Calculations Suggest a Pathway for the Transverse Diffusion of a Hydrophobic Peptide Across a Lipid Bilayer

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ABSTRACT Alamethicin is a hydrophobic antibiotic peptide 20 amino acids in length. It is predominantly helical and partitions into lipid bilayers mostly in transmembrane orientations. The rate of the peptide transverse diffusion (flip-flop) in palmitoyl-oleyl-phosphatidylcholine vesicles has been measured recently and the results suggest that it involves an energy barrier, presumably due to the free energy of transfer of the peptide termini across the bilayer. We used continuum-solvent model calculations, the known x-ray crystal structure of alamethicin and a simplified representation of the lipid bilayer as a slab of low dielectric constant to calculate the flip-flop rate. We assumed that the lipids adjust rapidly to each configuration of alamethicin in the bilayer because their motions are significantly faster than the average peptide flip-flop time. Thus, we considered the process as a sequence of discrete peptide-membrane configurations, representing critical steps in the diffusion, and estimated the transmembrane flip-flop rate from the calculated free energy of the system in each configuration. Our calculations indicate that the simplest possible pathway, i.e., the rotation of the helix around the bilayer midplane, involving the simultaneous burial of the two termini in the membrane, is energetically unfavorable. The most plausible alternative is a two-step process, comprised of a rotation of alamethicin around its C-terminus residue from the initial transmembrane orientation to a surface orientation, followed by a rotation around the N-terminus residue from the surface to the final reversed transmembrane orientation. This process involves the burial of one terminus at a time and is much more likely than the rotation of the helix around the bilayer midplane. Our calculations give flip-flop rates of $\sim 10^{-7}$/s for this pathway, in accord with the measured value of 1.7×10^{-6}/s.

INTRODUCTION

Alamethicin, an antibiotic peptide 20 amino acid residues in length, produced by the fungus *Trichoderma viride*, is one of the best studied models for peptide-membrane interactions (Cafiso, 1994). The sequence of alamethicin, Ac-UPUAUAQUVUGLUPVUUQQF-OH (where Ac is acetyl; U is α-amino isobutyric acid, and F-OH is phenylalaninol), reveals its hydrophobic nature, and structural studies indicate that it is predominantly α-helical both in solution (Fox and Richards, 1982; Banerjee and Chan, 1983; Esposito et al., 1987; Yee and O'Neil, 1992) and in bilayers (North et al., 1995; Schwarz et al., 1986).

The slightly amphipathic nature of alamethicin suggests that the peptide should be adsorbed onto lipid bilayers in a surface orientation (Fig. 1 *A*, state *c*). However, experimental (Barranger-Mathys and Cafiso, 1996; North et al., 1995; Huang and Wu, 1991; Lewis and Cafiso, 1999) and computational (Kessel et al., 2000) studies indicate that while surface orientations may be accessible to alamethicin, the peptide has predominantly transmembrane orientations (Fig. 1 *A*, states *a* and *e*).

Using NMR spectroscopy, Cafiso and his co-workers have recently studied the transverse diffusion of alamethicin between the two opposite transmembrane orientations shown in Fig. 1 *A* (states *a* and *e*), i.e., a flip-flop motion, across palmitoyl-oleyl-phosphatidylcholine (POPC) vesicles (Jayasinghe et al., 1998). The flip-flop rate was found to be 1.7×10^{-6}/s, much lower than the rate of a diffusion-controlled process, indicating the existence of an energy barrier. Alamethicin usually assumes a transmembrane orientation, with its N-terminus partially buried in the bilayer hydrocarbon region and with the polar C-terminus exposed to the aqueous solution (Barranger-Mathys and Cafiso, 1996; Kessel et al., 2000). Therefore, it was reasoned that the free energy barrier for the flip-flop of alamethicin across the bilayer should be dominated by the free energy penalty of insertion of the C-terminus of the peptide into the bilayer hydrocarbon. An analysis of hydrogen-bonding interactions, observed in molecular dynamics simulations, further supports this hypothesis: the polar C-terminus of alamethicin is anchored to the bilayer/water interface via formation of multiple hydrogen bonds (Tieleman et al., 1999b).

The flip-flop rate study of Cafiso and his co-workers is particularly intriguing because they used the Goldman–Engelman–Steitz hydropathy scale (Engelman et al., 1986) and estimated the free energy of insertion of the C-terminus into the lipid bilayer to be about half the experimentally derived value, i.e., they found many orders of magnitude difference between the theoretical and measured flip-flop rate constants (Jayasinghe et al., 1998). The purpose of the current work is to give a more exact theoretical analysis of the diffusion process.

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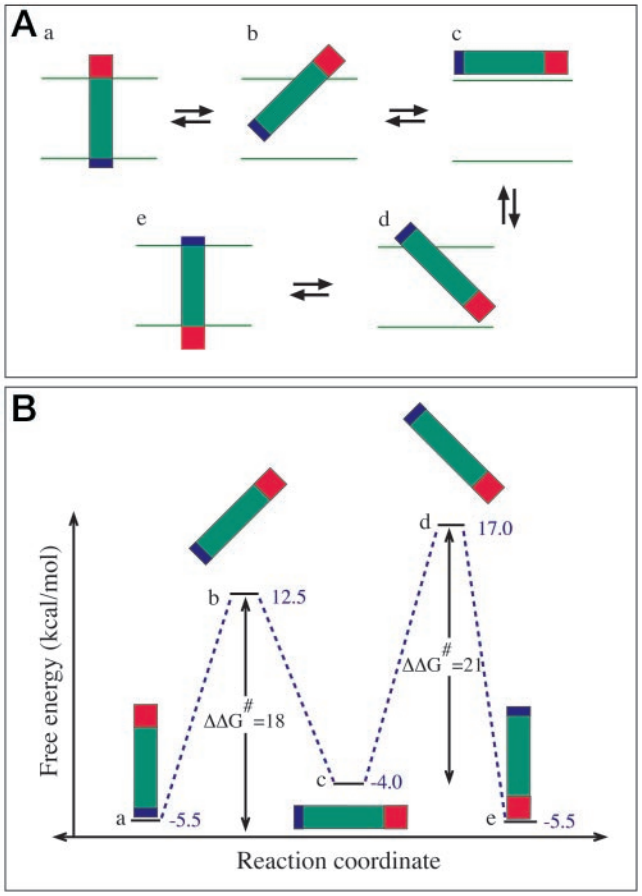

FIGURE 1 (*A*) A schematic representation of the two suggested paths for alamethicin flip-flop. Alamethicin is schematically depicted as a rectangle. The central hydrophobic region of the peptide is in green. The highly polar C-terminus of the peptide is solid red and the less polar N-terminus is solid blue. The borders of the hydrophobic core of the lipid bilayer are marked by the two horizontal lines. The two paths differ from one another in the order of occurrence of the configurations: $a \rightarrow e$ versus $e \rightarrow a$. Both paths involve the following configurations, each of which may be obtained from the previous one by rotation: (*a*) The initial (or final) transmembrane orientation of the peptide in the bilayer, with the C-terminus facing upwards and the N-terminus downwards. (Local membrane-thinning effects, described previously (Kessel et al., 2000), were included in the calculations, but are omitted from the picture for clarity.) (*b*) An intermediate configuration, in which the N-terminus of the peptide is buried in the lipid bilayer, while the C-terminus remains at the water-bilayer interface. In this orientation, the long axis of the peptide (N- to C-terminus) is tilted ~45° with respect to the normal to the bilayer plane. The path between configurations *a* and *b* involves rotation around the C-terminus residue. (*c*) An intermediate configuration, in which the peptide is adsorbed onto the surface of the lipid bilayer. The path between configurations *b* and *c* involves further rotation around the C-terminus residue. (*d*) An intermediate configuration, in which the C-terminus of the peptide is buried in the lipid bilayer, while the N-terminus remains at the water-bilayer interface. This orientation is the reciprocal of the orientation described in *b*; the long axis of the peptide (N- to C-terminus) is tilted ~135° with respect to the bilayer normal. The path between configurations *c* and *d* involves rotation around the N-terminus residue. (*e*) The final (or initial) transmembrane orientation of the peptide in the bilayer, with the C-terminus facing downwards and the N-terminus upwards. This transmembrane orientation is the reciprocal of orientation *a*. The path between configurations *d* and *e* involves further rotation around the N-terminus residue. (*B*) The calculated free energy values of the alamethicin-membrane system in different orientations of alamethicin along the two suggested transmembrane diffusion paths. Alamethicin is schematically depicted as in *A*. The intermediate states are marked from *a* to *e*, corresponding to the annotations in *A*, and the free energy value of each is written in blue. The free energy values associated with configurations *a*, *c*, and *e* were taken from Kessel et al. (2000) and the values associated with configurations *b* and *d* are reported in this study. The values of the free energy barriers of the most probable path, from *a* to *e* are marked in black. (See text for details.)

Very recently we studied the energetics of alamethicin-bilayer interactions using a continuum solvent approach (Kessel et al., 2000). In the present study we used the model to calculate the free energy of the alamethicin-membrane system at different configurations in a search for the most probable path for transmembrane flip-flop of the peptide and to estimate the free energy barrier of the process. The obvious flip-flop path involves the rotation of the peptide around its center of mass, which coincides approximately with the bilayer midplane. However, our calculations have shown that this process is characterized by a very high free energy barrier (~30 kcal/mol), resulting from burying the two termini in the bilayer simultaneously (Fig. 2 *B* of Kessel et al., 2000). Here we consider an alternative pathway, presented in Fig. 1 *A*, in which the flip-flop involves the sequential rather than simultaneous immersion of the polar termini of the peptide in the hydrocarbon region of the bilayer.

THEORETICAL BACKGROUND

The flip-flop rate of alamethicin across lipid bilayers, k, is the reciprocal of the average flip-flop time of an ensemble of alamethicin molecules, τ_{path}:

$$k = 1/\tau_{path} \tag{1}$$

Because the obvious flip-flop path, involving the rotation of the peptide around its center of mass, is unlikely, we focused on the more plausible option, i.e., a sequential rotation of the peptide around one terminus at a time, as described in Fig. 1 *A*. This flip-flop path involves two free energy barriers, each associated with inserting one of the peptide termini from the aqueous phase into the bilayer. If we denote the average time for crossing the barriers by τ_1 and τ_2, their sum is the total average time, τ_{path}:

$$\tau_{path} = \tau_1 + \tau_2 \tag{2}$$

The average migration time of each of these free energy barriers is given by

$$\tau = 2\pi k_B T/((F_1F_2)^{0.5}D)e^{\Delta\Delta G/k_BT} \tag{3}$$

where k_B is the Boltzmann constant and T is the absolute temperature (Schulten, et.al, 1981; Wilson and Pohorille, 1996). D is the diffusion coefficient of the peptide in a

uniform medium. For lack of experimental data on D for a flip-flop motion (in a uniform media), we relied on measurements based on lateral motion in the membrane plane of alamethicin in egg phosphatidylcholine and dioleoylphosphatidylcholine membranes (Barranger-Mathys and Cafiso, 1994) and gramicidin C in dimyristoylphosphatidylcholine multibilayers (Tank et al., 1982). Based on these measurements, $D \cong 10^9$ Å^2/s.

F_1 and F_2 in Eq. 3 are the force constants, i.e., the second derivatives of the free energy of the system with respect to the rotation angle, in the orientations separated by the free energy barrier. The peptide can rotate around many axes and the calculations presented in Results indicate that the values of F_1 and F_2 are not very sensitive to the choice of the rotation axis.

$\Delta\Delta G$ in the exponent of Eq. 3 is the free energy difference between the peptide-membrane system above ($\Delta G_{(2)}$) and below ($\Delta G_{(1)}$) the barrier (Fig. 1 *B*):

$$\Delta\Delta G = \Delta G_{(2)} - \Delta G_{(1)} \quad (4)$$

ΔG is the free energy of transfer of alamethicin from the aqueous phase to a given configuration in the lipid bilayer.

Choice of configurations

Our calculations depend strongly on the choice of the alamethicin-membrane configurations. In principle, we should have sampled and averaged over all possible configurations, but this is not computationally feasible. Instead, we relied on the available experimental data and on our experience from the previous computational study (Kessel et al., 2000) to deduce the most crucial configurations. The experimental evidence suggests the stability of alamethicin in transmembrane (Barranger-Mathys and Cafiso, 1996; Huang and Wu, 1991; North et al., 1995) and surface (Banerjee and Chan, 1983) configurations and we deduced the exact configurations (Fig. 1 *A*, configurations *a*, *c*, and *e*) from our previous calculations, which involved sampling around each of these configurations (Kessel et al., 2000).

The most difficult decision in the study was the choice of the tilted configurations, in which either the C- or the N-termini are immersed in the bilayer (Fig. 1 *A*, configurations *b* and *d*). We arbitrarily chose tilt angles of 45° and 135° between the principle axis of the helix (from the N- to the C-terminus) and the normal to the bilayer plane. We then calculated the solvation free energy at different peptide-membrane configurations at these two angles and chose the configuration associated with the smallest desolvation free energy penalty for each of them. The configurations are depicted in Fig. 2, *left* and *right*, and the calculation details are given in Results below. The flip-flop rate depends exponentially on the free energy difference (Eq. 1), and the uncertainty concerning the tilted configurations is the main source of error in our calculations. This issue is addressed in the Discussion.

The rate of lipid motions in the bilayer has been estimated from theoretical (e.g., Essmann and Berkowitz, 1999) and experimental (e.g., Blume, 1993) studies. The wobbling motion of the lipid molecule, in which the molecular long axis changes its orientation within a restricted angular range, has been estimated as $\sim 10^7$/s, and the spinning motion of the molecule around the long axis has been estimated as $\sim 10^8$/s. These values are significantly faster than the measured rate of alamethicin flip-flop ($\sim 10^{-6}$/s (Jayasinghe et al., 1998)), so we can safely assume that the lipids adapt to each orientation of the peptide in the bilayer membrane.

Calculation of ΔG

The free energy difference between alamethicin in the membrane and in the aqueous phase (ΔG) can be broken down into a sum of differences of the following terms: the electrostatic (ΔG_{elc}) and nonpolar (ΔG_{np}) contributions to the

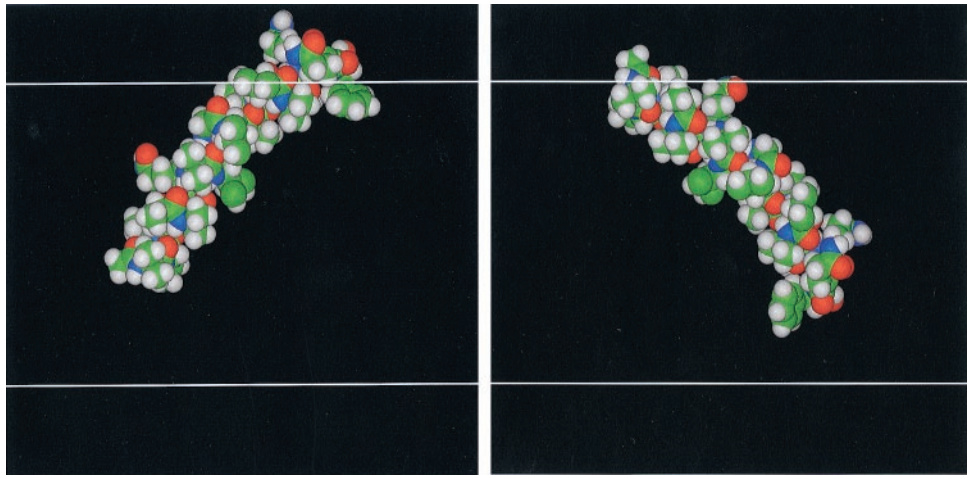

FIGURE 2 A schematic representation of (*left*) configuration *b* and (*right*) configuration *d* of alamethicin from Fig. 1 *A*. The space-filling model of the peptide is displayed with INSIGHT (Molecular Simulations, San Diego, CA). Carbon atoms are green, hydrogen atoms are white, oxygen atoms are red, and nitrogen atoms are blue. The two white lines represent the boundaries of the hydrocarbon region of the lipid bilayer.

solvation free energy, peptide conformation effects (ΔG_{con}), peptide immobilization effects (ΔG_{imm}), and lipid perturbation effects (ΔG_{lip}) (Engelman and Steitz, 1981; Jahnig, 1983; Jacobs and White, 1989; Milik and Skolnick, 1993; Fattal and Ben-Shaul, 1993; Ben-Tal et al., 1996a; White and Wimley, 1999):

$$\Delta G = \Delta G_{elc} + \Delta G_{np} + \Delta G_{con} + \Delta G_{imm} + \Delta G_{lip} \quad (5)$$

Note that alamethicin is voltage sensitive and it is possible to include energetic terms for the voltage dependence (e.g., Biggin et al., 1997). We avoided doing so because no voltage was applied in the experiments of Jayasinghe et al. (1998). The methodology for evaluating each of these terms has been described recently (Kessel et al., 2000) and here we give only a brief overview.

We estimated ΔG_{lip} and ΔG_{imm} based on Fattal and Ben-Shaul (1993) and Ben-Shaul et al. (1996) and calculated $\Delta G_{elc} + \Delta G_{np} = \Delta G_{sol}$ exactly as in Kessel et al. (2000). The peptide was represented in atomic details; each atom was assigned a radius and a partial charge. The hydrocarbon region of the bilayer was represented as a slab of low dielectric constant of 2 embedded in the high dielectric constant of water (80). The Poisson equation was numerically solved and ΔG_{elc} was calculated. ΔG_{np} was calculated by multiplying the water-accessible surface area of the peptide that is buried in the hydrocarbon region by an experimentally derived surface tension coefficient.

Experimental and theoretical studies indicate that the conformation of alamethicin is predominantly α-helical both in water and in lipid bilayers. However, CD measurements suggest an increase in helix content upon membrane binding (Schwarz et al., 1986). Recent molecular dynamics simulations carried out by Tieleman et al. (1999a,b) have indicated that the conformation of the C-terminus of alamethicin is relatively stable when the peptide is membrane associated, but flexible when in water. This suggests that the transfer of alamethicin from water to the lipid bilayer may involve significant conformational changes in the C-terminus of the peptide, resulting in a free energy change (ΔG_{con}). The energetics of polyalanine α-helices in the aqueous phase has been the subject of both 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 a polyalanine helix of $\sim$10 residues involves a free energy value close to zero. This suggests that the conformational flexibility of the C-terminus of alamethicin in water should involve only a negligible free energy change. We therefore used $\Delta G_{con} = 0$.

RESULTS

Free energy calculations of different alamethicin-membrane configurations

Fig. 1 *A* shows the two hypothetical flip-flop paths used in our calculations, and Fig. 1 *B* shows the calculated free energy values of each alamethicin-bilayer configuration in the paths. The free energy values associated with the transmembrane and surface configurations *a*, *c* and *e* were taken from Kessel et al. (2000). These calculations involved a relatively extensive sampling in search of the peptide-membrane configurations associated with the most negative ΔG value.

Each of the two paths of Fig. 1 *A* involves the insertion of the polar N- and C-termini of the peptide into the lipid bilayer, one at a time, and the free energy barrier associated with these configurations. Membrane insertion of each of the polar termini involves a large electrostatic free energy penalty, a part of which may be balanced by nonpolar free energy contributions from the hydrophobic core of the peptide. We searched for the configurations associated with the smallest possible desolvation free energy penalty. To this end, we calculated the dependence of ΔG_{sol} on the distance between the geometrical center of alamethicin and the bilayer center at the constant tilt angels of 45° and 135° between the principle axis of the peptide (N- to C-terminus) and the bilayer normal. The configurations associated with the local minima of ΔG_{sol} (hence in ΔG) are depicted in Fig. 2, *left* and *right*. The configuration of Fig. 2, *left* (i.e., configuration *b* of Fig. 1 *A*) was obtained when the N-terminus was buried inside the lipid bilayer and the ΔG value associated with it is 12.5 kcal/mol. The configuration of Fig. 2, *right* (i.e., configuration *d* of Fig. 1 *A*) was obtained when the C-terminus was buried inside the lipid bilayer and the ΔG value associated with it is 17 kcal/mol. The difference in the ΔG values of the configurations of Fig. 2, *left* and *right* results from differences in the electrostatic free energy penalty associated with the transfer of the C- and N-termini of alamethicin from the aqueous phase into the bilayer; the C-terminus is much more polar than the N-terminus (e.g., Fig. 3 *A* of Kessel et al., 2000).

To get an estimate of the sensitivity of the analysis to the choice of configurations *b* and *d*, we tried several configurations and our results indicate that even a dramatic change of $\sim$10° in the orientation of alamethicin yields a free energy change of $\sim$2.5 kcal/mol or less (Fig. 3).

Estimates of the force constants

We calculated the dependence of the free energy on the rotation angle near the configurations of extreme free energy (Fig. 1 *A*, configurations *a*, *b*, *c*, *d*, and *e*) to estimate the free energy curvatures (or force constants) of Eq. 3. Fig. 3 shows the free energy as a function of the rotation angle around an arbitrary axis in the membrane surface for configuration *d*, which is associated with the highest free energy barrier for the flip-flop. In this configuration, the peptide is situated in the lipid bilayer with its C-terminus immersed in the bilayer and its N-terminus protruding into the aqueous solution. The rotations were carried out around the N-terminal residue of the peptide.

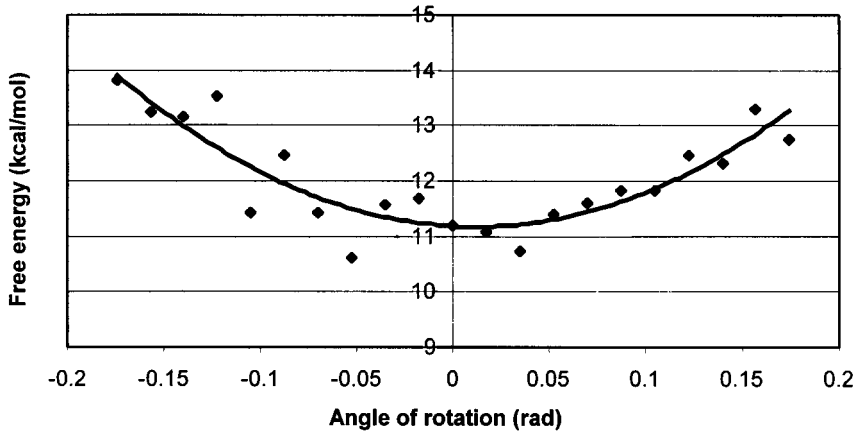

FIGURE 3 The solvation free energy of alamethicin, in the vicinity of configuration *d* as a function of rotation around its N-terminus residue. The results of the calculations are marked with diamonds and the solid parabolic curve represents the best polynomial fit. Our estimate of F_d is based on the curvature of the parabolic curve. (See text for details.)

A polynomial fit shows that the free energy values of Fig. 3 can best be fit by a parabolic curve, from which we estimated $F_d = 150$ kcal/(mol (rad)^2). The deviations from the curve are partially due to computational errors, but for the most part they are due to the solvation and desolvation of chemical groups on the peptide. In this respect they reflect the detailed atomic structure of the peptide. Our calculations showed that the value of F_d obtained by rotating the peptide around the perpendicular axis is essentially the same (data not shown).

The values of the force constants are given in units of kcal/(mol (rad)^2), which is inconsistent with the units of the diffusion coefficient *D*. The reason for this is that we used an estimate of *D* derived from measurements of peptide lateral motion in the membrane plane rather than from measurements of its flip-flop, as mentioned above. To convert the units of the force constants to match our estimated diffusion coefficient, we assumed that the main contribution to the free energy comes from the termini. Thus, we converted the angle of rotation (α) to the translation (ρ) of the C-terminus of the helix on the circumference of an imaginary circle formed by rotating the helix around the N-terminus, using the geometrical relation: $\rho = h \sin(\alpha)$, where *h* is the helix length. Using this relation, $F_d = 0.1$ kcal/(mol (Å)^2). We repeated the calculations of Fig. 3 for each of the orientations *a–e* and the results are: $F_a = 8.7$, $F_b = 0.2$, $F_c = 8.4$, $F_d = 0.1$, and $F_e = 8.7$ kcal/(mol (Å)^2).

Estimates of the transmembrane flip-flop rate

Each of the two putative paths of Fig. 1 *A* involves two free energy barriers: steps *a*→*c* and *c*→*e* in the forward path, or steps *e*→*c* and *c*→*a* in the backward path. Using the calculated free energy values of the different alamethicin-bilayer configurations separated by these barriers, and the set of force constants associated with these configurations, we calculated the average migration time of the peptide through the barriers and the transmembrane flip-flop rate, as described in Theoretical Background above. The calculated values are shown in Table 1. The calculations indicate that the preferred path for alamethicin flip-flop is *a*→*e*, and that the associated flip-flop rate is $\sim 10^{-7}$/s, compared with the measured value of 1.7×10^{-6}/s (Jayasinghe et al., 1998). The calculations also show that the rate-determining step for the flip-flop is *c*→*e*, i.e., crossing the free energy barrier associated with configuration *d*.

TABLE 1 Free energy difference ($\Delta\Delta G$), average time (τ), and rate (k) of the two flip-flop paths depicted in Fig. 1

Path*	Barrier†	$\Delta\Delta G^{\ddagger}$ (kcal/mol)	$\tau_{barrier}$§ (s)	τ_{path}¶ (s)	k_{path}‖ (s^{-1})
a*→*e	*a*→*c*	18.0	3.9×10^4	**9.1×10^6**	**$\sim 10^{-7}$**
	c*→*e	**21.0**	**9.1×10^6**		
e→*a*	*e*→*c*	22.5	1.0×10^8	1.0×10^8	$\sim 10^{-8}$
	c→*a*	16.5	3.2×10^3		

The calculations were carried out as described in Theoretical Background.

*The suggested migration path, as depicted in Fig. 1.

†The free energy barriers of both directions of the path; *a*, *c*, and *e* mark the alamethicin-membrane configurations separated by the free energy barriers, corresponding to the scheme in Fig. 1 *A*.

‡The free energy difference between the alamethicin-membrane system above and below each free energy barrier (Eq. 4).

§The average migration time of each of the two barriers in the path (Eq. 3).

¶The average migration time of the full path (Eq. 2).

‖The flip-flop rate (Eq. 1). Data on the preferred path are shown in bold.

The free energy penalty of inserting the backbone carbonyl and the terminus OH groups into bilayers

It is evident from Fig. 1 *B* and Table 1 that the flip-flop rate is determined by the penalty of transferring the polar C-terminus across the bilayer, i.e., the free energy of configuration *d*. To facilitate a closer examination of the energetics of this step, we calculated the free energy change for the membrane insertion of the polar groups at the C-terminus that are most likely to influence the energetics of the insertion, i.e., the unpaired backbone carbonyl groups at the C-terminus of the peptide and the C-terminus's OH group. The insertion free energy of the carbonyl groups was calculated as the difference between the free energy of configuration *d* of Fig. 1 and the free energy of the same configuration treating these carbonyl groups as neutral, i.e., setting the partial atomic charges to zero. Likewise, the insertion free energy of the terminal OH group was calculated as the difference between the free energy of configuration *d* of Fig. 1 and the free energy of the same configuration treating this group as neutral. (In this respect it is noteworthy that the terminal OH group is protonated and uncharged (Jayasinghe et al., 1998).) We found the free energy penalties for the insertion of the unpaired carbonyl groups and of the OH group to be 8 kcal/mol and 4 kcal/mol, respectively (Table 2). The insertion of the C-terminus of alamethicin into the bilayer also involves the insertion of the Gln18, Gln19, and Phol20 side-chains and we estimated the corresponding free energy as described in the Discussion below.

DISCUSSION

A number of approximations were used in this study. The underlying assumption in the calculations is that the lipid motions are significantly faster than the flip-flop of alamethicin and that the lipids can therefore adapt to each orientation of alamethicin in the membrane. All the available experimental and theoretical evidence supports this assumption as mentioned in Theoretical Background above. We thus estimated the transmembrane flip-flop rate of alamethicin by choosing different peptide-membrane configurations, representing critical steps in the process. The choice of configurations, although based on free energy considerations, is somewhat arbitrary. This is especially true for the choice of the configurations at the top of the free energy barriers (Fig. 1 *A*, configurations *b* and *d*). The flip-flop rate constant depends exponentially on the free energy (Eq. 3) and the error estimate given below shows that the choice of these configurations is likely to be the main source of error in our study.

TABLE 2 Group decomposition of the free energy of membrane insertion of the polar C-terminus of alamethicin

	Group	ΔG_{tot} (kcal/mol)
Side-chains*	Gln18	5.4†
	Gln19	5.4†
	Phol20	−1.5†
Backbone‡	Terminal OH	4.0§
	Carbonyl groups of Gln18 and Gln19	8.2§
Total¶	Estimated	21.5¶
	Calculated	21.0‖

*The side-chain groups of Gln18, Gln19, and Phol20.
†Estimates from Kessel and Ben-Tal, 2000.
‡The backbone groups of Gln18, Gln19, and the terminal hydroxyl group of the peptide.
§The free energy of transfer of the C-terminal unpaired carbonyl groups and the terminal OH group from water to the hydrocarbon region of the lipid bilayer.
¶The total free energy of transfer of the C-terminus from water to the hydrocarbon region of the lipid bilayer, estimated as the sum of the contributions of the individual groups.
‖The "exact" value of the free energy barrier as obtained from the calculations of Fig. 1 and Table 1.

Several other approximations of the peptide-membrane system were used in our model, and these have already been discussed previously (Kessel et al., 2000). The main approximation of this model is the description of the lipid bilayer as a slab of low dielectric constant. This representation obscures all atomic detail about alamethicin-bilayer interactions. It also neglects the polar headgroup region, which is, presumably, the site of alamethicin adsorption onto the bilayer. This region, whose dielectric constant is estimated to be between 25 and 40 (Ashcroft et al., 1981), was assigned a value of 80, identical to that of water, in our model. In our previous study (Kessel et al., 2000) we used the same model to calculate the free energy of transfer of alamethicin from the aqueous phase into a lipid bilayer and, despite the approximations, the calculated value was nearly identical to the measured value of Lewis and Cafiso (1999). Although such perfect agreement between the calculations and measurements may be fortuitous, it should also hold for this study, because the same system is studied in both. Therefore, we believe that the free energy of transfer of alamethicin from the aqueous phase into the bilayer at a given configuration is accurately calculated using the model. Likewise, our estimate of the force constants (F_1 and F_2 in Eq. 3) should be fairly accurate, because they are based on the calculated free energy of transfer of the peptide from the aqueous phase into the bilayer at different configurations. The main source of error in the calculations is, therefore, the choice of configurations *b* and *d* of Fig. 1 *A*, which is admittedly arbitrary. In fact, of these two configurations, *d* is associated with the highest free energy barrier for the flip-flop and is therefore the more crucial. It is evident from Fig. 2 that the free energy depends only weakly on the exact choice of configuration *d*; even a dramatic rotation of ~10° from *d* yields a free energy change of ~2.5 kcal/mol or less. We therefore estimate the error in $\Delta\Delta G$ to be no more than 2.5 kcal/mol, which translates to a factor of ~60 in the rate constant *k*.

Another source of error in *k* is our estimate of the diffusion coefficient *D*. As discussed in Theoretical Background

above, there is no direct measurement of D of peptide rotation in a uniform hydrocarbon-like medium, so we had to rely on values associated with lateral motion of peptides in the membrane plane. Taking all these uncertainties together, we estimate that our calculated value of k should be accurate to within $\sim$2 or 2.5 orders of magnitude.

Calculations toward an estimate of the value of k should have been based on intensive sampling of alamethicin conformations and configurations in the lipid bilayer. To make such sampling feasible, one usually has to rely on highly approximated (preferably analytical) expressions of the free energy of the system (e.g., Milik and Skolnick, 1996). We chose a different approach, carrying out a small number of relatively accurate calculations at carefully selected alamethicin-bilayer configurations representing key points in the flip-flop path. One may argue that the configurations were chosen simply to fit with the experimental data, which was already available when we started the calculations. This is not the case. The transmembrane and surface configurations *a*, *c* and *e* of Fig. 1 *A* were chosen based on the available experimental data and on previous calculations (Kessel et al., 2000). Thus, the value of ΔG below the free energy barriers should be well defined. The only arbitrary choice that we had to make concerned the configurations in which the N- and C-termini of alamethicin were buried in the bilayer. These configurations (*b* and *d* of Fig. 1 *A*) determine the value of ΔG above the barrier. In fact, even the choice of these configurations is not completely arbitrary. After arbitrarily choosing peptide tilt angles of 45° and 135°, respectively, we searched for the local minima in the solvation free energy penalty to obtain the configurations of Fig. 2, *left* and *right*. Finally, we carried out calculations (e.g., Fig. 3) to test the sensitivity of ΔG to the exact choice of the configurations and showed that it is not very sensitive.

The careful selection of configurations that are crucial for the flip-flop path is most likely the reason why the value of k found in our calculations is close to the measured value ($\sim 10^{-7}$/s vs. 1.7×10^{-6}/s). The most likely error anticipated when using our approach is to overlook configurations in which either the N- or the C-terminus is immersed in the bilayer, which are associated with small desolvation free energies compared with the values obtained in the configurations of Fig. 2, *left* and *right*. This would lead to an overestimate of the free energy barrier in the flip-flop motion; i.e., our calculated value of the free energy barrier should be an upper bound to the true value and the calculated value of k should be regarded as a lower bound to the true value. Thus, it is reassuring that the calculated value is somewhat smaller than the measured one. The overall agreement between the calculated and measured values of k suggests that the flip-flop path of alamethicin is similar to the path of Fig. 1 *A*. In this respect, our model provides a molecular interpretation of the measurements of Jayasinghe et al. (1998).

Our calculations suggest that the main free energy barrier of alamethicin flip-flop results from the insertion of the (highly polar) C-terminus of the peptide into the bilayer. We investigated this suggestion by calculating the free energy of insertion of the individual C-terminal groups of alamethicin into the bilayer. These groups consist of Gln18, Gln19, Phol20, and the C-terminal OH group. We have estimated the insertion free energy of the Gln18, Gln19, and Phol20 side-chains into the bilayer using a hydropathy scale derived from calculations of the insertion free energy of polyalanine-like α-helices (Kessel and Ben-Tal, 2000). The insertion of the C-terminus of alamethicin into the bilayer also involves the exposure of unpaired carbonyl groups to the hydrophobic region of the bilayer. We calculated the free energy of insertion of the carbonyl and OH groups as described in Results. As mentioned above, Cafiso and his co-workers used the GES hydropathy scale (Engelman et al., 1986) to estimate the free energy of insertion of the Gln18, Gln19, and Phol20 side-chains and of the C-terminal OH group into the lipid bilayer to be $+8$ kcal/mol. They also considered the insertion of three unpaired carbonyl groups at the C-terminus and estimated the corresponding free energy value to be $+6$ kcal/mol. Thus, a total value of $+14$ kcal/mol was obtained. However, their estimate is considerably lower than the experimentally derived value (Jayasinghe et al., 1998).

Our calculated free energy penalty of the insertion of each of the polar groups at the C-terminus of alamethicin is shown in Table 2. These values differ from the estimates of Cafiso and co-workers. First, our estimate of the free energy of insertion of the side chains of Gln18, Gln19, and Phol20 and of the terminal OH group is $\sim$5 kcal/mol higher than the value used by Jayasinghe et al. (1998), which was based on the GES hydropathy scale. Second, alamethicin's structure suggests that there are only two rather than three unpaired carbonyl groups at the C-terminus. Our free energy calculations indicate that the insertion of these two groups into the bilayer involves a free energy penalty of $\sim+4$ kcal/mol per group. Thus, our estimate of the free energy of insertion of the unpaired carbonyl groups is $\sim$2 kcal/mol higher than the 6 kcal/mol estimate of Cafiso and co-workers. Overall, our estimate of the group decomposition of the free energy barrier due to insertion of the C-terminus into the membrane, 21.5 kcal/mol, compares very well with the value obtained in the "exact" calculations of Fig. 1 *B* (21 kcal/mol).

Schwarz et al. (1986) used fluorescence spectroscopy to study the kinetics of alamethicin incorporation into dioleylphosphatidyl choline (DOPC) and dimyristoylphosphatidylcholine (DMPC) vesicles. Their interpretation of the results suggests an essentially one-step incorporation process. This process includes an intermediate state, where the peptide is positioned on the membrane surface, pending its insertion into the bilayer. The average insertion time of alamethicin into DOPC and DMPC bilayers as measured in their study

was ~0.4 μs and ~2.3 μs, respectively. The association of alamethicin with the lipid bilayer, as suggested by Schwarz and co-workers, is also part of the transmembrane flip-flop path (Fig. 1 *B*). The membrane-adsorbed state is described by configuration *c*, and the insertion of the peptide into the bilayer, via its N-terminus, is described by the change from configuration *c* to *a*. Our calculations indicate, as seen in Fig. 1 *B* and in Table 1, that the membrane adsorption of the peptide is diffusion controlled ($\Delta G = -4$ kcal/mol), whereas its insertion into the bilayer involves a free energy barrier of 16.5 kcal/mol, with an average time of $\sim 3 \times 10^3$ s. Thus, we suggest that the time measured by Schwarz and co-workers is for the adsorption of alamethicin on the bilayer surface rather than the insertion into the bilayer.

In conclusion, various theoretical tools, such as molecular dynamics simulations, are used to investigate membrane proteins and peptides. However, these methods usually use explicit description of the investigated system and are, consequently, time costly. In contrast, continuum solvent models are simpler and time saving but may neglect important features of the system. We have recently used continuum solvent model calculations to investigate the thermodynamics of alamethicin-membrane systems (Kessel et al., 2000) and obtained results that were in good agreement with experimental data. In the present study, we have extended the model to investigate the kinetics of these systems and again the measured values fall well within the computational error. These two studies, in addition to earlier studies on polyalanine α-helices interactions with lipid membranes (Ben-Tal et al., 1996a,b) and on the membrane permeability of monensin-cation complexes (Ben-Tal et al., 2000), demonstrate the power of continuum solvent models, and the simple slab model in particular, in the study of peptide-membrane systems. These models can often provide a means of obtaining a molecular interpretation of available experimental data.

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