Correlation between the Free Energy of a Channel-Forming Voltage-Gated Peptide and the Spontaneous Curvature of Bilayer Lipids[†]

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ABSTRACT: The aqueous-membrane partitioning of alamethicin, a voltage-gated channel-forming peptide, was measured as a function of the membrane spontaneous curvature. EPR spectroscopy was used to measure the partitioning of the peptide in lipid compositions formed from dioleoylphosphatidylcholine (DOPC) and varied percentages of dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylethanolamine-*N*-methyl (DOPE-Me), or dioleoylphosphatidylethanolamine-*N*,*N*-dimethyl (DOPE-Me_2). When the mole fraction of DOPE in mixtures of DOPC/DOPE is increased the binding of alamethicin decreases, and the increase in binding free energy is found to be linearly dependent upon the mole fraction of DOPE in the mixture. Addition of DOPE-Me or DOPE-Me_2 also increases the binding free energy, except that the effect is reduced relative to that of DOPE. The free-energy increase per mole fraction of DOPE was found to be 1400 cal/mol, whereas for DOPE-Me and DOPE-Me_2 the free-energy changes were 980 and 630 cal/mol, respectively. When the free-energy changes for alamethicin binding are compared with the previously determined spontaneous curvatures for mixtures of DOPC/DOPE and DOPC/DOPE-Me, the free energy of binding is found to be linearly dependent upon the spontaneous curvature of the bilayer lipids. The effects of membrane lipid unsaturation on the partitioning of alamethicin were also measured and are qualitatively consistent with this conclusion. The sensitivity to spontaneous curvature and the cooperativity that is seen in the binding curves for alamethicin are postulated to be a result of a localized thinning of the bilayer promoted by this peptide.

Lipids form heterogeneous lamellar or nonlamellar phases as a result of a large number of noncovalent interactions, including hydrophobic, electrostatic, steric, and hydrogen-bonding interactions. For lipids such as phosphatidylcholines, PC, the lamellar bilayer phase is the preferred phase, while for phospholipids such as phosphatidylethanolamines, PE, inverted phases such as inverted hexagonal phases (HII) are often preferred. Although they tend to form nonlamellar (or nonbilayer) phases, many biological membranes are composed of a significant fraction of lipids such as PE. In the lamellar phase, the presence of these lipids produces a stress within the bilayer that can be characterized by a membrane property referred to as the spontaneous curvature (*1*). The spontaneous curvature represents the curvature that one monolayer of a bilayer would assume if it were allowed to bend freely, independent of the constraints imposed by the bilayer. The energy, *E*, that is inherent in this elastic bending is given by

$$E = \frac{k_c}{2}\left(\frac{1}{R} - \frac{1}{R_0}\right)^2 \quad (1)$$

where *R* represents the radius of curvature of the lipid phase (which is infinite for a bilayer) and R_0 represents the spontaneous radius of curvature. Here k_c represents the bending modulus for the bilayer. The spontaneous curvature is just the reciprocal of the spontaneous radius of curvature, $C_0 = 1/R_0$. It should be noted that this type of bilayer strain can also be expressed as a gradient (along the bilayer normal) of the lateral pressure within the membrane (*2*).

There has been considerable interest in this membrane property in recent years, as it appears to be carefully regulated in at least some organisms. For example, the mycoplasma *Acholeplasma laidlawii* alters the composition of the headgroups attached to its membrane phospholipids in response to altered levels of acyl chain saturation, and in this way maintains the spontaneous curvature or elastic stress of its plasma membrane at a constant value (*3*). The reasons for maintaining this curvature stress are not clear, but this finding indicates that some mechanism, probably involving membrane proteins, must facilitate this sensitivity to spontaneous curvature. Direct evidence that this membrane property modulates the activity of membrane proteins can be found in the literature, and evidence for its role in the regulation of the G-protein coupled receptor rhodopsin (*4*−*7*), the ion channel gramicidin (*8*, *9*), and the voltage-gated ion channel alamethicin (*10*) has been reported.

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[1] Abbreviations: CD, circular dichroism; EPR, electron paramagnetic resonance; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, dioleoylphosphatidylethanolamine-*N*-methyl; DOPE-Me_2, dioleoylphosphatidylethanolamine-*N*,*N*-dimethyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DLnPC, dilinoleoylphosphatidylcholine; HII, hexagonal II phase; MeA, α-methylalanine; POPC, palmitoyloleoylphosphatidylcholine.

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The mechanisms by which the spontaneous curvature of the bilayer lipid modulates membrane protein behavior remains unclear. For gramicidin, there is evidence that the spontaneous curvature modulates the interactions of tryptophan residues with the membrane lipid (*9*). For proteins containing helical bundles such as rhodopsin, structural changes in the orientation of helices have been observed during activation (*11*) and they could result in a greater area expansion of the protein within the headgroup domain than within the hydrocarbon. This differential area change during rhodopsin activation could explain the apparent sensitivity of the protein to the spontaneous curvature.

Alamethicin is a small 20 amino acid peptide that forms voltage-gated ion channels in planar bilayers and vesicles. The conductance state of alamethicin has been shown to depend on the spontaneous curvature of the bilayer (*10*). In mixtures of dioleoylphosphatidycholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE), an increase in the fraction of DOPE (which makes R_0 smaller) results in higher conductance states and more stable channels. Alamethicin has also been observed to seed the formation of nonbilayer phases at surprisingly low concentrations of peptide (*12*). Therefore, alamethicin appears to interact with the curvature stress of the lamellar system. This observation is not entirely unexpected, because a peptide channel that is sensitive to the spontaneous curvature would also be expected to modulate the spontaneous curvature.

In the present study, we investigate the energy of alamethicin bound to membranes in a monomeric form as a function of the spontaneous curvature. This is accomplished by measuring the aqueous-membrane phase partitioning of a spin-labeled analogue of alamethicin in mixtures of DOPC−DOPE, as well as DOPC mixtures containing monomethyl and dimethyl PEs. We show that the binding free energy for the peptide is linearly related to the spontaneous curvature, and on the basis of previous structural work, we discuss a likely mechanism by which this curvature dependence might arise.

MATERIALS AND METHODS

Materials. The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methyl (DOPE-Me), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*,*N*-dimethyl (DOPE-Me_2), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLnPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The lipids were used without further purification and stored in chloroform until use. The paramagnetic molecule 3-carboxy-PROXYL and native alamethicin were purchased from Sigma Chemical Co. (St. Louis, MO) and used directly to produce the spin-labeled alamethicin analogue which was HPLC purified as described below.

Synthesis of a C-Terminal Spin-Labeled Alamethicin Analogue. The C-terminal spin-labeled analogue of alamethicin was produced by coupling 3-carboxy-PROXYL to the C-terminal phenylalaninol of the crude commercially available alamethicin product using *N*,*N*-dicyclohexylcarbodiimide (DCC) and (dimethylamino)pyridine in a manner similar to that previously described (*13*, *14*). The spin-labeled product was then purified on a reverse-phase semipreparative C18 HPLC column with a gradient of 40% A/60% B to 10% A/90% B, where A is 0.05% trifluoroacetic acid (TFA) in water and B is 0.05% TFA in acetonitrile (CH_3CN). Native alamethicin contains four different peptides resulting from amino acid substitutions of Ala or MeA (α-methylalanine) at position 6 and Glu or Gln at position 18. The major spin-labeled fraction (F1) with Ala 6 and Gln 18, having the sequence Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-Val-MeA-Gly-Leu-MeA-Pro-Val-MeA-MeA-Gln-Gln-Phol, was used throughout these experiments. The retention time for the spin-labeled alamethicin fraction F1 under these conditions was 17 min, and the peptide had a purity in excess of 98% as determined by HPLC. The peptide was identified by electrospray mass spectrometry, which yielded predominantly the triply charged ion at an *m*/*z* of 711.12, corresponding to a mass of 2130.4.

Preparation of Vesicles. Large unilamellar phospholipid vesicles were prepared by drying aliquots of lipid in cholorform under a vacuum for at least 12 h, hydrating the lipid in the appropriate buffer solution (100 mM KCl, 10mM MOPS, pH 7, unless otherwise noted), vortexing and freeze−thawing the lipid suspension five times, and manually extruding the sample 20 times at room temperature through 0.1 μm polycarbonate filters using a commercially available extruder (Lipex Biomembranes Inc., Vancouver, B.C.). After the final extrusion, the phospholipid vesicles were stored for a maximum of one week at 4 °C under an argon atmosphere. Final lipid concentrations were determined from the vacuum-dry weight of the phospholipid used to make the lipid suspension.

Lipid vesicles were prepared in a series of lipid mixtures containing DOPC and DOPE in order to vary the spontaneous curvature of the lamellar phase. In these mixtures, DOPE was added to a maximum mole fraction of 0.6. At this concentration of DOPE, the lipid mixture forms a lamellar phase with a bilayer to nonbilayer transition that is significantly above room temperature (*15*). In addition to DOPE, mixtures of DOPC containing DOPE-Me and DOPE-Me_2 were also produced. Lipid vesicles containing one and four unsaturated bonds formed of POPC and DLnPC, respectively, were also prepared.

EPR Spectroscopy. EPR spectra were obtained using an X-band Varian (Sunnyvale, CA) E-line Century Series spectrometer. Lipid titrations were performed in a standard X-band (TM) cavity using a modulation amplitude of 1.25 G P-P and a 10 mW incident microwave power. To characterize the membrane-aqueous partitioning of alamethicin in lipid vesicles, we added spin-labeled alamethicin at a final concentration of approximately 20−30 μM to buffer and titrated with lipid. At these concentrations of peptide, alamethicin is monomeric in the buffer solution used. The titration was carried out using a modified quartz sample cell, which allowed lipid to be added into the sample without the removal of the cell from the cavity (*14*). EPR spectra were acquired and processed using the IBM-compatible software package EPR Data Acquisition System, Version 2.2 (Philip D. Morse, II and the University of Illinois College of Medicine, Urbana, IL).

Analysis of the EPR Spectra. The partitioning of the peptide, λ, is defined here as the ratio of the number of membrane-associated to aqueous peptides, N_B/N_F. The value

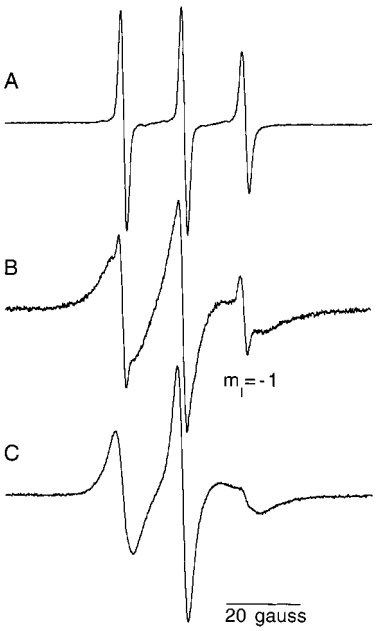

FIGURE 1: EPR spectra of spin-labeled alamethicin at a concentration of approximately 40 μM (A) in aqueous solution, (B) in the presence of extruded lipid vesicles formed from DOPC at a concentration of approximately 0.2 mM lipid, and (C) in the presence of lipid vesicles formed from DOPC at a concentration of 20 mM.

of λ was determined as described previously from the high-field resonance amplitude of the EPR spectrum (*16*, *17*), except that the spectra were analyzed using a program written in MATLAB (The Math Works, Inc.) that allowed deconvolution of the composite spectra into bound and aqueous spin-label populations. The partitioning, λ, is related to the lipid concentration C_L and the partitioning coefficient, β, by the following:

$$\frac{1}{\lambda} = \frac{2}{\beta A_L}\left(\frac{1}{C_L} - \bar{V}_L\right) \qquad (2)$$

where V_L is molar volume of the phospholipid and A_L is the area per lipid molecule. The partition coefficient, β, has units of length and is equivalent to $K\delta$, where K is the binding constant and δ is the effective thermodynamic thickness of the membrane-binding domain of alamethicin. In these experiments, values of 2.33 cm^3/g and 5.2 $\times$ 10^6 cm^2/g were used for V_L and A_L, respectively (*14*, *18*). For δ we used a value of 30 Å, since the peptide is fully inserted into the membrane under the conditions used for this experiment.

RESULTS

Alamethicin Binding Curves Are Not Linear and the Peptide Shows an Enhanced Binding at Higher Peptide−Lipid Ratios. Shown in Figure 1 are EPR spectra of the spin-labeled alamethicin in aqueous solution and in the presence of phospholipid bilayers formed from DOPC. The membrane-associated EPR spectrum (Figure 1C) is similar to those seen previously and is characteristic of a nitroxide associated with a highly mobile, monomeric peptide with an apparent rotational correlation time of about 3 ns. At lower concentrations of lipid (Figure 1B) the spectra are a composite of both membrane-associated and aqueous peptide, and as indicated above (see methods), these composite EPR spectra were used to determine the partitioning of the peptide between membrane and aqueous phases.

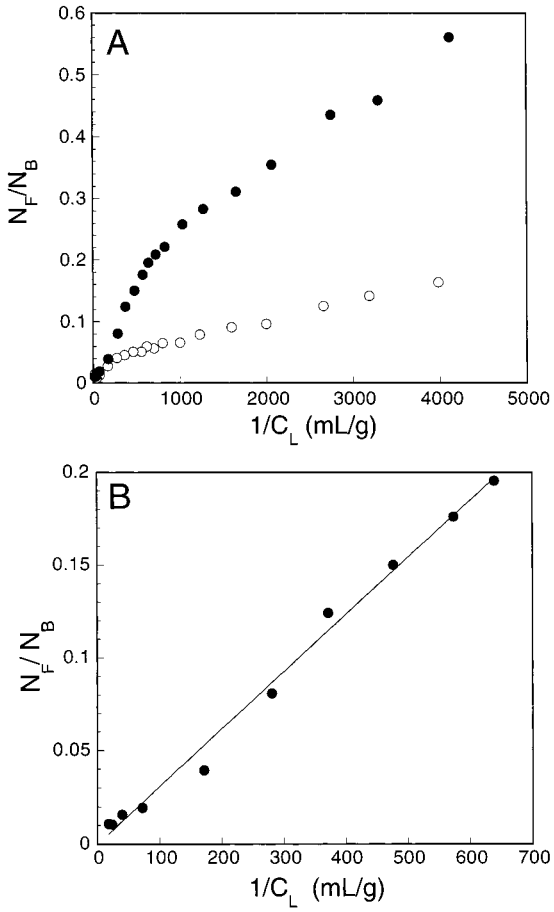

FIGURE 2: A reciprocal binding plot of alamethicin to bilayers composed of DOPC (○) and DOPC−DOPE (40:60) (●). (A) A complete binding curve from a peptide−lipid ratio of less than 1:2000 up to a ratio of approximately 1:14. The binding curves exhibit a cooperative transition at peptide−lipid ratios of about 1:50 to 1:100. (B) Binding curve for alamethicin to DOPC−DOPE (40:60) in the limit of low peptide concentration where the mole fraction of peptide is less than about 0.01. The binding free energy was determined by fitting binding data in this low concentration limit to eq 2.

Figure 2A shows binding data obtained for alamethicin in the presence of both pure DOPC and a mixture of DOPC−DOPE (40:60). As indicated above, the slope of this curve is proportional to the reciprocal of the membrane binding constant. These curves have significantly different slopes, indicating that alamethicin binds more weakly to membranes containing DOPE. As seen previously using both EPR and CD, these binding curves are also not linear over this range of bound peptide concentrations, but show an increase in membrane binding above approximately 1 mol % membrane-bound peptide (*14*, *19*). Through this concentration range, the further addition of alamethicin produces an increase in membrane affinity of approximately (700−800 cal/mol). Previous work has demonstrated that this apparent cooperative change in binding to DOPC is not the result of aggregation and that the peptide remains monomeric over a wide concentration range (*14*, *20*).

The EPR spectra for alamethicin bound to DOPC and mixtures of DOPC and DOPE above and below 1 mol % membrane bound peptide are shown in Figure 3. As found

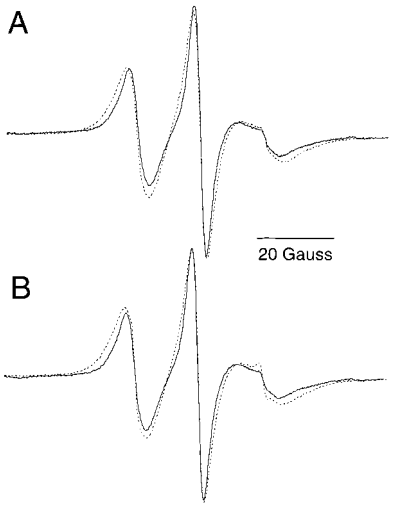

FIGURE 3: EPR spectra of spin-labeled alamethicin at peptide-to-lipid ratios of 1:200 (solid line) and 1:40 (dashed line) in membranes composed of (A) DOPC and (B) DOPC−DOPE (40:60). The lipid concentration was 20 mM, and essentially all of the peptide is membrane-associated. These peptide−lipid ratios correspond to membrane concentrations of peptide above and below the point where alamethicin undergoes a cooperative binding transition. The spectra indicate that peptide aggregation is not the source of this cooperative behavior (see text).

previously in DOPC, no large change in line shapes for these spectra is seen as a function of concentration (*20*). The slight increase in line widths shown was seen previously and can be quantitatively accounted for by the expected collision frequency between alamethicin monomers. In the presence of DOPE, the spectra shown are identical to those found in DOPC and indicate that the dynamics of the label at the C-terminal end of the peptide is not affected by the inclusion of DOPE. The absence of any change in nitroxide motion and the absence of significant collisional exchange or dipole−dipole coupling indicates that the peptide in lipid mixtures of DOPC−DOPE are also monomeric.

The Free Energy of Alamethicin Binding Is Linearly Dependent upon Spontaneous Curvature. To determine the free energy of binding of alamethicin to bilayers composed of DOPC and mixtures of DOPC−DOPE, we examined data from binding curves such as those shown in Figure 2A in the dilute limit where the lipid−peptide ratio was typically greater than 100:1. Figure 2B shows the partitioning of alamethicin in DOPC−DOPE (40:60) for membrane mole fractions less than 1 mol %. Within this concentration range, the binding constant for the peptide could be determined from a linear fit of the data using eq 2. The binding constant for the association of alamethicin to mixtures of DOPC−DOPE as a function of the concentration of DOPE is shown in Figure 4A. In these systems, the addition of DOPE produces a decrease in the membrane affinity of the peptide or an increase in the free energy of binding. Shown in Figure 4B is a plot of the change in free energy $\Delta\Delta G$ as a function of the membrane mole fraction of DOPE. Within experimental error, the increase in free energy is linearly dependent upon the mole function of DOPE added to DOPC.

If the decrease in binding of alamethicin that is produced by adding DOPE to DOPC is a result of changes in the membrane curvature stress, lipids such as DOPE-Me and DOPE-Me_2 would also be expected to reduce the membrane binding of alamethicin but produce smaller changes on a molar basis than those seen with DOPE addition. The binding of alamethicin to membranes composed of DOPC and DOPE-Me or DOPE-Me_2 was measured and was also found to decrease with the addition of either PE derivative. The changes in the free energy of binding were quantitated and the results are shown in Figure 4B, along with data for DOPC−DOPE membranes. Also shown in Figure 4B are linear fits to the data. Within experimental error there is a linear change in the free energy of binding as a function of the mole fraction of DOPE, DOPE-Me, or DOPE-Me_2. In addition, the largest changes are induced by DOPE (the smallest headgroup), and the smallest changes are induced by DOPE-Me_2. For these lipid mixtures, the change in the free energy of binding to DOPC was found to be approximately 1400, 980, and 630 cal/mol per mole fraction of added DOPE, DOPE-Me, or DOPE-Me_2, respectively.

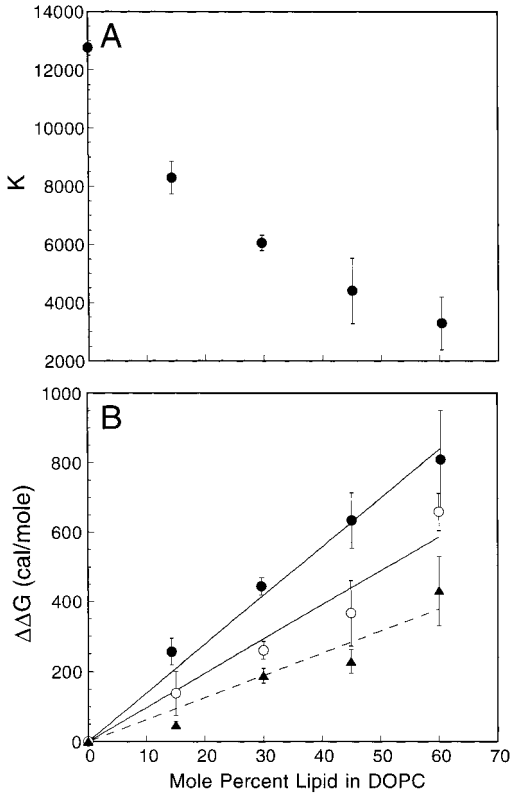

FIGURE 4: (A) The binding constant *K*, determined from eq 2 where $\beta = K\delta$, is plotted as a function of the mole fraction of DOPE in DOPC. (B) Change in the free energy of binding $\Delta\Delta G$ with the addition of DOPE (●), DOPE-Me (○), or DOPE-Me2 (▲) to membranes composed of DOPC. The error bars represent standard deviations from three separate binding measurements on a single lipid sample. This does not include the experimental error associated with sample reproducibility, which we estimate to be approximately 10%.

For the lipids DOPC, DOPE, and DOPE-Me, the water cylinder center-to-center spacings have been measured for mixtures of lipid and alkane (*10*). From these spacings the spontaneous curvatures for the DOPC−DOPE and DOPC−DOPE-Me_2 mixtures used here were estimated as described previously. The changes in free energy of binding, $\Delta\Delta G$, were then plotted in Figure 5 for both lipid systems as a function of the spontaneous curvature. As is readily apparent in Figure 5, binding measurements for both lipid systems fall on a single line, providing strong evidence that there is a linear relationship between peptide binding and membrane curvature stress.

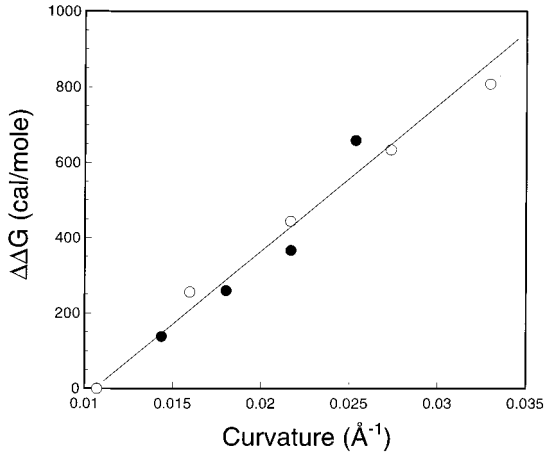

FIGURE 5: Plot of binding free energy $\Delta\Delta G$ as a function of the spontaneous curvature for mixtures of DOPC−DOPE and DOPC−DOPE-Me. The membrane curvature of the DOPC−DOPE mixture, C_{mix}, is given by the following: $C_{mix} = XC_{DOPE} + (1 - X)C_{DOPC}$, where X is the mole fraction of DOPE, $C_{DOPC} = 0.0107$ Å^{-1}, and $C_{DOPE} = 0.0476$ Å^{-1}. The membrane curvature of the DOPC−DOPE-Me mixture was calculated in a similar fashion using $C_{DOPE-Me} = 0.0351$ Å^{-1}.

Table 1: Binding of Alamethicin to Membranes of Varied Saturation

lipid	no. double bonds	K	$\Delta G°$ (cal/mol)
POPC	1	34000	−6220
DOPC	2	17100	−5810
DLnPC	4	14390	−5706

Increasing the Lipid Unsaturation Decreases the Binding Affinity of Alamethicin. Changes in the membrane curvature stress can also be produced by changes in the level of the membrane acyl chain unsaturation. As the level of lipid unsaturation is increased, the chain volume within the hydrocarbon domain increases, and the membrane curvature stress is increased (*21*). Increasing the level of unsaturation should have the same qualitative effect as increasing the PE content of the membrane. The binding of alamethicin was measured to membranes composed of palmitoyloleoyl PC (POPC) (1 double bond) and dilinoleoyl PC (DLnPC) (4 double bonds), and these affinities were compared to that obtained for DOPC (2 double bonds). The binding affinities obtained in the limit of low peptide−lipid ratios are shown in Table 1. As seen in Table 1, the binding affinity of alamethicin decreases as the level of lipid unsaturation is increased, consistent with the idea that the binding decreases seen in the presence of PE are the result of changes in the curvature stress of the bilayer. This decrease in binding with increasing unsaturation is qualitatively consistent with previous results obtained using CD spectroscopy (*19*).

DISCUSSION

The binding measurements presented here demonstrate that the membrane affinity of alamethicin decreases as the mole fraction of PE is increased in mixtures of DOPC and DOPE. This is consistent with previous work using CD, indicating that there is a decrease in membrane binding of alamethicin to membranes formed from PE (*19*). When mixtures of DOPE-Me or DOPE-Me$_2$ with DOPC are examined, these PE derivatives are also found to reduce the membrane affinity of alamethicin; the effect these lipids produce is diminished as the substitution on the choline nitrogen is increased. When the spontaneous curvatures for mixtures of DOPC−DOPE and DOPC−DOPE-Me are estimated, they are found to directly correlate with the binding free energy of alamethicin, and the aqueous-membrane partition free energy $\Delta\mu_b$ increases as the intrinsic membrane curvature stress is increased. Membranes formed from PE differ from PC in several respects. For example, the PC headgroup is much more hydrophobic than the PE headgroup, and other noncovalent interactions might account for the energy differences seen in the presence of PE. However, the data shown here provide strong support for the idea that membrane curvature stress is the source of the binding free-energy differences seen for alamethicin in membranes containing PE.

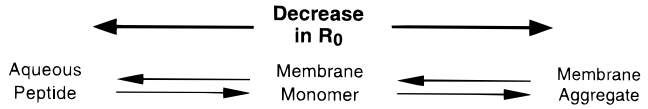

FIGURE 6: Equilibria exhibited by alamethicin in the presence of lipid bilayers. Alamethicin in the aqueous phase is in equilibrium with peptide in the membrane. Most of the membrane-bound peptide is in a monomeric form, but a fraction of the peptide can assemble to form a conductive pore. A decrease in R_0, corresponding to a more negative membrane spontaneous curvature, shifts the equilibrium by increasing the energy of the monomeric membrane-bound form relative to either aqueous peptide or membrane-bound aggregate.

Previous work has demonstrated that alamethicin responds to the curvature stress of bilayers. Alamethicin seeds the formation of nonbilayer phases (*12*), and the channel conductance is dependent upon the membrane spontaneous curvature. It is interesting to note that, as the level of PE is raised in the membrane, the lifetime of the channel increases and single channel recordings show that the peptide reaches higher conductance states (*10*). These higher conductance states are thought to be the result of larger channel aggregates that result from the association of alamethicin monomers into a conductive pore. Indeed recent work on alamethicin dimers provides convincing evidence for the correlation between aggregation and conductance states (*22*). Thus, an increase in the curvature stress appears to stabilize the multimeric channel at the same time it decreases the membrane binding of the peptide. These seemingly contradictory observations are easily explained and might even be expected. For alamethicin, previous work using EPR indicates that the vast majority of alamethicin is in a monomeric state within the bilayer (*14*, *20*), at least in the absence of voltage, but small levels of the peptide must be able to associate in order to form a conductive channel. As indicated in Figure 6, these results suggest that as the spontaneous radius of curvature decreases the equilibrium between aqueous, membrane monomer and membrane aggregate is altered to favor either aqueous peptide or peptide multimer. Indeed, if peptide-membrane interactions are made less favorable with increasing curvature stress, conversion of the monomer into an aggregate would be favored because the size of the lipid−peptide interface per monomer would be reduced.

Why should the binding of alamethicin decrease as the curvature stress is increased? Structural studies indicate that alamethicin is largely inserted into the membrane hydrocarbon core when lipids are fully hydrated (*23*−*25*), and a fraction of the binding free energy will be due to the work necessary to expand the bilayer upon insertion. There are

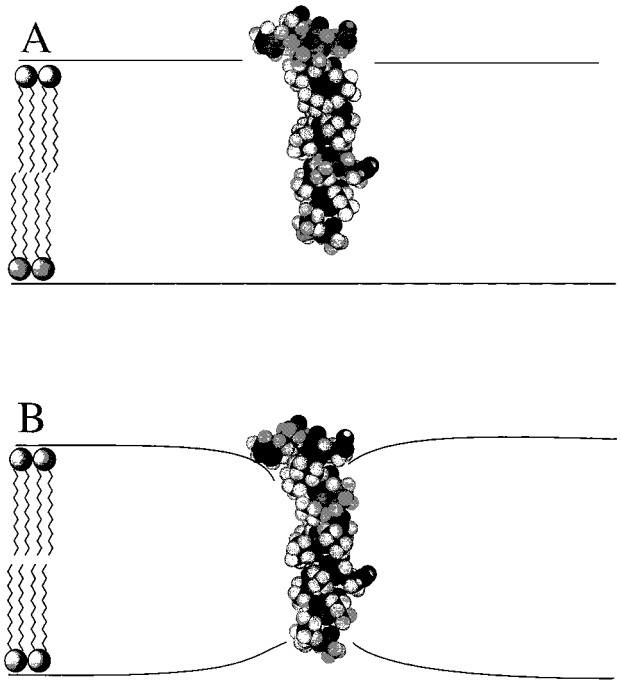

FIGURE 7: (A) Position of alamethicin as determined by site-directed spin-labeling (*24*). In this structure, the N-terminus fails to completely cross the membrane interface, and a C-terminal nitroxide label lies approximately 3−4 Å on the aqueous side of the membrane interface. (B) The placement of the C-terminus of alamethicin results in an effective hydrophobic mismatch, which promotes a deformation of the bilayer. The generation of membrane regions having positive curvature could account for the binding sensitivity to membrane spontaneous curvature.

several ways in which the peptide might respond to the curvature stress. For example, if the peptide produced a greater lateral expansion in the hydrocarbon region than in the headgroup domain upon insertion, the peptide free energy within the membrane would increase as R_0 became smaller. Magnetic resonance studies of alamethicin in membranes suggest that the peptide has a nearly linear α-helical structure. In this configuration, the overall shape of the alamethicin monomer is roughly cylindrical, although the C-terminal phenylalanine gives this protein a slightly conical shape (see Figure 7). Thus, the shape of the alamethicin monomer does not provide an explanation for its sensitivity to R_0. It has also been suggested that proteins, when inserted into bilayers, will perturb the membrane thickness even if there is a match between the hydrophobic domain of the protein and the bilayer thickness (*26*). If alamethicin produced this type of distortion, its binding would be expected to be sensitive to the magnitude of the membrane spontaneous curvature.

Another mechanism by which alamethicin might perturb the bilayer is illustrated in Figure 7. On the basis of structural data obtained from EPR, the peptide is positioned so that the N-terminus is placed within the opposing hydrocarbon. Thus, it fails to completely cross the bilayer (*24*). It is interesting to note that recent work on alamethicin indicates that the C-terminus may be strongly associated with the membrane interface (*27*), and the length of the helical segment associated with the hydrocarbon may indeed be fixed. This is not an energetically favorable position, as it leaves several polar groups at the N-terminus with unsatisfied hydrogen bonds. A likely outcome of this arrangement would be the distortion of the bilayer in order to hydrate the N-terminus. Indeed recent diffraction studies (*28*) suggest that alamethicin at modest concentrations results in a thinning of the bilayer, a process that might occur in order to hydrate the N-terminus. Such a distortion is indicated in Figure 7B and would require that the lipids in the vicinity of the peptide assume a positive curvature, a process that becomes less energetically favorable as the fraction of PE is increased and the value of R_0 is made smaller. This type of membrane distortion is expected to occur as a result of a hydrophobic mismatch between a membrane-bound peptide or protein where the hydrocarbon thickness is not sufficient to accommodate the protein (*29*−*31*). In the case of alamethicin, the peptide is of sufficient length to span the bilayer hydrocarbon, but because it is not completely inserted, as shown in Figure 7, an effective hydrophobic mismatch arises.

It should be noted that the increased membrane binding of alamethicin observed here (see Figure 2) and elsewhere (*14*, *19*) occurs near the membrane concentration where alamethicin is observed to thin the bulk membrane phase (*25*, *28*). As discussed above, this is not due to aggregation. We speculate that at low peptide concentrations the binding of alamethicin includes an additional free-energy contribution due to the energy necessary to distort the bilayer (as shown in Figure 7B), thereby doing work against the curvature stress. At higher concentrations of bound peptide, the membrane has largely thinned, and the free energy for binding alamethicin is lowered because the bilayer no longer needs to be distorted. Thus, a local bilayer thinning by alamethicin not only is reasonable given what is known structurally about the peptide but also could explain the sensitivity of the peptide to spontaneous curvature and the cooperativity seen in the binding curves. It also explains previous measurements which indicate that alamethicin prefers to bind to thinner membranes (*19*). Finally, we note (data not shown) that in thinner membranes formed from dilauroylphosphatidylcholine, the increase in binding affinity (Figure 2) is no longer observed. Again, this observation is consistent with the idea that the change in the binding affinity of the peptide results from a change in bilayer thickness and a change in the energy penalty due to the curvature stress.

Clearly the explanations given above are not the only explanations for the sensitivity of alamethicin binding to curvature, and it is possible that other structural features of the peptide−membrane interaction result in the sensitivity to curvature seen here. In any case, a more detailed structure of membrane-associated alamethicin will be needed to fully account for the results.

An estimate can be made of the differences in strain energy due to the addition of DOPE or DOPE-Me to DOPC using eq 1, and these energies range from 1368 to 800 cal/mol lipid. It is interesting to note that these energies are roughly equivalent to the binding energy differences that are measured for alamethicin to these bilayer mixtures (1400 and 980 cal/mol). There does not appear to be any reason that these energies should be similar, since the change in binding energy for alamethicin will depend on the number of lipids the peptide affects as well as the magnitude of the distortion it makes. Nonetheless, the similar size of these energies is consistent with our conclusion that the membrane curvature stress is a likely source of the energy differences seen for alamethicin in mixtures of PE and substituted PEs.

In summary, the binding free energy of alamethicin to DOPC decreases as the mole fraction of DOPE or substituted DOPEs in the lipid mixture is increased. For DOPE and DOPE-Me, the binding free energy of alamethicin to membranes containing these lipids is directly related to the bilayer curvature stress defined by the bilayer spontaneous curvature. One likely explanation for this effect is a local thinning of the bilayer by alamethicin, a process that may also explain why the binding of this peptide is cooperative. Simple helical peptides such as alamethicin provide interesting and useful models to determine how membrane proteins couple to this important bilayer property.

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