delta G_x^0 \equiv m_{bil}^0 - m_{water}^0 = -RT \ln K_x \tag{2}$$

The subscripts x serve to remind us that mole fraction units are being used. K_x is the mole fraction partition coefficient given by

$$K_x = \frac{[P]_{bil} / ([L] + [P]_{bil})}{[P]_{water} / ([W] + [P]_{water})} \tag{3}$$

where $[P]_{bil}$ and $[P]_{water}$ are the bulk molar concentrations of peptide attributable to peptide in the bilayer and water phases, respectively, and $[L]$ and $[W]$ are the molar concentrations of lipid and water.[32] Eq. (3) assumes that all lipid in the bilayer vesicles used in measurements of partitioning is accessible to the peptide. If only the outer leaflet of the bilayer is accessible, one should replace $[L]$ by $[L]/2$ for large unilamellar vesicles (LUV). Unfortunately, there is no general way to establish with certainty the transbilayer distribution of peptides. The practical consequence of this uncertainty is that the *absolute* value of the free energy of transfer is uncertain. In the absence of direct experimental evidence, we use $[L]$ rather than $[L]/2$ as standard practice. If one chooses to assume that particular peptides cannot penetrate the inner leaflet of the vesicle bilayer, then one should add −0.41 kcal/mol to the free energy calculated using Eq. (2).

[29]G. N. Lewis and M. Randall, "Thermodynamics." McGraw-Hill, New York, 1961.
[30]N. Ben-Tal, A. Ben-Shaul, A. Nicholls, and B. Honig, *Biophys. J. 70,* 1803 (1996).
[31]C. Tanford, "The Hydrophobic Effect: Formation of Micelles and Biological Membranes." John Wiley & Sons, New York, 1973.
[32]W. C. Wimley and S. H. White, *Anal. Biochem. 213,* 213 (1993).

It is always true in partitioning experiments that $[W]$ = 55.3M >> $[P]_{water}$ and generally one keeps the concentration of peptide in the bilayer low in order to avoid concentration-dependent partition coefficients so that $[L]$ >> $[P]_{bil}$. Therefore, to high accuracy one may write

$$K_x = \frac{[P]_{bil}/[L]}{[P]_{water}/[W]} \tag{4}$$

Equilibrium dialysis and other measurements (see later) can be used for determining K_x and therefore free energies of transfer. Primary data of equilibrium dialysis consist of multiple measurements of the concentrations of lipid and peptide in the two halves of a dialysis cell at equilibrium over a range of values of aqueous peptide and lipid concentrations. In other types of partitioning experiments (see later), it is sometimes convenient to measure partitioning by titrating a starting peptide solution with lipid vesicles and calculating the fraction f_p of the total amount of peptide partitioned into the lipid vesicles as a function of lipid concentration $[L]$. Given that $[P]_{total} = [P]_{bil} + [P]_{water}$, one can easily show that

$$f_p = \frac{K_x[L]}{[W] + K_x[L]} \tag{5}$$

K_x can be determined by least-squares fitting of Eq. (5) to plots of f_p against $[L]$.

The infinite dilution (Henry's Law) standard states of Eq. (2) were chosen so that the laws of dilute solutions prevail. One must therefore establish that the partition coefficients and free energies measured are in fact the infinite dilution values. This is done by examining the dependence of K_x on the membrane concentration (peptides per lipid) of a peptide through measurements of partitioning over a wide range of aqueous peptide and lipid concentrations and selecting as infinite dilution values those that are independent of membrane peptide concentration.[10] Examples of three types of dependence of K_x on the membrane concentration of peptide are shown in Fig. 3: simple concentration-independent partitioning,[10,52,73,75] cooperative increases in partitioning due to peptide aggregation on the membrane,[32a] and anti-cooperative decreases in partitioning due to electrostatic repulsion[33-36] arising from

[32a]W. C. Wimley, K. Hristova, A. S. Ladokhin, L. Silvestro, P. H. Axelsen, and S. H. White, *J. Mol. Biol. 277*, 1091 (1998).
[33]S. McLaughlin and H. Harary, *Biochemistry 15,* 1941 (1976).
[34]G. Schwarz and G. Beschiaschvili, *Biochim. Biophys. Acta 979,* 82 (1989).
[35]T. E. Thorgeirsson, Y. G. Yu, and Y.-K. Shin, *Biochemistry 34,* 5518 (1995).
[36]E. Kuchinka and J. Seelig, *Biochemistry 28,* 4216 (1989).

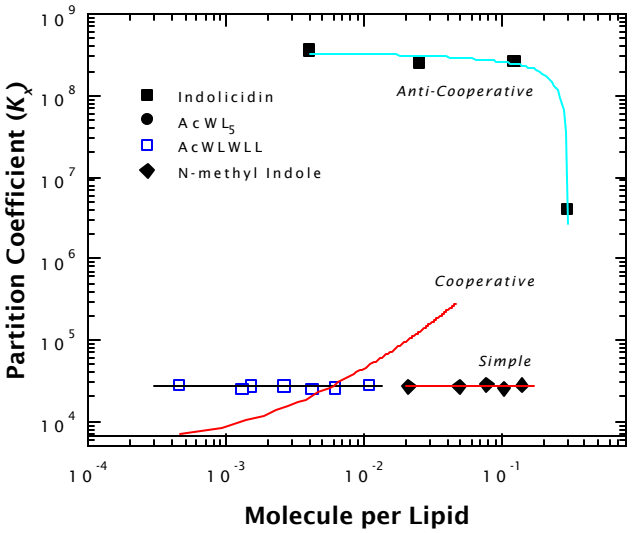

FIG. 3. Partition coefficients (K_x) of several compounds plotted against membrane concentration (molecule per lipid). The partitioning behaviors of four compounds are shown. A pentapeptide[10,73] AcWLWLL and a small molecule[52,75] *N*-methylindole bind in a manner that has no detectable concentration dependence over the measurable range; they bind ideally. The other two compounds provide examples of nonideal partitioning. The peptide[10,73] $AcWL_5$ binds cooperatively because it aggregates on the membrane,[32a] whereas the 13-residue peptide indolicidin[55] binds anticooperatively because of electrostatic repulsion at high concentration. Except for the indolicidin data,[55] data presented here have not been published previously in this form.

the partitioning of highly charged peptides. Notice that partition coefficients can vary with concentration by factors of 10 or more (each "power of 10" change in K_x corresponds to a change of 1.36 kcal/mol in free energy). Concentration-dependent partitioning can also be revealed if the data are plotted according to Eq. (5) as shown by the simulations presented in Fig. 4 that are based upon the three types of partitioning behavior shown in Fig. 3.

One should keep in mind that the bilayer is not a simple uniform slab of hydrocarbon liquid, but rather consists of interfacial and hydrocarbon core regions. X-ray and neutron diffraction measurements[16] and molecular dynamics simulations[37,38] show that the interfacial layers are each about 15 Å thick so that together they equal the 30-Å thickness of the HC core. Peptides can partition into either or both layers. This has no effect on partition coefficient measurements, which are strictly thermodynamic in nature, but knowledge of the location of partitioned peptides with respect to bilayer thickness is necessary if the partitioning experiments are to be interpreted in molecular terms.[39-41]

[37]K. Tu, D. J. Tobias, J. K. Blasie, and M. L. Klein, *Biophys. J. 70,* 595 (1996).
[38]S.-W. Chiu, M. Clark, V. Balaji, S. Subramaniam, H. L. Scott, and E. Jakobsson, *Biophys. J. 69,* 1230 (1995).
[39]S. H. White, G. I. King, and J. E. Cain, *Nature (London) 290,* 161 (1981).
[40]R. E. Jacobs and S. H. White, *Biochemistry 28,* 3421 (1989).
[41]T. E. Thorgeirsson, C. J. Russell, D. S. King, and Y.-K. Shin, *Biochemistry 35,* 1803 (1996).

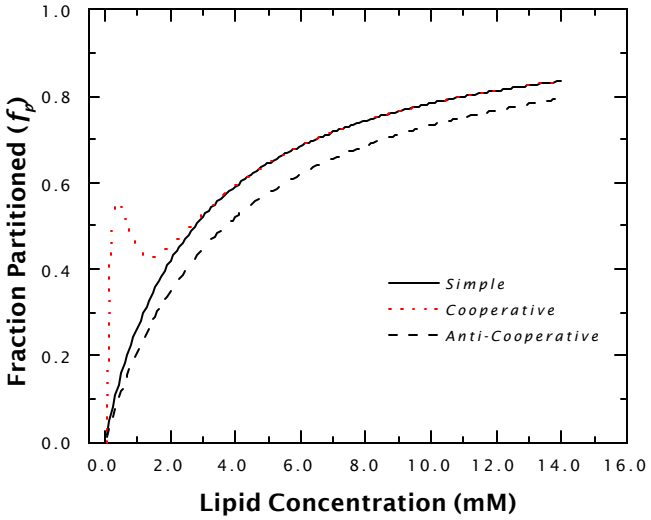

FIG. 4. Simulated fractions of peptide partitioned (f_p) as a function of lipid concentration that demonstrate simple, cooperative, and anticooperative partitioning behavior. The simulations are based on the results shown in Fig. 3. The partitioning is presented here by plotting the fraction of the peptide partitioned (f_p) against the concentration of lipid. This type of plot typically results from experiments in which a starting peptide solution is titrated with lipid vesicles. The simple concentration-independent partitioning is described by Eq. (5). Cooperative partitioning occurs if the peptide aggregates on the membrane. Anticooperative partitioning typically results from electrostatic repulsion arising from the partitioning of highly charged peptides. See text and also Fig. 7.

Experimental Determination of Partition Coefficients

Methods for determining the partitioning of peptides into membranes generally fall into two broad categories: (1) Physical separation of bound and free molecules, including such methods as equilibrium dialysis, centrifugation, membrane filtration, and various kinds of chromatographic techniques. These methods provide a direct measurement of the fraction of peptide molecules that are bound. (2) Titration methods that rely on the measurements of a certain property of the system that changes upon binding without physical separation of bound and free molecules. Various spectroscopic methods along with titration calorimetry belong to this category. For simple interactions, these two complementary categories, if implemented correctly, will produce accurate measurements of partition coefficients. However, the amount of information and effort required to obtain the correct answer will differ. An approach that falls outside of these categories is the use of measurements of electrophoretic mobility (or ζ potential). Partition coefficients are obtained from ζ potentials using the Gouy-Chapman-

Stern theory.[33,42-44] This approach is similar to separation methods in that titration is not required. Unlike separation methods, however, they do not provide a direct measurement of the fraction of molecules bound.

The fundamental differences between the two categories can be illustrated with the help of simulations of simple membrane partitioning determined either by a separation technique (Fig. 5A) or a spectroscopic titration technique (Fig. 5B). The three curves of Fig. 5A give the fraction of peptide partitioned f_p, ranging from 0 to 100%, for three partition coefficients that differ from each other by an order of magnitude. Note that the curves do not intersect, so that a single point on the plane defines partitioning in a unique way and is sufficient for calculating the partition coefficient using Eq. (5). If data obtained by a separation technique do not fall on a simple-partitioning curve, then partitioning is cooperative, anticooperative, or otherwise nonideal (Fig. 4). These possibilities are discussed in greater detail later.

For spectroscopic titration techniques (Fig. 5B), the fraction of partitioned molecules is not immediately known. Instead, one measures a spectroscopic response, such as fluorescence intensity or absorbance dichroism, for example, that is assumed to be proportional to the fraction f_p . The proportionality constant must be defined by the maximum signal change at full binding, but usually is not known *a priori*. As a result, a point on the plane of Fig. 5B can be attributed to a curve with an arbitrary partition coefficient unless the spectroscopic measurement has been properly calibrated by titrating over a range of lipid concentrations and measuring the spectroscopic response at each concentration. The two sets (3 curves in each) of simulated curves in Fig. 5B (wide and narrow lines) have the same partition coefficients and line styles as those in Fig. 5A, but differ in maximal signal changes. Some of the curves appear closer to each other at lower lipid concentrations, some at higher. The curves of Fig. 5B were adjusted arbitrarily to produce an intersection at a particular point. This illustrates the fact that the curve for any partition coefficient can pass through any arbitrary point. To define the partitioning uniquely, one must measure the signal over a broad range of lipid concentrations and use all the measurements to recover the maximal spectroscopic response as well as the partition coefficient. The implicit assumption in such a procedure is that the partition coefficient is the same for all points. This assumption is justified for stoichiometric binding to discrete sites (e.g. protein-ligand binding) with the obvious exception of cooperativity between discrete binding sites. However, for membrane partitioning, this assumption is not necessarily correct because of the possible existence of nonideality and

[42]R. M. Peitzsch and S. McLaughlin, *Biochemistry 32,* 10436 (1993).

[43]J. Y. Kim, M. Mosior, L. A. Chung, H. Wu, and S. McLaughlin, *Biophys. J. 60,* 135 (1991).

[44]S. McLaughlin, *in* "Current Topics in Membranes and Transport" (F. Bronner *et al.*, eds.), p. 71. Academic Press, New York, 1977.

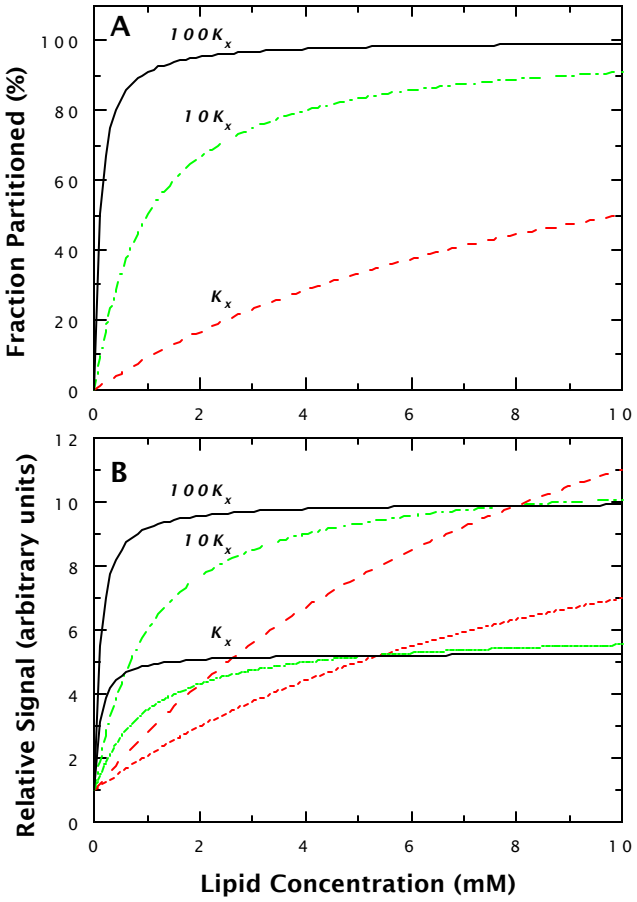

FIG. 5. Simulations of (**A**) the fraction of peptide partitioned and (**B**) relative spectroscopic signal as a function of lipid concentration. These simulations illustrate the differences between membrane partitioning as assessed by (**A**) separation and (**B**) titration techniques for three partition coefficients (K_X) related by 10-fold multiples. Separation techniques provide actual fractions of bound peptide whereas titration techniques measure certain spectroscopic or calorimetric response assumed to be proportional to partitioning. Each point on plot A defines partitioning in a unique way. However, for each point on plot B, multiple solutions exist. The two sets of three curves each in (B) correspond to the same values of K_X as in (A), but have different maximal signals (see text).

aggregation effects that change gradually with the number of bound molecules per lipid (examples are given in the following sections). Therefore, the demands on the quality of data and on the range of lipid and peptide concentrations covered are much more stringent for titration techniques than for separation methods.

Separation Methods: Equilibrium Dialysis

The general principle underlying separation methods is that measurement of any two of the three concentrations in $[P]_{total} = [P]_{bil} + [P]_{water}$ defines the partition coefficient explicitly [Eqs. (4) and (5)]. Although centrifugation,[36,45,45a] filtration,[40,43,46] or chromatography[11] can be used to separate the membranes with partitioned peptide from the aqueous phase, this article discusses in detail only equilibrium dialysis[32] and the centrifugation methods.[32a,45a] For any of the methods, the free peptide concentration $[P]_{water}$ is generally compared to the total concentration $[P]_{total}$ determined prior to the separation of the membranes. The sampling of total and free peptide is done directly in equilibrium dialysis, and there is an explicit assumption that the free peptide has the same concentration on both sides of the dialysis membrane. Separation methods have an advantage over spectroscopic ones because the partitioning is measured more directly and can be used even when there is no measurable spectroscopic change or when fractional binding is small. A single measurement at one lipid concentration is sufficient to determine the binding constant uniquely (Fig. 5A). In addition, unlike spectroscopic methods, separation methods are obviously not influenced by optical scattering artifacts arising from the membranes and can consequently be used over a wider range of partition coefficients. This is an important issue for weakly binding peptides because the high lipid concentrations that are required to get good binding can preclude determination of the maximal spectroscopic change and thereby make the spectroscopic data useless (see later).

The basic principle of equilibrium dialysis[32] is that two half cells, one containing a small volume of lipid solution and the other containing buffer, are separated by a membrane that is permeable to peptide but impermeable to lipid vesicles. Large unilamellar vesicles (LUV) are preferable to small unilamellar vesicles (SUV) because SUV are metastable[47,48] and generally have anomalous partition coefficients caused by high bilayer curvature.[49-51] The assembled dialysis cells are rotated in a thermostat until equilibrated, generally 8 to 24 hr. We generally use a 20-

[45]N. Ben-Tal, B. Honig, R. M. Peitzsch, G. Denisov, and S. McLaughlin, *Biophys. J. 71,* 561 (1996).

[45a]C. A. Buser and S. McLaughlin, *in* "Methods in Molecular Biology" (D. Bar-Sagi, ed.), p. 267. Humana Press, Totowa, NY, 1998.

[46]G. Montich, S. Scarlata, S. McLaughlin, R. Lehrmann, and J. Seelig, *Biochim. Biophys. Acta 1146,* 17 (1993).

[47]C. F. Schmidt, D. Lichtenberg, and T. E. Thompson, *Biochemistry 20,* 4792 (1981).

[48]M. Wong, F. H. Anthony, T. W. Tillack, and T. E. Thompson, *Biochemistry 21,* 4126 (1982).

[49]S. F. Greenhut, V. R. Bourgeois, and M. A. Roseman, *J. Biol. Chem. 261,* 3670 (1986).

[50]J. Seelig and P. Ganz, *Biochemistry 30,* 9354 (1991).

[51]D. A. Plager and G. L. Nelsestuen, *Biochemistry 33,* 7005 (1994).

cell equilibrium dialysis apparatus and Spectrapor 4 dialysis disks (Spectrum Medical, Los Angeles, CA). After equilibration, the vesicle half cell contains bound and free peptide whereas the buffer half cell contains only free peptide. For analysis of partitioning energies, we assume that the presence of lipid has no large effect on the activity of the free peptide in solution and thus that the molar concentration of free peptide $[P]_{water}$ is the same in both cells. Consequently, the difference between the concentrations in the two cell halves is the concentration of the bound peptide $[P]_{bil}$. Given the known lipid concentration $[L]$, the partition coefficient is calculated using Eq. (4). The major disadvantage of equilibrium dialysis is that long equilibration times are required. However, this disadvantage is ameliorated by the fact that long equilibration times make it more likely that equilibration has actually been reached in the binding experiment. Regardless of the method used, one must establish experimentally that equilibrium has been achieved.

All separation methods require that the peptide concentration be measured in the presence and absence of membranes, but the measurements need only be of relative peptide concentration. There are numerous useful methods for doing this, such as radioisotope labeling, spectroscopy, and chemical analysis. We prefer to use quantitative reverse-phase HPLC for the determination of peptide concentrations because of its superior sensitivity and reproducibility and because peptides do not need to be radiolabeled. Furthermore, HPLC allows the simultaneous assessment of peptide purity and chemical integrity during the course of an experiment and, in addition, the simultaneous measurement of the partitioning of multiple peptides in a single sample. A complete discussion of this technique has been published elsewhere.[32] Briefly, a 0.45 × 5-cm plastic HPLC column (Upchurch Scientific, Temecula, CA) hand-packed with C_8 reversed-phase silica (Rainin, Emeryville, CA) is used for all measurements. Buffers are typically water and acetonitrile with 0.1% trifluoroacetic acid, but occasionally water with 0.1% ammonium acetate and 80% acetonitrile + 0.1% aqueous ammonium acetate are used as buffers. A flow rate of 3 ml/min is used. Run times are approximately 12 min total with most peptides eluting in 3–6 min, during which time the baseline is linear. We have shown, under these conditions, that as little as 50 ng of peptide can be quantitated routinely and that the presence of milligrams of lipid has no measurable effect on the peak area or retention times of hydrophobic peptides. Very similar methodology can be used with ion-exchange column chemistry. Thus, HPLC has proven to be a very effective tool for studying peptide–bilayer interactions.[10,32,52-55]

[52]W. C. Wimley and S. H. White, *Biochemistry 32,* 6307 (1993).
[53]W. C. Wimley, M. E. Selsted, and S. H. White, *Protein Sci. 3,* 1362 (1994).
[54]K. Hristova, M. E. Selsted, and S. H. White, *Biochemistry 35,* 11888 (1996).
[55]A. S. Ladokhin, M. E. Selsted, and S. H. White, *Biophys. J. 72,* 794 (1997).

The equilibrium dialysis method just described works well only for $K_x < 10^7$ for reasons described later. However, the range of the method can be extended to above 10^7 if there is another type of lipid available for which the partition coefficient is measurable.[55] The basic idea is that vesicles formed from strongly binding lipids (L_S) are dialyzed against vesicles made from weakly binding lipids (L_W). If the partition coefficient K_W is known, then K_S can be determined from

$$K_S = K_W \frac{([P]_{L_S} - [P]_{water})/[L_S]}{([P]_{L_W} - [P]_{water})/[L_W]} \tag{6}$$

This method has been used successfully to measure partitioning of the 13-residue antimicrobial peptide indolicidin,[55] which binds to anionic vesicles with a partition coefficient of greater than 10^8 (Fig. 3).

Separation Methods: Centrifugation

The principle of centrifugation methods is that the total peptide concentration in a mixture of peptide and lipid is assayed, the suspension is centrifuged to sediment the vesicles with bound peptide, and then the concentration of free peptide in the supernatant is assayed. Because $[P]_{bil} = [P]_{total} - [P]_{free}$, the mole fraction partition coefficient of Eq. (4) can be written as

$$K_x = \frac{([P]_{total} - [P]_{free})/[L]}{[P]_{free}/[W]}, \tag{7}$$

where $[P]_{total}$ and $[P]_{free}$ are the aqueous peptide concentrations measured before addition of vesicles and after the centrifugation, respectively.

The difficulty with these methods is that the density of lipid is very close to the density of water so that the vesicles do not sediment readily in a moderate centrifugal field. This problem was solved by Ben-Tal *et al.*[45] by loading the vesicles with a dense sucrose solution.[45a] This approach works well, provided that the peptides do not cause leakage of the contents of the liposomes.

To avoid this potential problem, we adapted an idea introduced by Fox *et al.*[56] Specifically, we use LUV made from 1-oleoyl-2-(9,10-dibromostearoyl)phosphatidylcholine (OBPC), which has density of 1.2 g/cm^3 and thus sediments easily in a centrifugal field.[32a] Although OBPC has been found to be isomorphous with DOPC in X-ray diffraction

[56]C. F. Fox, J. H. Law, N. Tsukagoshi, and G. Wilson, *Proc. Natl. Acad. Sci. U.S.A. 67*, 598 (1970).

studies,[57] we have found that some peptides can have a slightly different partition coefficient in OBPC LUV compared to POPC LUV.[32a] This presumably can happen if a peptide can penetrate into the hydrocarbon core.

An unanticipated problem occurred during the development of this approach. As shown in Fig. 6, the partition coefficient of AcWLWLL (determined by equilibrium dialysis[10] to be $\approx$ 30,000) depends strongly on the centrifugal field and the lipid concentration. The deviation from the expected value is obviously a result of high centrifugal fields. The maximum centrifugation speed for which the measurement of K_x is still correct depends on the lipid concentration: the maximum speed is lower for low lipid concentrations than for high lipid concentrations. The exact mechanism of this phenomenon is not clear, but it may be due to the fact that the strong force fields alter the LUV. This could occur in at least two ways: (1) Liposomes may undergo elastic deformations such as area expansion and thereby expose more of the hydrocarbon region for partitioning. (2) The LUV may rupture and then reseal, exposing both lipid surfaces to the peptide. This latter possibility should, however, only increase K_x by a factor of 2 if the peptides do not distribute naturally on both sides of the membrane prior to centrifugation. Fig. 6 shows that K_x can increase by three- to fivefold. Whatever the cause, with low enough centrifugation speeds, a constant value of K_x is obtained over a wide range of lipid concentrations. It is not yet known if this speed effect is problematic for sucrose-loaded vesicles.[45a] A way to distinguish whether the centrifugation speed affects measurements of K_x is to compare K_x at different lipid concentrations. If the value increases with decreases in lipid concentration, then most probably the measurements are affected by high centrifugation speeds.

We have developed the following protocol for measuring the partition coefficients for lipid concentrations in the range of 0.2 - 10 m*M*. The protocol assumes that peptide concentrations are determined using the HPLC method of Wimley and White,[52] which permits the peptide concentration to be measured accurately in the presence of high concentrations of LUV. An aliquot of concentrated LUV is added to a peptide solution in a centrifuge tube and incubated until equilibrium is achieved. Our experience is that equilibrium is achieved in a few minutes for peptides that show simple partitioning (Fig. 3); equilibrium may take hours (overnight is usually safe) for peptides that show cooperative partitioning. Following equilibration, an aliquot of the lipid–peptide solution is withdrawn for HPLC analysis of total peptide. (Assaying the whole sample in this way rather than assaying the starting peptide solution allows for possible peptide adsorption to the centrifuge tube).

[57]M. C. Wiener and S. H. White, *Biochemistry 30*, 6997 (1991).

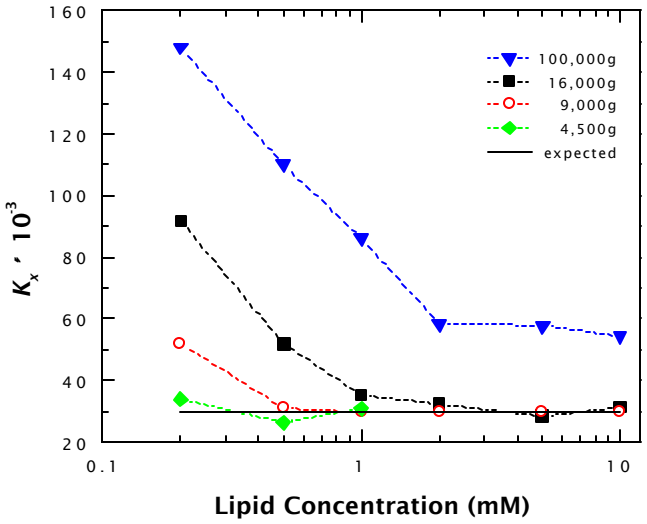

FIG. 6 Dependence of the partition coefficient (K_X) of AcWLWLL on the centrifugal field and lipid concentration. The deviation from the expected dialysis value[10] of ≈30,000 is attributable to high force fields. The deviation increases as the centrifugal force (speed) of the Biofuge A increases from 4500*g* (◆) to 16000*g* (■). The maximum speed for which the measurement of K_X is still correct depends on the lipid concentration: For low lipid concentrations, the maximum speed is lower than for high lipid concentrations. The results deviate dramatically from the correct value at very high mean centrifugal fields (▼) on the order of 100,000*g*, obtained using an Airfuge (Beckman Instruments, Fullerton, CA).

The samples are then sedimented using a Biofuge A centrifuge (Heraeus-Christ GmbH, Osterode, Germany). The sedimentation is done at 23°C progressively by spinning at 4500, 9000, and then 16000*g* for 30 min each so that the liposomes are sedimented gently at first and then spun into a tight pellet that is not disturbed when the supernatant is gently pulled out with a microliter syringe. Finally, the peptide concentration of the supernatant is assayed by HPLC. We have used this method to measure the partition coefficient of $AcWL_5$ which forms large aggregates on the membrane[32a] (Fig. 3). The experimental uncertainties, which were unusually large for this particular peptide in equilibrium dialysis experiments, were much smaller in centrifugation experiments. The larger uncertainties may result from a combination of highly cooperative aggregation and long diffusion paths in the equilibrium dialysis measurements.

Titration Methods: Spectroscopic

Spectroscopic techniques are well known to provide a sensitive tool for studies of the formation of various macromolecular assemblies. There are a number of excellent papers and reviews available devoted to

subjects related to linear-response spectroscopic techniques,[58] evaluation of multiple equilibria,[59] and numerical procedures for data analysis.[60-63] We outline here important aspects of the spectroscopic determination of binding in general, including stoichiometric binding as well as some that are specific to membrane partitioning.

Linear-Response Spectroscopic Techniques

Consider the simple case of a two-state equilibrium (the general case is described by Toptygin and Brand[58]) that can be followed by a change in a certain spectroscopic parameter S that changes from a value S_{min} in the absence of binding to S_{max} when all of the molecules are bound. The fractional change in the spectroscopic parameter f_{signal}, defined as $f_{signal} = (S - S_{min})/(S_{max} - S_{min})$, is often used to characterize the extent of completion of the binding reaction. The fraction of bound molecules $f_{bound} = P_{bound}/P_{Total}$ will coincide with f_{signal} only if S is a linear-response function. For that case, the signal observed can be represented as a linear combination of molar fractions of bound and free molecules weighted by their corresponding molar spectroscopic characteristics, s_{bound} and s_{free}:

$$S / P_{Total} = f_{bound} \cdot s_{bound} + f_{free} \cdot s_{free} = f_{bound} \cdot s_{bound} + (1 - f_{bound}) \cdot s_{free} \qquad (8)$$

Not all spectroscopic data satisfy this equation. For example, the following spectroscopic parameters are not linear-response functions and therefore cannot be utilized for measurements of binding: position of maximum of fluorescence spectrum and related parameters such as center of weight, center of the chord at any intensity level, or ratio of intensities on the wings; fluorescence polarization (or anisotropy); intensity at the maximum of the spectrum; the phase f and modulation m from frequency domain fluorimetry; fluorescence decay curve collected to a constant peak; and transmittance from spectrophotometric measurements. Parameters that are linear-response functions and can be utilized for measurements of binding include the following: fluorescence quantum yield; steady-state intensity at a constant wavelength I_{ss}; $I_{ss} m \cos f$ and $I_{ss} m \sin f$; fluorescence decay curve collected to a constant time; vertical and horizontal components of fluorescence intensity (and of any other angle too); and absorbance from spectrophotometric measurements. Ellipticity measured by circular dichroism spectroscopy is

[58]D. Toptygin and L. Brand, *Anal. Biochem. 224,* 330 (1995).

[59]T. M. Lohman and W. Bujalowski, *Methods Enzymol. 208,* 258 (1991).

[60]P. J. Munson and D. Rodbard, *Anal. Biochem. 107,* 220 (1980).

[61]M. L. Johnson, *Anal. Biochem. 148,* 471 (1985).

[62]C. A. Royer and J. M. Beechem, *Methods Enzymol. 210,* 481 (1992).

[63]Z.-X. Wang, N. R. Kumar, and D. K. Srivastava, *Anal. Biochem. 206,* 376 (1992).

assumed to be a linear-response function. However, according to Tilstra and Mattice[64] (p. 262), "this assumption is often made because it is simple, rather than because there is strong evidence in its support" at least for β-sheet–coil transitions. The existence of an isointensity point in titration data (e.g., Fig. 1) can serve as an indication of the suitability of such data for quantitation of binding using Eq. (8). This is also an indication of a two-state transition.

In addition to general rules that apply to all binding experiments, there are some peculiarities relevant to membrane partitioning. There is an explicit assumption that s_{bound} does not depend on the completion of binding or on the number of bound molecules per lipid vesicle. However, in the case of fluorescence, the intensity can be a nonlinear function of bound molecules due to the nonradiative homotransfer of energy or other self-quenching mechanisms. This possibility is often overlooked during the analysis of complex membrane partitioning.

Analysis of Titration Data

Three parameters characterize the spectroscopic signal S of a solution containing lipid at concentration L and peptide at concentration P_{total}: the spectroscopic responses s_{free} and s_{bound} of free and bound peptide, respectively, and the partition coefficient K. These are related through

$$S(L) = P_{Total} \cdot \left[f_{bound}(K,L) \cdot s_{bound} + (1 - f_{bound}(K,L)) \cdot s_{free} \right] \tag{9}$$

where the dependence of f_{bound} on L and K is given by Eq. (5) with $f_{bound} \equiv f_p$. P_{Total} can be estimated from independent measurements (as usually done for CD), or it can be eliminated from the equation by normalizing data with respect to the signal observed at zero lipid concentration when $f_{bound} = 0$ (as usually is done for fluorescence). For quantitative analyses, one can fit Eqs. (8) and (9) to measured $S(L)$ data by numerical minimization methods. Because s_{free} is usually known directly from experiment, there will be only two fitting parameters: K and s_{bound}.

All of the parameters of Eq. (9) must be considered even for qualitative examinations of partitioning. For example, consider a peptide for which fluorescence increases on membrane partitioning. If the peptide exhibits higher fluorescence in one lipid system than in another, this does not necessarily mean that its partitioning is higher in the first case because s_{bound} may be different for the two lipids. This has been observed for the antimicrobial peptide indolicidin, which partitions more strongly into palmitoyloleoylphosphatidylglycerol (POPG) vesicles than

[64]L. Tilstra and W. L. Mattice, *in* "Circular Dichroism and the Conformational Analysis of Biomolecules" (G. D. Fasman, ed.), p. 261. Plenum Press, New York, 1996.

into palmitoyloleoylphosphocholine (POPC) vesicles, but its fluorescence is higher in POPC.[55] Similarly, changes in peptide fluorescence that accompany changes in temperature, pH, or salt concentration cannot be attributed to changes in partitioning until the effects of the variables on the fluorescence of both free and partitioned peptide are known.

Measuring Fluorescence in Membrane Systems

Fluorescence titration is a widely used and generally excellent method for determining partition coefficients. However, the titration of a peptide solution with highly scattering membranes can introduce two significant artifacts that must be taken into account. First, scattering of excitation light into the fluorescence region can be mistaken for the fluorescence signal of the peptide. This background problem is usually accounted for by subtracting the total signal obtained from a peptide-free membrane solution from the signal obtained for the peptide + membrane solution. This procedure is valid, however, only if the absorbance of the fluorophore is vanishingly small. Because this condition is usually not met in fluorescence titration experiments, the membrane-scatter correction is excessive so that incorrect values of intensity and spectral position are obtained. This pitfall can be avoided by scaling the background signal empirically such that the subtraction results in a flat baseline on the wing of the excitation peak where no fluorescence is expected (usually 285-295 nm for tryptophan fluorescence excited at 280 nm). The selection of the scaling factor can be facilitated by exciting at shorter wavelengths (260-270 nm), which reduces scattering in the region of the fluorescence emission peak while broadening the useful wing of the scattering peak. We generally obtain scaling factors in the range of 0.6 to 0.9.

A second, and often overlooked, artifact arises from the loss of fluorescence intensity due to scattering of the excitation beam and to scattering of the fluorescence out of the detector path. We are unaware of any reliable mathematical procedure to account for the resulting loss of intensity. Corrections[65,66] for the well-known inner-filter effect due to optical absorbance are not applicable because the randomly scattered exciting light is still capable of exciting fluorescence. This problem is solved by correcting data using empirical factors obtained from titration experiments using a model fluorophore that does not interact with the membranes. For experiments based on tryptophan fluorescence, the tryptophan zwitterion is a good model fluorophore.[55] Using it, we have found that the correction factor, at moderate lipid concentrations, can be as large as 30%, depending on geometry, optical path, and membrane type and concentration.

[65]J.R. Lakowicz, "Principles of Fluorescence Spectroscopy." Plenum Press, New York, 1983.

[66]E.A. Permyakov, "Luminescent Spectroscopy of Proteins." CRC Press, Boca Raton, FL, 1993.

Multistate Equilibria in Water and Bilayer

Many membrane-active peptides can aggregate in the aqueous and/or membrane phases. Melittin, for example, can be monomeric or tetrameric depending upon the conditions of the aqueous phase.[67,68] The very simple peptide acetyl-Trp-Leu_5 ($AcWL_5$) is monomeric in the aqueous phase but forms aggregates[10] when partitioned into the membrane[32a] (Fig. 3). Such multistate equilibria significantly complicate the spectroscopic determination of partitioning. Aggregation in solution is relatively easy to account for because monomer-oligomer equilibrium can first be studied in the absence of membranes in order to establish the spectroscopic responses for all of the water soluble forms prior to partitioning experiments. We discuss here the interesting and challenging case of aggregation on the membrane and show how the behavior of a system can appear very different when it is examined with three different techniques: direct measurements of partitioning, fluorescence titration, and circular dichroism titration.

A spectroscopic description of the cooperative partitioning of a peptide such as $AcWL_5$ into lipid vesicles requires the use of Eq. (9) modified to take into account the spectroscopic properties of the bound aggregates (*BA*) as well as bound monomer (*BM*) and free monomer (*FM*). Using f_i and s_i to represent the fractions and linear-response spectroscopic parameters, respectively, of the peptides in the three forms (i = *FM*, *BM*, or *BA*), Eq. (9) becomes

$$S(L) = P_{Total} \cdot (f_{FM} \cdot s_{FM} + f_{BM} \cdot s_{BM} + f_{BA} \cdot s_{BA}) \tag{10}$$

Unlike a two-state process described by Eq. (9), the fractional change in spectroscopic signal no longer necessarily coincides with the fraction of bound molecules. A significant and challenging complication of aggregating systems such as $AcWL_5$ is that self-assembly on the membrane can occur.[32a] Although slow aggregation can make spectroscopic titration impractical, we use the observed properties of $AcWL_5$ to illustrate the spectroscopic behavior of on-membrane aggregation. We assume that the ellipticities of the *FM* and *BM* forms correspond to random coil, whereas the *BA* form exhibits features of a β structure. Fluorescence measurements, however, indicate that both the *BM* and *BA* forms have similar intensities that differ from *FM*. For simplicity, assume that fluorescence intensities are given by $s_{FM}^{Fl} = 1$ and $s_{BM}^{Fl} = s_{BA}^{Fl} = 2.9$ and that the per residue ellipticities (10^4 deg cm^2 dmol^{-1}) at 198 nm by $s_{FM}^{CD} = s_{BM}^{CD} = -2.2$ and $s_{BA}^{CD} = 4.0$. Assume P_{Total} = 10 μM.

[67]S. C. Quay and C. C. Condie, *Biochemistry 22,* 695 (1983).

[68]J. C. Talbot, J. Dufourcq, J. De Bony, J.-F. Faucon, and C. Lussan, *FEBS Lett. 102,* 191 (1979).

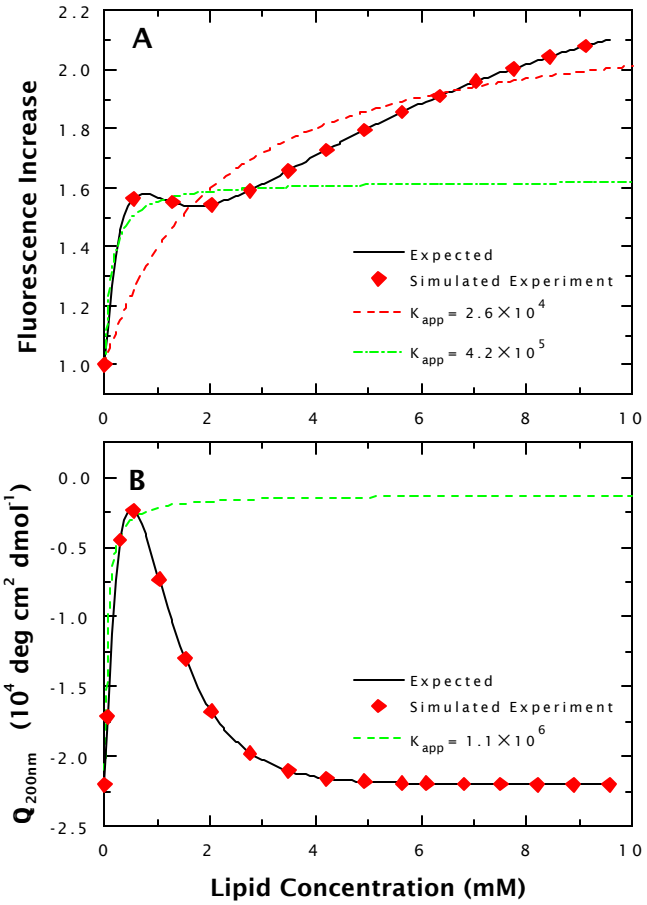

Figure 7. Simulations of (**A**) the fluorescence and (**B**) ellipticity at 200 nm (Θ_{200nm}) as a function of lipid concentration for membrane partitioning of the peptide $AcWL_5$. Partitioning, as determined by equilibrium dialysis (Fig. 3), is a complex process that involves free and membrane-bound monomers and membrane-bound aggregates.[32a] Two-state models are incapable of describing simulated experimental data. However, because, in reality, reequilibration occurs slowly, it is not always possible to determine whether models with apparent partition coefficients K_{app} that describe initial data (dashed and dot-dashed lines) are adequate (see text). Note that the CD and fluorescence responses to additions of membranes differ dramatically.

The results of the simulation of partitioning of $AcWL_5$ peptide into POPC vesicles as followed by tryptophan fluorescence is shown in Fig. 7A. There is a sharp initial fluorescence increase associated with cooperative binding due to aggregate formation arising from the fact that the peptide:lipid ratio is very high for the initial lipid additions. Further additions of lipid reduce the peptide:lipid ratio so that aggregation is suppressed. Because all membrane-bound forms are assumed to have the same fluorescence intensity, the fractional fluorescence change will coincide in this particular case with the total fraction of bound peptide in both forms. As expected, a simple two-state model (dashed line) does

not describe the entire data set. Furthermore, if the spectroscopic observations were limited to the initial lipid concentrations (up to 4 m*M*), then it might not be obvious that a two-state model with a high partition coefficient (dotted line) does not adequately describe the system either, especially given real experimental uncertainties.

The spectroscopic response to binding is very different for the circular dichroism data as shown in Fig. 7B. Ellipticity of $AcWL_5$ increases dramatically for early lipid titrations due to the formation of aggregates for high peptide:lipid ratios. Further titrations, however, lead to decreases in the signal because the formation of membrane-bound monomers is promoted by lower peptide:lipid ratios. A very important point is that the disruption of aggregates can take hours. If one does not wait long enough for reequilibration, then one might see the maximum signal appear to be essentially constant, suggesting (incorrectly) that partitioning is very high over a wide range of lipid concentrations (dotted curve).

Titration Methods: Calorimetric

Titration calorimetry[51,69,70] is being utilized more frequently for studying the partitioning of peptides into membranes because of the commercial availability of high-sensitivity titration calorimeters. In this method, the calorimeter measures the heat generated or absorbed as a solution of vesicles is titrated into a peptide solution. The sum of the individual titration events measures the total heat change, which in turn is a measure of the amount of peptide bound to the membranes. An advantage of titration calorimetry is that it does not require the sometimes complex interpretation of spectral changes (see later). Furthermore, relatively high lipid concentrations can be used. The disadvantage of the method is that it is considerably less sensitive than most spectroscopic methods. In addition, like spectroscopic methods, it can suffer from the fact that the partitioning of peptides can be highly nonideal during the first few lipid titration steps because of the high peptide:lipid ratios.

Some Experimental Details

The previous discussion suggests that techniques based on physical separation of partitioned and free molecules have a significant advantage in the determination of free energies of transfer because they provide unambiguous information. This is especially important for complex multistate equilibria. However, titration methods canbe more precise and

[69]G. Beschiaschvili and J. Seelig, *Biochemistry 31,* 10044 (1992).
[70]F. Hanakam, G. Gerisch, S. Lotz, T. Alt, and A. Seelig, *Biochemistry 35,* 11036 (1996).

and provide additional valuable information on conformational and thermodynamic changes associated with partitioning. The use of both types of measurements is essential for achieving a complete description of membrane partitioning. As is generally true of experimental science, reliability of result depends upon attention to details.

Choice of Vesicle System

Small unilamellar vesicles are used frequently in partitioning studies because their relatively small optical scattering simplifies measurements of partitioning by spectroscopic titration methods. However, we strongly recommend using LUV because SUV are metastable.[47,48] Furthermore, free energies of transfer depend strongly on vesicle curvature so that partition coefficients can be anomalous for the smallest vesicles.[49-51] The effects of optical scattering from LUV can be minimized by careful attention to the placement of the sample relative to the detector and to the choice of wavelengths.

Assessing Nonideality in Titration Experiments

One of the most critical assumptions for the analysis of spectroscopic or calorimetric titration experiments is that the partition coefficient and the spectroscopic parameter for the bound peptide be independent of the concentration of bound peptide. Data presented in Fig. 3 show that the binding of peptides to vesicles can become cooperative or anticooperative at high peptide:lipid ratios. In titration experiments, the earliest lipid additions have the greatest statistical influence on the measurements of partition coefficients. Unfortunately, these earliest additions also have the highest likelihood of nonideal behavior because of the high peptide:lipid ratio. One simple method for assessing the possibility of nonideal partitioning is to repeat the titrations at different starting peptide concentrations. Large variations in the apparent partition coefficients should serve as a warning of nonideal behavior. Another approach is to perform an inverse titration experiment in which a peptide solution is titrated into a low concentration of membranes so that the experiment begins at low peptide:lipid ratios. The region of linear response in such an experiment will define the experimental conditions under which the binding is "ideal"; deviations from linearity will help define the nature of the nonideality.

Range of Detectable Partition Coefficients

The range of measurable partition coefficients varies significantly between methods and depends in each case on a number of experimental details. Nonetheless, in all cases it is determined by the limits of the

concentration of peptide and lipid that are experimentally accessible and experimentally meaningful. Spectroscopic methods have a smaller range because of the limits imposed by the scattering artifacts caused by the lipid vesicles. These artifacts are generally present for lipid concentrations in the 1-10 m*M* range that are frequently used in experiments. We estimate the lower limit of measurable K_x values to be ~100,000 for CD spectroscopy and ~20,000 for fluorescence spectroscopy. The lower limit for titration calorimetry, based on the sensitivity of current instruments, is ~10,000. A much wider range of concentrations can be used in separation methods because of the absence of scattering artifacts. The maximum lipid concentration practically attainable for LUV preparations is about 100 m*M*, which allows mole fraction partition coefficients of about 100 to be determined.

There is also an upper limit on the value of K_x than can be determined under conditions of Eq. (4) because of the inherent conflict between the low lipid concentrations needed to obtain a detectable concentration of free peptide and the low peptide concentration needed to keep the peptide:lipid ratio low. This upper limit is approximately 10^5 for titration calorimetry, 10^6 for circular dichroism, and 10^7 for most other methods. Overall, titration methods have dynamic ranges of accessible partition coefficients of approximately two to three orders of magnitude, whereas separation methods typically cover at least five orders of magnitude.

Peptide Solubility

The very property, namely hydrophobicity, that favors the partitioning of peptides into membranes also places severe constraints on their aqueous solubility. Fig. 8 shows how partitioning and aqueous solubility are related for a family of simple hydrophobic peptides of the form $AcWL_n$, where $n = 1–6$. The partitioning of the highly water-soluble shortest member, AcWL, is so weak that it is barely detectable by equilibrium dialysis and HPLC and is completely undetectable by any other technique. As the length of the peptides goes up, partitioning increases and solubility decreases concomitantly. However, the solubilities of the longer peptides decrease much more rapidly than the partition coefficients increase, resulting in the slope of −0.32 shown in Fig. 8. Thus, for nonpolar peptides, the strong competition between membrane partitioning and aqueous precipitation is more likely to drive precipitation than strong membrane partitioning. The experimental consequences of the resulting solubility–partitioning limits are obvious and make it clear that intelligent principles are needed for the design of membrane-binding peptides that are sufficiently soluble in water to be experimentally useful.

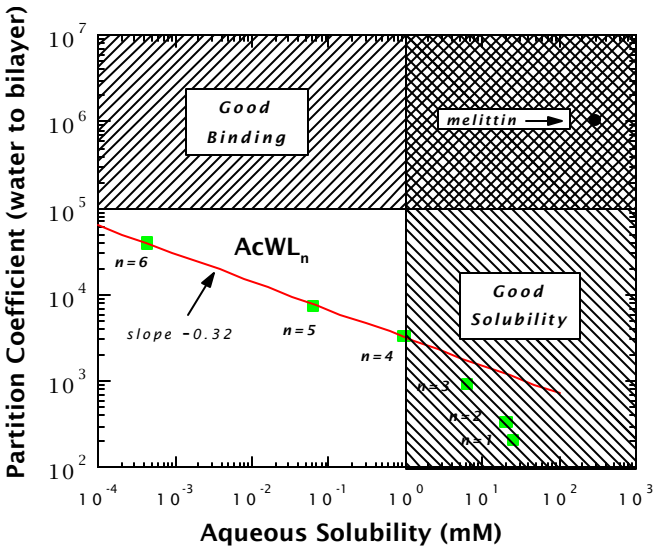

Figure 8. Mole fraction partition coefficients plotted against aqueous phase solubility of AcWL$_n$ peptides (W. C. Wimley and S. H. White, unpublished). Measurement of the partition coefficients was done by equilibrium dialysis and HPLC as described in the text and elsewhere.[10,32] Solubility was measured by filtering visibly turbid samples of the peptides in order to obtain a clear saturated solution. Concentrations were determined by HPLC. As these peptides get longer, the solubility decreases rapidly, but the partition coefficients increase much more slowly. As a result, they become insoluble before they are hydrophobic enough to bind strongly to membranes.

Peptide Aggregation

For longer hydrophobic peptides there is another possible consequence of hydrophobicity: aggregation or self-association in the aqueous phase that does not lead to precipitation. The peptide melittin, for example, shown as a single point in Fig. 8, has many hydrophobic residues and binds strongly to membranes, but is apparently more soluble than the weaker binding AcWL$_6$ by a factor of 10^6 or more. This is due, in part, to the fact that melittin self-associates in water to form highly water-soluble tetramers.[67,68] Other hydrophobic peptides form less ordered aggregates,[10,71-73] but in any case, the presence of aqueous phase aggregation will undermine any attempt to determine the energetics of peptide–membrane interactions. Consequently, knowledge of the aggregation state of hydrophobic peptides in water is of the utmost importance in the analysis of partitioning energetics.

Aggregation of peptides in water can be assayed in numerous complementary ways that include filtration and centrifugation, dynamic and steady-state light scattering, circular dichroism, infrared and fluores-

[71]E. John and F. Jähnig, *Biophys. J. 63,* 1536 (1992).

[72]V. G. Tretyachenko-Ladokhina, A. S. Ladokhin, L. M. Wang, A. W. Steggles, and P. W. Holloway, *Biochim. Biophys. Acta 1153,* 163 (1993).

[73]W. C. Wimley, T. P. Creamer, and S. H. White, *Biochemistry 35,* 5109 (1996).

cence spectroscopy, fluorescence quenching, and fluorescence anisotropy. The most sensitive method may be fluorescence. Tryptophan fluorescence is especially sensitive to aggregation because its fluorescence characteristics depend strongly on its local environment. We typically assesses aggregation in the following manner: Visibly turbid solutions of peptide are filtered through 0.22-μm nylon syringe filters to obtain saturated solutions. For the $AcWL_n$ peptides, the saturating concentrations obtained in this way are shown in Fig. 8. The saturated solutions are diluted if necessary and the tryptophan fluorescence spectra measured and compared to that of acetyltryptophan in solution. Aggregates generally, but not always, have very different tryptophan fluorescence than monomeric peptides. Tryptophan fluorescence can be quenched or enhanced by aggregation and it generally shifts to shorter wavelengths. In addition, changes in quenching by aqueous phase quenchers, such as the anion iodide and the cation cesium, can reveal aggregation. As an example, $AcWL_6$ in solution has a blue-shifted spectrum that is 30% more intense than monomeric $AcWL_5$ or any other monomeric peptide. In addition, its ability to be quenched with iodide is much smaller than for $AcWL_5$ or any other monomeric peptide. Furthermore, these properties are concentration dependent for $AcWL_6$, which is not the case for monomeric peptides. Together, these fluorescence experiments clearly indicate the presence of aggregates of $AcWL_6$ in aqueous solution and serve as a paradigm for examining the aggregation of other peptides.

Acknowledgments

This research was supported by grants GM-46823 (to S.H.W.) and AI-22931 (to Dr. Michael Selsted) from the National Institutes of Health. We thank Dr. Stuart McLaughlin for useful comments on the manuscript.

[74]Z. Oren and Y. Shai, *Biochemistry 36,* 1826 (1997).
[75]W. C. Wimley and S. H. White, *Biochemistry 31,* 12813 (1992).