Continuum Solvent Model Calculations of Alamethicin-Membrane Interactions: Thermodynamic Aspects

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ABSTRACT Alamethicin is a 20-amino acid antibiotic peptide that forms voltage-gated ion channels in lipid bilayers. Here we report calculations of its association free energy with membranes. The calculations take into account the various free-energy terms that contribute to the transfer of the peptide from the aqueous phase into bilayers of different widths. The electrostatic and nonpolar contributions to the solvation free energy are calculated using continuum solvent models. The contributions from the lipid perturbation and membrane deformation effects and the entropy loss associated with peptide immobilization in the bilayer are estimated from a statistical thermodynamic model. The calculations were carried out using two classes of experimentally observed conformations, both of which are helical: the NMR and the x-ray crystal structures. Our calculations show that alamethicin is unlikely to partition into bilayers in any of the NMR conformations because they have uncompensated backbone hydrogen bonds and their association with the membrane involves a large electrostatic solvation free energy penalty. In contrast, the x-ray conformations provide enough backbone hydrogen bonds for the peptide to associate with bilayers. We tested numerous transmembrane and surface orientations of the peptide in bilayers, and our calculations indicate that the most favorable orientation is transmembrane, where the peptide protrudes ~4 Å into the water-membrane interface, in very good agreement with electron paramagnetic resonance and oriented circular dichroism measurements. The calculations were carried out using two alamethicin isoforms: one with glutamine and the other with glutamate in the 18th position. The calculations indicate that the two isoforms have similar membrane orientations and that their insertion into the membrane is likely to involve a 2-Å deformation of the bilayer, again, in good agreement with experimental data. The implications of the results for the biological function of alamethicin and its capacity to oligomerize and form ion channels are discussed.

INTRODUCTION

Alamethicin is a 20-amino acid antibiotic peptide, produced by the fungus *Trichoderma viride*, that forms voltage-gated ion channels in lipid bilayers (Cafiso, 1994). It is the best studied of a class of membrane active peptides of fungal origin called peptaibols, which are rich in α-aminosobutyric acid (Aib) (Sansom, 1991). The small size of alamethicin makes it an attractive model for the study of voltage gating and peptide-membrane interactions. X-ray diffraction (Fox and Richards, 1982) and high-resolution NMR studies (Banerjee and Chan, 1983; Esposito et al., 1987; Yee and O'Neil, 1992) demonstrated that alamethicin is predominantly α-helical, and solid-state ^{15}N-NMR studies indicated that the helical structure of the N-terminal segment of alamethicin is maintained in dimyristoylphosphatidylcholine (DMPC) vesicles (North et al. 1995). Alamethicin is slightly amphipathic, and the ion channels are believed to be formed by parallel bundles of alamethicin helices surrounding a central transbilayer pore (Rink et al., 1994; He et al., 1995; Mak and Webb, 1995; Sansom, 1998)

Knowledge of the favorable conformation and orientation of alamethicin in its monomeric form in lipid bilayers and other details of the peptide-membrane interactions are important for understanding the assembly mechanism of the channel and the voltage gating phenomenon. Thus the alamethicin-bilayer system has been intensively studied using experimental and theoretical methods. Early NMR studies indicated that alamethicin is surface oriented (Banerjee et al., 1985), suggesting a gating mechanism involving a change in helix orientation (i.e., from surface oriented to transmembrane) (Baumann and Mueller, 1974), and a surface orientation is compatible with the slightly amphipathic nature of alamethicin. However, more recently, EPR spectroscopy of the peptide in egg PC vesicles (Barranger-Mathys and Cafiso, 1996), solid state NMR spectroscopy in DMPC dispersions (North et al., 1995), and oriented circular dichroism studies of alamethicin in multilayers of diphytanoylphosphatidylcholine (DPhPC) (Huang and Wu, 1991) have shown that it assumes a transmembrane orientation. These findings suggest that the vast majority of the alamethicin population is in transmembrane orientation even before the application of membrane voltage.

Many theoretical studies have attempted to understand the role of alamethicin as an ion channel and have focused on interpreting the experimentally observed current-voltage curves and the conductance behavior of single and multiple channels (e.g., Boheim, 1974; Baumann and Mueller, 1974; Sansom, 1991, 1993, 1998). However, theoretical investigation into the details of the alamethicin-bilayer interactions has only been undertaken recently, and the main contribution comes from molecular dynamics simulations by San-

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som and his co-workers (reviewed in Sansom, 1998). The first study (Biggin et al., 1997) was based on a simplified representation of the membrane as a "hydrophobic potential," adapted from the Monte Carlo simulations of peptide-membrane systems of Milik and Skolnick (1993, 1995), and the second (Tieleman et al., 1999a,b) involved an atomic description of the membrane. The results support the experimental observation that alamethicin is predominantly in an α-helix conformation, although it has a relatively flexible kink near Pro^{14}. They also suggest that while the polar C-terminus of alamethicin is anchored to the bilayer-water interface, the N-terminus is relatively free to move between the two sides of the membrane, and therefore the peptide fluctuates between transmembrane and surface orientations in lipid bilayers. The authors have concluded from their study that alamethicin is mainly surface oriented in the absence of membrane potential and that application of the potential enhances its likelihood of being in transmembrane orientation, where it can oligomerize to form ion-conducting channels. However, as the authors admit, the simplified model is not detailed enough to give conclusive results, while the all-atom simulations are not long enough to guarantee that significant changes in the alamethicin-bilayer interactions would not occur if the duration of the simulation were extended. In this paper we present an alternative theoretical approach.

Despite the intensive experimental and theoretical studies, the most favorable conformations and orientations of the peptide in the membrane are still unknown, and the free energy determinants of alamethicin insertion into bilayers are unclear. We used continuum solvent models to answer these questions. The calculations are based on a simplified representation of the lipid bilayer, as a slab of low dielectric constant embedded in the high dielectric constant of water, while alamethicin is described in atomic detail. We have recently used this model to calculate the free energy of insertion of polyalanine α-helices into lipid bilayers, and the results were in good agreement with the experimental data (Ben-Tal et al., 1996a; Ben-Shaul et al., 1996). Very recently, we also used it to calculate the permeability of monensin-cation complexes in biomembranes, and again the results were in good agreement with measurements (Ben-Tal et al., manuscript submitted for publication). Our calculations suggest that alamethicin assumes a transmembrane orientation in biomembranes and indicate that the transmembrane insertion of alamethicin into the lipid bilayer is likely to involve a slight deformation of the bilayer to match the width of the hydrocarbon region to the hydrophobic length of the peptide.

METHODS

The total free energy difference between a peptide in the membrane and in the aqueous phase (ΔG_{tot}) can be decomposed into a sum of differences of the following terms: the electrostatic (ΔG_{elc}) and nonpolar (ΔG_{np}) contributions to the solvation free energy, peptide conformation effects (ΔG_{con}), peptide immobilization effects (ΔG_{imm}), lipid perturbation effects (ΔG_{lip}), membrane deformation effects (ΔG_{def}), and effects due to changes in the pK_a of titratable residues (ΔG_{pKa}) (Engelman and Steitz, 1981; Jähnig, 1983; Honig and Hubbell, 1984; Jacobs and White, 1989; Milik and Skolnick, 1993; Fattal and Ben-Shaul, 1993; Ben-Tal et al., 1996a):

$$\Delta G_{tot} = \Delta G_{elc} + \Delta G_{np} + \Delta G_{con} + \Delta G_{imm} + \Delta G_{lip} + \Delta G_{def} + \Delta G_{pKa} \quad (1)$$

All of the experimental and theoretical studies indicate that the main contribution to the transfer free energy comes from the solvation free energy, ΔG_{solv}, defined as

$$\Delta G_{solv} = \Delta G_{elc} + \Delta G_{np} \quad (2)$$

ΔG_{solv} is the free energy of transfer of alamethicin from water to a bulk hydrocarbon phase. It accounts for electrostatic contributions resulting from changes in the solvent dielectric constant as well as for van der Waals and solvent structure effects, which are grouped in the nonpolar term and together define the classical hydrophobic effect. We calculate ΔG_{solv} by use of the continuum solvent model. The method has been described in detail in our earlier studies of the membrane association of polyalanine α-helices (Ben-Tal et al., 1996a) and monensin-cation complexes (Ben-Tal et al., manuscript submitted for publication). In the following subsections we present a brief outline, with emphasis on the minor changes we made to adapt the method to alamethicin.

Electrostatic contributions

The calculations are based on a continuum model in which electrostatic contributions are obtained from finite difference solutions to the Poisson-Boltzmann equation (the FDPB method) (Honig et al., 1993; Honig and Nicholls, 1995). Three-dimensional model structures of alamethicin (Fox and Richards, 1982) were retrieved from the Protein Data Bank (Brookhaven National Laboratory, entry no. 1AMT). Hydrogen atoms were added to the x-ray crystal structures, and the structures were energy minimized as described below. The NMR structures (Franklin et al., 1994) include hydrogen atoms and were not minimized. Alamethicin was represented in atomic detail, with atomic radii and partial charges defined at the coordinates of each nucleus. The charges and radii were taken from PARSE, a parameter set that was derived to reproduce gas phase-to-water (Sitkoff et al., 1994) and alkane-to-water (Sitkoff et al., 1996) solvation free energies of small organic molecules. We recently used it to study amide hydrogen bond formation (Ben-Tal et al., 1997), polyalanine α-helix insertion into

lipid bilayers (Ben-Tal et al., 1996a), helix-helix interactions in lipid bilayers (Ben-Tal et al., 1996b), and the permeability of monensin-cation complexes (Ben-Tal et al., manuscript submitted for publication).

In the FDPB calculations reported here, the boundary between alamethicin and the solvents (water or membrane) was set at the contact surface between the van der Waals surface of the complex and a solvent probe (defined here as having a 1.4-Å radius; Sharp et al., 1991). Alamethicin and the lipid bilayer were assigned a dielectric constant of 2, whereas water had a dielectric constant of 80. The system was mapped onto a lattice of 129^3 grid points, with a resolution of three points per Å, and the Poisson equation was numerically solved for the electrostatic potential. The electrostatic free energy was calculated by integration over the potential multiplied by the charge distribution in space.

Nonpolar contributions

The nonpolar contribution to the solvation free energy, G_{np}, was assumed to be proportional to the water-accessible surface area of alamethicin, A, as in the expression

$$G_{np} = \gamma A + b \tag{3}$$

We used the parameters $\gamma = 0.0278$ kcal/(mol Å^2) and $b = -1.71$ kcal/mol, which have been derived from the partitioning of alkanes between liquid alkane and water (Sitkoff et al., 1996) and have been successfully used in our previous studies (Ben-Tal et al., 1996a,b, 1997, and manuscript submitted for publication). The total area of alamethicin accessible to lipids in a particular configuration was calculated with a modified Shrake-Rupley (Shrake and Rupley, 1973) algorithm (Sridharan et al., 1992).

Molecule conformation effects

Experimental and theoretical studies indicate that the conformation of alamethicin is predominantly α-helical in both water and lipid bilayers. However, circular dichroism (CD) measurements suggest an increase in helix content upon membrane binding (Schwarz et al., 1986). Recent molecular dynamics simulations have demonstrated that the C-terminus is relatively flexible in water, suggesting that the transfer of alamethicin from water to the lipid bilayer may involve some conformational changes at the C-terminus (Tieleman et al., 1999a,b). Our calculations indicate that in the most favorable orientation of alamethicin in the lipid bilayer, the C-terminus of the peptide is partially excluded from the bilayer. The stability of polyalanine α-helices has been the subject of theoretical (Yang and Honig, 1995) and experimental (e.g., Wójcik et al., 1990) studies. These studies indicate that a complete helix-to-coil transition of polyalanine helix of ~10 residues involves a free energy value close to zero. By extrapolation, the free energy penalty resulting from conformational changes during the membrane association of alamethicin (ΔG_{con} in Eq. 1) should be insignificant and is thus neglected (see also the Discussion).

Because both experimental and theoretical studies indicate that alamethicin's conformation depends slightly on the environment and may change in the course of the insertion process, one may consider the minimization of the peptide structure at each step. While such an idea may seem attractive, it is risky because the available force fields were not parameterized for molecules that are in the water-membrane interface, and this exercise may yield unrealistic peptide conformations. Therefore, our approach was to use only experimentally determined structures.

Estimates of ΔG_{lip} and ΔG_{imm}

ΔG_{lip} is the free energy penalty resulting from the interference of the solute with the conformational freedom of the lipid bilayer chains, and ΔG_{imm} is the free energy penalty resulting from the confinement of the external translational and rotational motion of the solute inside the membrane. $\Delta G_{lip} = 2.3$ kcal/mol and $\Delta G_{imm} = 3.7$ kcal/mol were calculated for the insertion of polyalanine α-helices into the lipid bilayer (Ben-Tal et al., 1996a; Ben-Shaul et al., 1996), and we use these values for alamethicin, which is a helix of similar shape.

Estimates of ΔG_{def}

Insertion of a solute into a lipid bilayer may result in a deformation of the lipid bilayer to match the width of the hydrocarbon region to the hydrophobic length of the solute, following the "mattress model" (Mouritsen and Bloom, 1984). The deformation involves an energy penalty, ΔG_{def}, resulting from the compression or expansion of the lipid chains. ΔG_{def} has been calculated by several research groups using different methods, and the values are similar (e.g., Mouritsen and Bloom, 1984; Helfrich and Jakobsson, 1990; Fattal and Ben-Shaul, 1993; Ben-Shaul et al., 1996; Nielsen et al., 1998; Dan and Safran, 1998; May and Ben-Shaul, 1999). We rely on the calculations of Fattal and Ben-Shaul (1993), which are based on a statistical thermodynamic molecular model of the lipid chains.

Estimates of ΔG_{pKa}

The transmembrane insertion of a peptide may involve the unfavorable exposure of a titrateable residue to the hydrophobic region of the lipid bilayer. The high free energy penalty involved in the process may be lowered if the residue is neutralized by protonation (e.g., Honig and Hubbell, 1984). The protonation involves an energy penalty, ΔG_{pKa}, given by

$$\Delta G_{pKa} = -2.3kT(pK_a - pH) \tag{4}$$

where k is the Boltzmann constant, T is the absolute temperature, and K_a is the ionization equilibrium constant of glutamate. The pK_a and pH were assigned values of 4 and 7, respectively.

Models of alamethicin and the solvents

Alamethicin has two main isoforms, R_f30 and R_f50, that differ only in the residue at the 18th position. The sequence of R_f30 is Ac-U-P-U-A-U-A-Q-U-V-U-G-L-U-P-V-U-U-E-Q-F-OH, where Ac is acetyl, U is α-amino isobutyric acid, and F-OH is phenylalaninol. In R_f50, Glu^{18} (marked in bold) is replaced by Gln, and we refer to these isoforms as Glu^{18}-alamethicin and Gln^{18}-alamethicin, respectively.

Glu^{18}-alamethicin

The structure of Glu^{18}-alamethicin was determined by x-ray crystallography (Fox and Richards, 1982; PDB entry number 1AMT). The unit cell contains three monomers, and most of the calculations were done with monomer A of the x-ray crystal structure, because it is the most likely to partition into lipid bilayers as described below. Hydrogen atoms were added to the structure, and it was minimized using the Insight-II set of molecular modeling tools (MSI, San Diego, CA).

Gln^{18}-alamethicin

Two types of conformations for the Gln^{18}-alamethicin isoform were used. One is taken from NMR studies in sodium dodecyl sulfate (SDS) micelles (Franklin et al., 1994), and the other is based on the x-ray structure of Glu^{18}-alamethicin. The seven low-energy conformations determined from the NMR measurements were tested. However, because the calculations described below demonstrate that the peptide is unlikely to partition into the membrane in these NMR conformations, we modified the x-ray structure of Glu^{18}-alamethicin by replacing the OH group in the Glu^{18} side chain with an NH_2 group (Insight/Biopolymer), followed by minimization (Insight/Discover).

In the calculations, the peptides were described in atomic detail and were placed at different distances and orientations with respect to our model of the lipid bilayer. The bilayer was represented as a slab of ~30-Å width with a dielectric constant of 2, known from a combination of thickness and capacitance measurements (Fettiplace et al., 1971; Dilger and Benz, 1985). This is a very simplistic model of the membrane that has many limitations, as discussed by Ben-Tal et al. (1996a, and manuscript submitted for publication; see also this paper). Nevertheless, it is a standard model for the dielectric properties of the bilayer, and we use it because the experimental evidence suggests that the solvation free energy is the dominant contribution to the free energy of the system.

Our approach is based on a detailed atomic model of alamethicin and a rough slab model of the lipid bilayer, which may seem disproportional. However, this combination allows us to address thermodynamic questions that cannot be addressed using more balanced approaches. As we mentioned in the Introduction, the alternatives, i.e., either using detailed atomic models for both the peptide and the membrane (Tieleman et al., 1999a and 1999b) or using a rough model for both (Biggin et al., 1997), are inappropriate.

RESULTS

Alamethicin transfer across lipid bilayers

We calculated the free energy of transfer of alamethicin across lipid bilayers along two hypothetical pathways: vertical translocation, with the helix principal axis perpendicular to the membrane surface (Fig. 1 *A*), and horizontal translocation, with the helix principal axis parallel to the membrane surface (Fig. 1 *B*). The calculations were carried out using the Gln^{18} isoform of alamethicin, and the results are presented in Fig. 2.

Vertical translocation

The electrostatic, nonpolar, and solvation free energies as a function of the distance, h, between the geometric center of the peptide and the membrane midplane are presented in Fig. 2 *A*. The translocation starts at $h = -32$ Å, where the

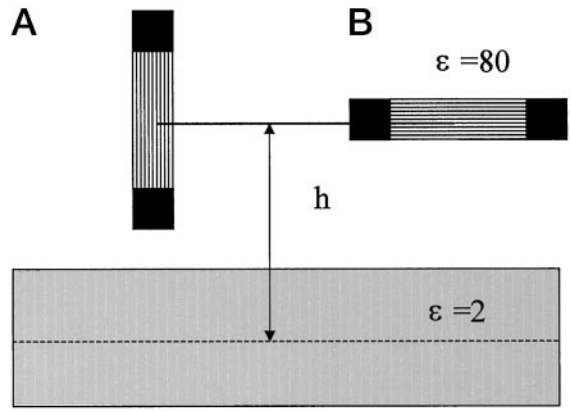

FIGURE 1 Modes of helix insertion. A schematic diagram showing two hypothetical insertion processes of alamethicin into the lipid bilayer. (*A*) A vertical insertion, in which the principal axis of the peptide is perpendicular to the membrane surface. (*B*) A horizontal insertion, in which the principal axis of the peptide is parallel to the membrane surface. Alamethicin is schematically depicted as a rectangle, with its central hydrophobic region in stripes and the polar termini in solid black. The hydrophobic core of the lipid bilayer is depicted by the shaded rectangle. The distance h is measured between the geometrical center of alamethicin and the midplane of the lipid bilayer.

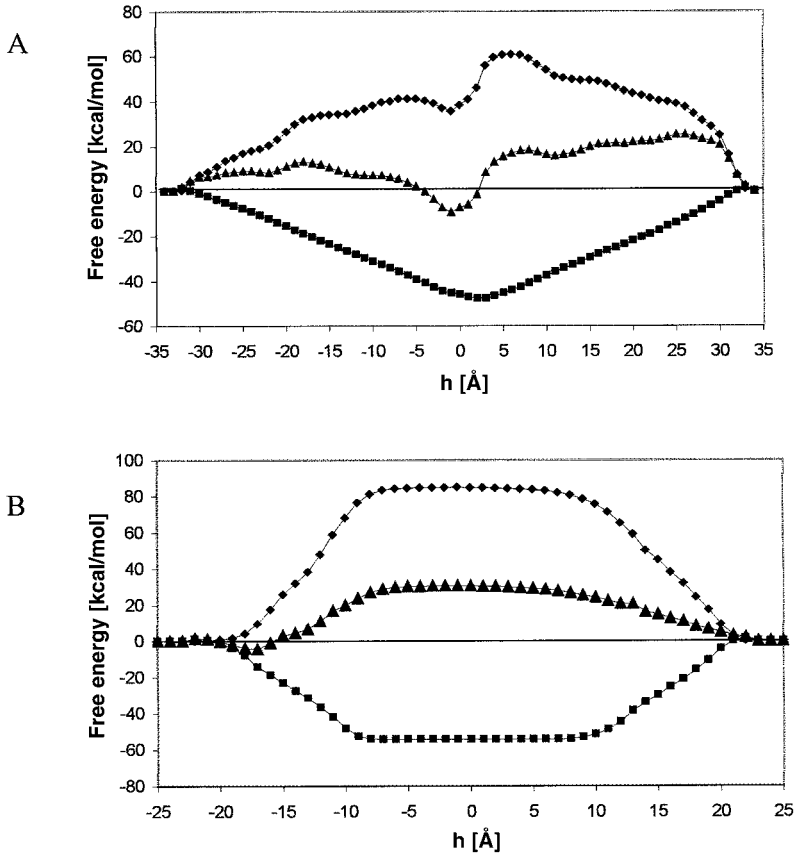

FIGURE 2 Insertion of alamethicin into a lipid bilayer in the vertical (*A*) and horizontal (*B*) orientations of Fig. 1. The electrostatic (♦), nonpolar (■), and solvation (▲) free energies of the peptide-membrane system are presented as a function of the distance *h* between the geometrical center of the helix and the membrane midplane. The zero of the free energy for each helix was chosen at $h = \infty$. The membrane width was 30 Å, and the model of Gln^{18}-alamethicin is taken as monomer A of the x-ray crystal structure. The calculations were carried out on a lattice of 129 points and a resolution of three grid points per Å as described in Methods.

peptide's N-terminus is just in contact with the lipid bilayer. At $h = 0$ the peptide is fully inserted into the bilayer, with its termini protruding evenly from both sides of the bilayer, and at $h = 33$ Å the peptide is at the other end of the membrane, with its C-terminus just in contact with the bilayer. The translocation process can be viewed as two independent insertion processes; one starts at $h = -32$ Å and involves the insertion of the N-terminus into the bilayer, and the other starts at $h = 33$ Å and involves the membrane insertion of the C-terminus. Both processes end at $h = 0$.

It is evident from the figure that the electrostatic penalty of insertion increases as the depth of insertion into the bilayer increases for both processes. However, the increase is larger for the C-terminus than for the N-terminus, and the reason for this is that the C-terminus is more polar than the N-terminus, as indicated in Fig. 3 *A*. When the helix termini begin to emerge from the far side of the bilayer (at $h = -7$ Å and $h = 8$ Å, respectively), the electrostatic free energy begins to decrease until it reaches the final value of ~36 kcal/mol. (at $h = -1$ Å).

The nonpolar contribution to the insertion free energy in each of the two processes increases in magnitude until the helix begins to emerge from the other side of the bilayer, reaching a final value of ~−46 kcal/mol. Thus the fully inserted alamethicin (Fig. 2 *A*, $h = -1$ Å) is predicted to be stabilized by $\sim 36 - 45 = -9$ kcal/mol relative to the isolated alamethicin in the aqueous phase. However, the insertion process involves a free energy barrier of ~10–20 kcal/mol, depending on the direction of insertion. This issue will be addressed further in the Discussion.

Horizontal translocation

The solvation free energy terms for the horizontal translocation of alamethicin into a lipid bilayer as a function of the distance, *h*, between the geometric center of the peptide and the membrane midplane are presented in Fig. 2 *B*. The translocation starts at $h = -20$ Å, where the relatively hydrophobic face of the helix is just in contact with the bilayer, and ends at $h = 22$ Å, where the helix is at the other end of the bilayer, with its relatively hydrophilic face just in contact with the bilayer. Again, the translocation process can be viewed as two independent insertion processes; one starts at $h = -20$ Å and involves the insertion of the hydrophobic face of the helix into the bilayer first, and the other starts at $h = 22$ Å and involves insertion of the hydrophilic face of the helix first. Both processes end at $h = 0$.

The electrostatic penalty for horizontal insertion is much greater than for vertical insertion because, in the former case, the two termini are inserted simultaneously and never

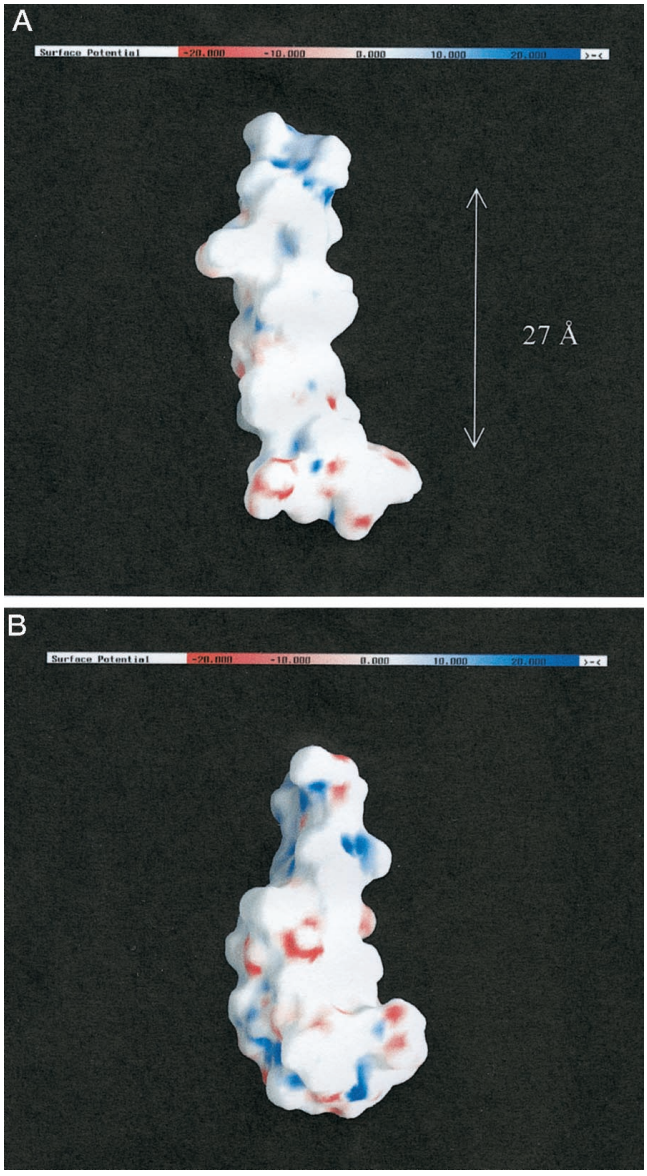

FIGURE 3 Surface electrostatic potential of alamethicin in two experimentally observed conformations. (*A*) The conformation found in the crystal structure and (*B*) a characteristic NMR conformation. The electrostatic potential (ϕ), calculated using DelPhi (Nicholls and Honig, 1991), is color-coded and displayed on the molecular surface with GRASP (Nicholls et al. 1991). Negative potentials ($0kT/e > \phi > -20kT/e$) are red, positive potentials ($0kT/e < \phi < 20kT/e$) are blue, and neutral potentials are white. The peptides are shown with their N-termini pointing up and their more polar C-termini pointing down. The arrow in *A* indicates the length of the hydrophobic core of the peptide. *A* and *B* are drawn using the same scale, and it is obvious that the *A* conformation is more elongated than *B*.

emerge from the bilayer. The nonpolar contributions are insufficient to fully balance the electrostatic penalty, and thus fully horizontal insertion is never observed. Nevertheless, the results indicate a solvation free energy minimum of about -8 kcal/mol when alamethicin is adsorbed at the water-bilayer interface with its hydrophobic face dissolved in the bilayer and its hydrophilic face in water (Fig. 2 *B*, $h = -17$ Å).

The conformation of membrane-associated alamethicin

Two different helical conformations of Gln^{18}-alamethicin have been experimentally determined: 1) conformations from x-ray diffraction in methanol (Fox and Richards, 1982) and 2) low-energy conformations observed using ^{1}H-NMR spectroscopy in SDS micelles (Franklin at al., 1994). We calculated the free energy of membrane-association of alamethicin in each of these conformations to find the most likely conformation in the bilayer.

The solvation free energy values for membrane association of the peptide in the x-ray crystal conformation (of monomer A) in transmembrane and surface orientations are presented in Fig. 2 *A* ($h = -1$ Å) and Fig. 2 *B* ($h = -17$ Å). The corresponding total free energy values (Eq. 1) are -5.3 kcal/mol and -4.2 kcal/mol, respectively. These values indicate that both the transmembrane and surface associations of the conformation are energetically favorable. Conversely, the membrane association of the peptide in each of the low-energy NMR conformations in transmembrane and surface orientations result in highly positive free energy values ($\sim +30$ kcal/mol and $\sim +10$ kcal/mol, respectively), indicating that alamethicin is unlikely to associate with lipid bilayers in any of these conformations. Our calculations show that the difference between the x-ray and NMR conformations arises mainly from the difference in the electrostatic contribution to the total free energy, suggesting that the polar groups are more exposed to the surrounding medium in the NMR conformation than in the x-ray conformation.

We determined the degree of polar group exposure in the x-ray and NMR conformations by calculating their surface electrostatic potentials. The graphic representation of these calculations, in Fig. 3, confirms the suggestion that the difference in transfer free energy between the x-ray and NMR conformations results from the difference in polar group exposure between the two conformations. In the x-ray conformations, the backbone polar groups of the central region of the peptide are paired in hydrogen bonds, and the polar regions are almost exclusively at the peptide termini, mainly the C-terminus. The peptide is long enough to span the entire length of the hydrocarbon region of the bilayer, while its polar regions are exposed to the water-membrane interface. In contrast, the NMR conformations are shorter and have numerous unsatisfied backbone hydrogen bonds along the entire length of the peptide. The peptide-membrane association involves the transfer of at least some of the unsatisfied hydrogen bonds of the peptide from the aqueous phase into the low-dielectric hydrocarbon region of the bilayer, which is energetically unfavorable.

We calculated the surface electrostatic potentials of the three alamethicin monomers of the x-ray structure. The

results indicate that monomer A is the most hydrophobic of them, and because probing calculations indicate that it is the most likely to partition into bilayers, we used it throughout this study.

The orientation of alamethicin in lipid bilayers

The results above indicate that alamethicin may partition into lipid bilayers in transmembrane and surface orientations. We sampled peptide-membrane configurations around each of these orientations to find the ones with the most negative solvation free energy. We carried out the calculations for the Gln^{18} and Glu^{18} isoforms of alamethicin; the total free energy values of the most favorable orientations are presented in Table 1. They demonstrate that the transmembrane orientation is energetically more favorable than the surface orientation for both isoforms.

The effect of alamethicin on the membrane curvature

The hydrophobic region of alamethicin is ~3 Å shorter than the width of the hydrocarbon region of the lipid bilayer (Fig. 3 *A*). Thus, a transmembrane orientation of the peptide may involve membrane deformation, following the "mattress model" mentioned above. To explore this possibility we calculated the solvation component to the free energy of transfer of alamethicin from the aqueous phase into lipid bilayers of different widths, in transmembrane orientations, and added estimates of ΔG_{def}, ΔG_{lip}, and ΔG_{imm} (as described in Methods) to get the total free energy (ΔG_{tot}). Again, we sampled configurations around the transmembrane orientation and report the values obtained for the orientation with the most negative ΔG_{tot} in each case. Table 2 shows the different free energy contributions to ΔG_{tot} of insertion of the Glu^{18}-alamethicin isoform into bilayers. The results show that the most negative total free energy value is observed when the membrane is 2 Å shorter than its native width of 30 Å, because of the decrease in ΔG_{solv}.

We repeated the calculations for the Gln^{18} isoform as well, and the calculated ΔG_{tot} values for the two isoforms are presented in Table 3. Notice that the most probable width of the hydrocarbon region of the membrane is 28 Å for both isoforms and that their free energy values are similar, suggesting that residue 18 protrudes into the aqueous phase. This issue will be studied further in the following subsection.

The average width of the lipid bilayer in the vicinity of alamethicin can be calculated from the distribution in Table 3, using the definition

$$d_{(ave)} = \sum_i (d_i\, e^{-\Delta G_i/kT}) / \sum_i (e^{-\Delta G_i/kT}) \qquad (5)$$

where ΔG_i is the total free energy of the peptide in its most negative transmembrane orientation in a bilayer of width d_i. The calculated values of the average width of the lipid bilayer for the insertion of the Gln^{18} and Glu^{18} isoforms are 27.7 Å and 27.6 Å, respectively. These values indicate that the transmembrane insertion of alamethicin in both isoforms into a lipid bilayer of native hydrocarbon region of 30 Å is likely to involve a ~2-Å deformation of the bilayer to match the hydrophobic length of the peptide (Fig. 3 *A*).

An important corollary of the calculations of Table 3 is the most likely configuration of the peptide-bilayer system, which is presented in Fig. 4 for the Gln^{18} isoform. The peptide protrudes ~4 Å into the water-bilayer interface, in perfect agreement with the EPR studies of Barranger-Mathys and Cafiso (1996).

The effect of transmembrane insertion of alamethicin on the pK_a of Glu^{18}

The Glu^{18} residue of the R_f30 isoform of alamethicin is the only titratable residue in the peptide, and it is negatively charged at neutral pH. Charge transfer from the aqueous phase into low dielectric regions is energetically costly, and Glu^{18} is likely to be protonated if it is buried in the membrane while the peptide is in a transmembrane orientation (e.g., Honig and Hubbell, 1984). If this is the case, membrane association of the R_f30 isoform should involve a significant and experimentally detectable pK_a shift of Glu^{18}. Table 4 shows the calculated free energy values of the transmembrane insertion of alamethicin in its natural and protonated forms into the lipid bilayer. It is evident that while the protonation of alamethicin lowers ΔG_{solv}, the total free energy value for the insertion of the protonated peptide is higher (i.e., less negative) in comparison with the corresponding value for the deprotonated peptide, because of the free energy penalty of protonation. Some of the free energy penalty can be avoided if the insertion of alamethicin into the membrane does not involve the transfer of the Glu^{18} side chain into the hydrophobic core of the bilayer. As demonstrated in the previous subsection, the transmembrane insertion of alamethicin is likely to result in a deformation of the lipid bilayer, causing a local thinning of the bilayer. In this configuration, the Glu^{18} side chain of the transmembrane peptide is likely to be exposed to the water-bilayer interface. If this is the case, the free energy of transfer of alamethicin

TABLE 1 Total free energy values (Eq. 1) for the transmembrane and surface orientations of alamethicin in a lipid bilayer with a hydrocarbon region of 30 Å

Orientation*	Gln^{18}-alamethicin† (kcal/mol)	Glu^{18}-alamethicin‡ (kcal/mol)
Transmembrane	−5.3	−3.6
Surface	−4.2	−3.0

*The orientation of the bilayer-associated peptide.
†The alamethicin isoform containing Gln at position 18.
‡The alamethicin isoform containing Glu at position 18.

TABLE 2 Effects of insertion of Glu[18]-alamethicin into a lipid bilayer on the membrane curvature

Membrane width* (Å)	ΔG_{solv}† (kcal/mol)	ΔG_{imm}‡ (kcal/mol)	ΔG_{lip}§ (kcal/mol)	ΔG_{def}¶ (kcal/mol)	ΔG_{tot}‖ (kcal/mol)
30	−9.6	3.7	2.3	0.0	−3.6
29	−9.9	3.7	2.3	0.0	−3.9
28	**−11.0**	**3.7**	**2.3**	**0.1**	**−4.8**
27	−10.9	3.7	2.3	0.2	−4.5
26	−10.0	3.7	2.3	0.4	−3.4

The free energy of insertion of alamethicin into lipid bilayers of different widths was calculated, and the corresponding elastic free energy of deformation, ΔG_{def}, was added to approximate insertion into a deformed membrane.
*The width of the hydrophobic core of the bilayer.
†The solvation free energy (Eq. 2).
‡The peptide immobilization free energy.
§The lipid perturbation free energy.
¶The membrane deformation free energy.
‖The total free energy (Eq. 1).
The values obtained for the most probable configuration are marked in bold characters.

into the deformed bilayer should be almost independent of the charge on the Glu[18] side chain. We tested this possibility by arbitrarily setting the partial atom charges of the Glu[18] side chain to zero and comparing the free energy value obtained for the modified and unmodified peptide, at the most favorable orientation of alamethicin in the deformed bilayer. For comparison, we repeated the calculations with the native bilayer as well; the results are presented in Table 5.

It is evident from Table 5 that the charge neutralization of the Glu[18] side chain of the peptide has little effect on the free energy of its insertion into the deformed lipid bilayer. In contrast, the free energy of insertion of the peptide into bilayers of native width decreases significantly when the charge on the Glu[18] side chain is neutralized. These results indicate that the Glu[18] side chain is mostly excluded from the hydrocarbon region of the bilayer at the most favorable orientation in the deformed bilayer (Fig. 4), while in bilayers of native width it is partially dissolved in the lipid medium.

The implication of these calculations is that the transfer of alamethicin into biomembranes probably does not involve a significant shift in the pK_a of the Glu[18] side chain, i.e., its pK_a should be ~4, unless it is affected by the polar headgroups of the lipids. For stearic acid, for example, the interaction with the polar headgroups causes an upward shift of the pK_a toward neutral pH (Esmann and Marsh, 1985; Horváth et al., 1988).

Convergence tests and error estimate

We repeated the calculations in Table 1, for the Gln[18] isoform of alamethicin, using different grid sizes (129^3,

TABLE 3 Total free energy values of the transmembrane insertion of alamethicin into native and deformed lipid bilayers

Membrane width* (Å)	ΔG_{tot}† (kcal/mol)	
	Gln[18]-alamethicin‡	Glu[18]-alamethicin§
30	−5.3	−3.6
29	−4.9	−3.9
28	**−5.5**	**−4.8**
27	−5.3	−4.5
26	−5.3	−3.4
Average width¶ (Å)	27.7	27.6

The free energy was calculated as demonstrated in Table 2, and the results for the Glu[18] isoform are taken directly from that table.
*The width of the hydrophobic core of the bilayer.
†The total free energy (Eq. 1).
‡Alamethicin containing Gln at position 18.
§Alamethicin containing Glu at position 18.
¶The average bilayer width calculated using Eq. 4.
The values obtained for the most probable configuration are marked in bold characters.

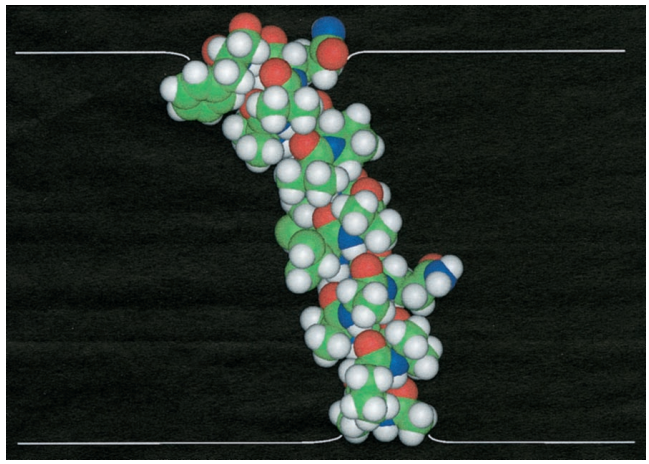

FIGURE 4 Schematic representation of the orientation of alamethicin in the 2-Å deformed bilayer. The space-filling model of the peptide is displayed with INSIGHT (Molecular Simulations, San Diego, CA). Carbon atoms are in green, hydrogen atoms are in white, oxygen atoms are in red, and nitrogen atoms are in blue. The two white lines represent the boundaries of the hydrocarbon region of the lipid bilayer.

TABLE 4 Free energy values for the transmembrane insertion of protonated and deprotonated alamethicin into a 30-Å lipid bilayer

Protonation state of Glu^{18}*	ΔG_{solv}† (kcal/mol)	ΔG_{imm}‡ (kcal/mol)	ΔG_{lip}§ (kcal/mol)	ΔG_{pKa}¶ (kcal/mol)	ΔG_{tot}‖ (kcal/mol)
Deprotonated	−9.6	3.7	2.3	0.0	−3.6
Protonated	−11.8	3.7	2.3	4.0	−1.8

*The protonation state of the Glu^{18} side chain.
†The solvation free energy (Eq. 2).
‡The peptide immobilization free energy.
§The lipid perturbation free energy.
¶The residue protonation free energy (Eq. 4).
‖The total free energy, i.e., the sum of the free energy terms of the previous columns.

161^3, and 209^3) and scales (three, four, and five grid points per Å) to test the convergence of our calculations. Our results show that the ΔG_{elc} calculations are converged to less than 0.2 kcal/mol, and because the error in ΔG_{np} is in essence zero, the error in ΔG_{sol} should be ~0.2 kcal/mol. However, the high precision of our calculations is due to the simplified model we use; the neglect of the polar headgroup region of the bilayer and the fixed conformation of alamethicin in our model may result in an error of ~1 kcal/mol in the absolute value of ΔG_{tot}, as discussed below.

While it is challenging to calculate the absolute value of ΔG_{tot}, the ability to calculate the relative value, e.g., the difference in ΔG_{tot} between two alamethicin-bilayer configurations, is sufficient for this study. The contributions of ΔG_{imm} and ΔG_{lip} as well as the contributions of the free energy terms that we neglected are likely to be more or less the same for each configuration, and the accuracy in the calculation of changes in ΔG_{tot} is probably ~0.2 kcal/mol. Thus, we feel safe to use the model even to investigate small changes such as these caused by membrane deformation.

DISCUSSION

We begin by discussing a number of approximations used in the study. The description of the lipid bilayer as a slab of low dielectric constant obscures all atomic details of alamethicin-bilayer interactions. However, as discussed in our previous work (Ben-Tal et al., 1996a, and manuscript submitted for publication) and in publications from other groups (e.g., Biggin et at., 1997; Bernèche et al., 1998), the slab model is the standard representation of the hydrocarbon region of lipid bilayers, and it is likely to provide a reasonable model for bilayer effects on electrostatic interactions. The greatest uncertainty in the model results from its complete neglect of the polar headgroup region, which is presumably the site of alamethicin adsorption to the bilayer. Because the dielectric constant in this region is estimated to be between 25 and 40 (Ashcroft et al., 1981), the polar headgroup region might be regarded, most appropriately, as part of the aqueous phase defined in this study. Still, the model does not take into account specific interactions between the polar groups of alamethicin and the headgroups of the lipid bilayer. We believe that these interactions are of secondary importance, and indeed, even a drastic change in the nature of all of the polar headgroups from phosphatidylcholine (PC) to phosphatidylserine (PS) gave an increase of only 1 kcal/mol in the binding of alamethicin to bilayers (Cafiso, unpublished observations).

TABLE 5 Free energy values for the insertion of charged and uncharged Glu^{18}-alamethicin into native and deformed lipid bilayers

Membrane width* (Å)	Glu^{18} side chain†	ΔG_{solv}‡ (kcal/mol)
30	Charged	−9.6
	Uncharged	−12.4
28	Charged	−11.0
	Uncharged	−12.0

The free energy of insertion of alamethicin into lipid bilayers of different widths was calculated, and the corresponding free energy of deformation (ΔG_{def}) was added to approximate insertion into a deformed membrane.
*The width of the hydrophobic core of the bilayer.
†The charge state of Glu^{18} side chain.
‡The solvation free energy.

The calculated solvation free energy values depend strongly on the value assigned to the inner dielectric constant and on the choice of the set of atomic partial charges and radii. However, PARSE yields accurate transfer free energies between water and liquid alkane for small organic molecules containing the amino acid backbone and side chains (Sitkoff et al., 1996). Therefore, it seems reasonable to assume that it provides a good approximation to the water-membrane solvation properties of peptides, such as alamethicin, that are constructed from the same chemical groups. Moreover, the nonpolar surface tension coefficient used in PARSE, which is deduced from the partitioning of nonpolar molecules between water and liquid alkane, is nearly identical to that reported recently for the transfer of nonpolar molecules into lipid bilayers (Buser et al., 1994; Thorgeirsson et al., 1996). Finally, the success of the model in reproducing experimental data of several biological systems (e.g., Ben-Tal et al., 1996a, and manuscript submitted

for publication; see also this paper) indicates its strength in cases where solvation effects dominate the energetics.

Another uncertainty in the model results from its neglect of conformational changes in alamethicin during its membrane association (ΔG_{con} in Eq. 1). As mentioned above, alamethicin has been found to adopt a predominantly α-helical conformation in methanol (Fox and Richards, 1982; Banerjee and Chan, 1983; Esposito et al., 1987; Yee and O'Neil, 1992), and the helical conformation of the N-terminal segment of the peptide is maintained in lipid bilayers (North et al., 1995). CD measurements of alamethicin in water and in dioleoylphosphatidylcholine (DOPC) suggest an increase in helix content upon membrane binding (Schwarz et al., 1986). Tieleman et al. (1999a,b) have recently carried out nanosecond molecular dynamics simulations to investigate the conformational stability of alamethicin in water, methanol, and a palmitoyloleoylphosphatidylcholine (POPC) bilayer. Their results indicate that the peptide is α-helical in methanol and bilayers. According to their study, the behavior of alamethicin in water is more complex. While most of the peptide is in an α-helical conformation, the C-terminal segment, comprising less than half of the peptide, undergoes substantial conformational changes. This suggests that the transfer of alamethicin from water to the lipid bilayer may be accompanied by significant conformational changes in the C-terminus of the peptide, with a resulting free energy penalty. However, our calculations indicate that in the most favorable orientation of alamethicin in the membrane, the C-terminus of the peptide, is at least partially excluded from the hydrophobic core of the bilayer. The free energy penalty resulting from conformational changes during the membrane association of alamethicin should, therefore, be insignificant.

SDS micelles are considered to be a membrane-like milieu, because of the similarity of some of their properties to those of lipid bilayers. Indeed, previous reports indicate that the structures of some peptides determined in SDS micelles are very similar to the structures in oriented lipid bilayers (e.g., Gesell et al., 1997; Bechinger et al., 1998). Thus, it is expected that the conformation of alamethicin in lipid bilayers will resemble one of the lowest energy conformations of the NMR structure, determined in SDS micelles (Franklin et al., 1994). Our results indicate that this is not the case. The NMR conformations are too irregular; they have many unsatisfied backbone hydrogen bonds, and their insertion into lipid bilayers involves a very large electrostatic solvation free energy penalty. There are two likely reasons why the NMR structure contains a number of unsatisfied hydrogen bonds. First, a large number of the Aib (MeA) residues are highly overlapped in the NMR spectrum, and fewer restraints are available than one would normally have for a 20-residue peptide. Thus the structure may be underdetermined. Second, in SDS, the peptide appears to fluctuate between linear and bent structures, each with a different hydrogen bond pattern (Franklin et al., 1994). The exchange of hydrogen bonds is highly unlikely when the peptide is membrane bound, and indeed, only the linear form can be observed when the peptide is membrane bound (Barranger-Mathys and Cafiso, 1996). Thus, in this particular case, SDS may not be a good mimic for the membrane, presumably because it allows the solvent more access to the helix. In contrast, the x-ray conformations have enough satisfied hydrogen bonds to partition into bilayers. Our calculations indicate that of all of the experimentally observed conformations of alamethicin, monomer A of the crystal structure is the most likely to be in lipid bilayers.

The published experimental and theoretical studies indicate that alamethicin is in transmembrane or surface orientation in lipid bilayers, and indeed these orientations were found to be lower in free energy than isolated alamethicin in the aqueous phase (Fig. 2 *A*, $h = -1$ Å and Fig. 2 *B*, $h = -17$ Å). We sampled numerous alamethicin-bilayer configurations around each of these orientations and found that the water-membrane-partition free energies of the Gln^{18} isoform are -5.5 kcal/mol for the transmembrane and -4.2 kcal/mol for the surface configurations. Similarly, we found that the corresponding values for the Glu^{18} isoform are -4.8 kcal/mol and -3 kcal/mol, respectively. Our calculations indicate conclusively that the transmembrane configuration is preferred over the surface configuration for both isoforms, in contrast with the simulations of Sansom and co-workers (Biggin et al., 1997), but in agreement with the vast majority of experimental data (Huang and Wu, 1991; North et al., 1995; Barranger-Mathys and Cafiso, 1996; Fringeli and Fringeli, 1979; Knoll, 1986; Latorre et al., 1981). Nevertheless, the small free energy difference of only 1.5–2 kcal/mol between the transmembrane and surface orientations suggests that an experimentally detected fraction of the alamethicin population is in the surface orientation. This population may be responsible for the observations of Banerjee et al. (1985).

Stankowski and Schwarz (1989) have measured a free energy value of ~ -4 kcal/mol for the transfer of Gln^{18}-alamethicin from the aqueous phase into DOPC bilayers, using CD spectroscopy. A more negative value of ~ -6 kcal/mol has recently been measured by us, using EPR spectroscopy for the same system (Lewis and Cafiso, 1999). The two studies indicate that the peptide is in a transmembrane orientation, and the source of the free energy difference between the two measurements is unknown. We calculated the free energy of insertion of alamethicin into a bilayer of native width of 27 Å, which is the width of the hydrophobic core of DOPC bilayers (Lewis and Engelman, 1983b; Wiener and White, 1992), to facilitate a direct comparison of the model with measurements (data not shown). The calculated free energy value of -5.7 kcal/mol is in nearly perfect agreement with the EPR measurements.

Fig. 3 *A* shows that the hydrophobic length of alamethicin is a little shorter than the native width of the hydrocarbon region of biomembranes (i.e., 30 Å), suggesting that the

transmembrane configuration of alamethicin may involve membrane deformation to match the hydrophobic region of the peptide. Our calculations demonstrate that this is indeed the case; the most favorable configuration of each of the two isoforms of alamethicin in lipid bilayers, shown in Fig. 4 for the Gln^{18} isoform, involves a 2-Å distortion of the membrane. The deformation facilitates the exclusion of the Glu^{18}/Gln^{18} side chain from the hydrocarbon region of the bilayer, and the results are in agreement with all of the available experimental data. It is in accord with the findings of Wu et al. (1995) and He et al. (1996) that a local thinning of the lipid bilayer may facilitate the transmembrane insertion of alamethicin. Similarly, it is in agreement with the observations of Lewis and Cafiso (1999) that the binding free energy of alamethicin to membranes is linearly dependent upon the membrane curvature.

The most favorable orientations of the two alamethicin isoforms in lipid bilayers are very similar, and each of them protrudes ~4 Å into the water-membrane interface, again in agreement with the EPR measurements of Barranger-Mathys and Cafiso (1996). However, the most favorable orientation of the peptide in our calculations (Fig. 4) is somewhat more tilted than the one inferred from these measurements (e.g., figure 5D of Barranger-Mathys and Cafiso (1996)).

Biological implications

Alamethicin is produced by fungi, and a key question is, how does the fungus protect itself from the toxic activity of alamethicin? A possible explanation is that the fungus possesses protective protein machinery, such as the bacterial ABC transport system, which renders bacteria immune to nisin, subtilin, and epidermin by inhibiting pore formation in the cytoplasmic membrane (Saris et al., 1996). The transmembrane insertion of alamethicin is accompanied by membrane deformation, which results in a free energy penalty. An alternative explanation for the relative immunity of the fungus to alamethicin is that its plasma membrane is wider than the bacterial membrane. The deformation of the fungal membrane will result, in this case, in a free energy penalty too great to be overcompensated for by the favorable nonpolar interactions between the peptide and the bilayer, and the peptide will not be inserted.

One of the suggestions for the voltage-gating mechanism of alamethicin channels is that the voltage controls the orientation of alamethicin in the bilayer, i.e., that alamethicin is predominantly in surface orientation before the application of the voltage and that the voltage causes the transmembrane orientation to dominate (Baumann and Mueller, 1974). The effect of the membrane potential was not taken into account in our study, and yet our calculations indicate that the transmembrane configuration of alamethicin is more likely than the surface configuration by a factor of 10–20. We therefore conclude that this is probably not the mechanism (Barranger-Mathys and Cafiso, 1996). Notice, however, that our calculations are for membranes of native hydrophobic width of 30 Å and that the surface orientation may be the most favorable in wider membranes.

An analysis of hydrogen-bonding interactions, observed in molecular dynamics simulations, has revealed that the polar C-terminus of alamethicin provides an "anchor" to the bilayer/water interface via formation of multiple hydrogen bonds (Tieleman et al., 1999b). The main conclusion from the study has been that the most likely mode of helix insertion into bilayers is via the N-terminus, which is believed to be the reason for the asymmetry of voltage activation of alamethicin channels. The polarity asymmetry between the C- and N-termini of alamethicin is evident in Fig. 3 *A*, and its effect on the preferred mode of insertion is manifested in Fig. 2 *A*. Membrane insertion via the N-terminus involves a free energy barrier of only ~10 kcal/mol and is, therefore, much more likely than insertion via the C-terminus, which involves a barrier about twice as high.

Evolutionary aspects

As mentioned above, the central hydrophobic region of alamethicin is shorter than the width of the hydrocarbon region of the lipid bilayer, and the transmembrane insertion of the peptide involves membrane deformation, resulting in a free energy penalty. The central hydrophobic region of alamethicin is confined by the polar N-terminus and by Gln/Glu^{18} in the C-terminus of the peptide. The addition of two hydrophobic residues to the hydrophobic segment (residues 1–17), or the replacement of Glu/Gln^{18} and Gln^{19} by hydrophobic residues could have improved the hydrophobic match between alamethicin and the lipid bilayer and thus further stabilized the monomer in the bilayer. Yet, the length of the central hydrophobic region of alamethicin is conserved throughout evolution, suggesting an advantage for a hydrophobic mismatch between alamethicin and bilayers. The formation of the ion channel results from aggregation of the alamethicin monomers. The aggregation reduces the peptide-bilayer interactions, and the deformation of the membrane should, therefore, also decrease. Thus, a peptide such as alamethicin, which is hydrophobically mismatched with the lipid bilayer, is likely to aggregate and form ion channels to reduce its unfavorable interactions with the lipid bilayer. This hypothesis is supported by studies that demonstrate a stabilization of the multimeric channel (Keller et al., 1993) and a decrease in the membrane binding of the monomer (Lewis and Cafiso, 1999) in membranes with increased phosphatidylethanolamine (PE) concentrations (i.e., membrane with increased negative curvature stress). The involvement of the hydrophobic mismatch in protein aggregation has also been found in the case of bacteriorhodopsin, by the use of electron microscopy

(Lewis and Engelman, 1983a), which suggests a general pattern.

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