Membrane Structure of Voltage-Gated Channel Forming Peptides by Site-Directed Spin-Labeling†

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ABSTRACT: Three spin-labeled derivatives of the voltage-gated peptide alamethicin were prepared with nitroxides at the C-terminal phenyalaninol, and at positions 9 and 15 in the amino acid sequence. In addition, three spin-labeled derivatives of an analog of alamethicin where α-methylalanine residues are replaced by leucine were prepared with nitroxide labels at the same positions. Continuous wave power saturation EPR spectroscopy was used to examine the effect of molecular oxygen and water soluble paramagnetic reagents on the saturation behavior of the labeled peptides. Using the gradients of these species which exist through the membrane−solution interface, distances for these nitroxide derivatives from the membrane-solution interface were estimated. The distances show that alamethicin is inserted along the bilayer normal with the C-terminus of the peptide lying in the aqueous solution 3 or 4 Å from the membrane interface. In this configuration alamethicin does not completely cross the bilayer, and the N-terminus of alamethicin is within the membrane hydrocarbon approximately 16 Å from the phosphate groups on the opposing interface. The analog where leucines replace α-methylalanines shows a similar conformation, except that the entire peptide is translated 3−4 Å deeper into the membrane than is native alamethicin. The distances that are measured for alamethicin using EPR are consistent with a linear high resolution NMR structure determined in SDS and the X-ray crystal structure. The membrane position and structure of alamethicin found here limit the likely models for voltage-gating of this peptide.

Alamethicin is a 20 amino acid peptide produced by the fungus *Tricoderma veride*, which induces a voltage-dependent conductance in planar bilayers and in vesicles (for recent reviews see: Wooley & Wallace, 1992; Sansom, 1993; Cafiso, 1994). Because of its interesting channel behavior and small size, it is has provided a tractable model for voltage-dependent gating phenomena in biological membranes. In addition, it has been explored as a model for protein−membrane interactions and helix−helix association (Schwarz et al., 1986; Rizzo et al., 1987; Woolley & Wallace, 1993).

Alamethicin has the sequence Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-Val-MeA-Gly-Leu-MeA-Pro-Val-MeA-MeA-Gln-Gln-Phol and contains eight α-methylalanine (MeA)[1] residues which appear to promote 3_{10} helical configurations in shorter peptides (Toniolo & Benedetti, 1991). In its crystal structure, alamethicin is predominantly α-helical, except for some 3_{10} character toward the C-terminal end of the peptide (Fox & Richards, 1982). The peptide overall is quite hydrophobic, but in the crystal structure it displays a weak laterally amphipathic character with two Gln residues lying on one face of the helix. As with many other channel forming peptides, alamethicin is envisioned to conduct by forming an aggregate of laterally amphipathic helices. High resolution NMR studies in methanol confirm that the peptide is helical (Banerjee & Chan, 1983; Esposito et al., 1987; Yee & O'Neil, 1992), and recent studies in detergent micelles provide evidence that the peptide bends about the central portion of the helix (Franklin et al., 1994). The tendency of this peptide to bend has been confirmed in methanol by examining paramagnetic enhancements of nuclear relaxation produced by a spin-label attached to the C-terminus of the peptide (North et al., 1994). The region having a high degree of conformational flexibility is near MeA 10 which is four residues away from proline at position 14. The carbonyl group of MeA 10 would normally hydrogen bond to the amide nitrogen at position 14.

There have been relatively few structural studies on the position of the peptide in the membrane, and most of the studies directed at examining peptide orientation have utilized optical spectroscopy. NMR studies indirectly on the membrane lipids suggest that alamethicin is surface oriented (Banerjee et al., 1985). However, studies using optical techniques indicate that alamethicin is inserted and oriented along the bilayer normal (Vogel, 1987; Huang & Wu, 1991). These studies also indicate that the hydration and the peptide:lipid ratio can alter the orientation of alamethicin. One earlier measurement based on IR spectroscopy reported that the peptide was extended (nonhelical) and aggregated in the membrane at full hydration (Fringeli & Fringeli, 1979). However, this extended structure and aggregation are not supported by more recent work (Archer et al., 1991;

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[1] Abbreviations: MeA, α-methylalanine; NiAA, nickel(II) acetylacetonate; MTSSL, methanethiosulfonate spin-label; BMSL, bromomethyl spin-label; TFA, trifluoroacetic acid; egg PC, egg yolk phosphatidylcholine; DIPCDI, diisopropylcarbodiimide; DMF, dimethylformamide; L1, analog of alamethicin where all MeA residues are replaced by Leu; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); T_1, spin−lattice relaxation time; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; CD, circular dichroism; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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Table 1: Alamethicin Analogs Synthesized[a]

analog	sequence
Alam-Phol	Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-Val-MeA-Gly-Leu-MeA-Pro-Val-MeA-MeA-Gln-Gln-Phol
Alam-V15C	Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-Val-MeA-Gly-Leu-MeA-Pro-**Cys**-MeA-MeA-Gln-Gln-Phe-OMe
Alam-V9C	Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-**Cys**-MeA-Gly-Leu-MeA-Pro-Val-MeA-MeA-Gln-Gln-Phe-OMe
L1	Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Gln-Gln-Phe
L1-V15C	Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-**Cys**-Leu-Leu-Gln-Gln-Phe-OMe
L1-V9C	Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-**Cys**-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Gln-Gln-Phe-OMe

[a] The "native" sequences, Alam-Phol and L1, were derivatized with a spin-label on their C-terminus, while Alam-V15C, Alam-V9C, L1-V15C, and L1-V9C were derivatized with the sulfhydryl reactive label MTSSL (see text).

Barranger-Mathys & Cafiso, 1994; Franklin et al., 1994). Information on the orientation and conformational flexibility of alamethicin has been actively pursued because it provides insight into the mechanisms of gating in this peptide channel.

Solid state NMR measurements in this laboratory indicate that the N-terminal segment of alamethicin is α-helical and inserted into the membrane (North et al., 1995). Here, we report a complementary series of measurements made by site-directed spin-labeling of alamethicin and analogs of alamethicin. An analog of alamethicin where leucines replace MeA (L1) has been shown to exhibit alamethicin-like activity and is easier to produce synthetically (Molle et al., 1989). We use spin-labels on alamethicin and these alamethicin-like analogs to determine the position and likely structures of these peptides within the membrane interior. These data are consistent with the data obtained from solid state NMR and optical measurements, but show that the C-terminus lies outside the membrane−solution interface. The leucine-based analogs have a similar conformation but take up a deeper position within the bilayer. These measurements are discussed in terms of voltage-gating mechanisms for alamethicin.

EXPERIMENTAL PROCEDURES

Materials. Fmoc protected amino acids as well as the alkoxybenzyl alcohol resin were purchased from BaChem Bioscience Inc. (Bubendorf, Switzerland). The Tentagel S AC resin with Phe attached was purchased from Rapp Polymere (Teubingen, Germany). The paramagnetic molecules 3-carboxy-PROXYL, 3-(aminomethyl)-PROXYL, chromium oxalate, and nickel(II) acetylacetonate (NiAA) were purchased from Sigma (St. Louis, MO) and 1-oxy-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methylmethanethiosulfonate spin-label (MTSSL) and 1-oxy-2,2,5,5-tetramethyl-3-(bromomethyl)-Δ^3-pyrroline (BMSL) were purchased from Reanal (Budapest, Hungary). The acyl fluoride of MeA, Fmoc-MeA-F, was synthesized as described previously (Wenschuh et al., 1994). Egg yolk phosphatidylcholine (egg PC) was isolated from fresh hens eggs according to the procedure of Singleton et al. (1965) and stored in a chloroform solution under argon at −20 °C.

Synthesis of Peptides. A list of the peptides synthesized based on native alamethicin and the leucine analog of alamethicin (L1) are listed in Table 1. The C-terminal labeled alamethicin, Alam-Phol-SL, was synthesized as described previously from native alamethicin (Archer et al., 1991) and purified on a reverse phase C18 HPLC column where the gradient was 40% A/60% B, to 10% A/90% B, where A is 0.05% trifluoroacetic acid (TFA) in water and B is 0.05% TFA in CH_3CN. The two analogs of native alamethicin Alam-V9C and Alam-V15C were synthesized using solid phase peptide synthesis and an Fmoc protection scheme as described previously (Stewart & Young, 1984). The Fmoc amino acids were used in 5-fold excess and were coupled using diisopropylcarbodiimide (DIPCDI) and hydroxybenzotriazole hydrate. In order to enhance the efficiency for coupling MeA, these residues were coupled as Fmoc protected acyl fluorides (Wenschuh et al., 1995). This residue coupled in 15 min using 3 equiv of both the amino acid and diisopropylethylamine. To acetylate the N-terminus, the peptide was treated with a 20% solution of acetic anhydride and 10% diisopropylethylamine in DMF for 15 min before cleavage. The peptides were cleaved with 5% triethylsilane (Et_3SiH), 5% phenol, and 5% H_2O in 50/50 TFA/CH_2Cl_2 for 30 min and isolated as the *tert*-butylthio protected cysteine. Before spin-labeling, they were deprotected by stirring overnight in degassed 1-butanol containing 5% tri-*n*-butylphosphine. To form the methyl ester at the C-terminus, peptides were dissolved in methanol at a concentration of approximately 25 mg/mL, and 10 μL of TFA was added and the mixture stirred overnight at room temperature. To spin-label the peptides, a 3-fold excess of MTSSL was added to a methanol solution containing approximately 0.06 M peptide, and the mixture was then stirred for several hours. The solutions were purified with a Vydac analytical reverse phase column C18 218TP104 using isocratic conditions of 10% A/90% B and a flow rate of 1 mL/min, where A was 0.1% aqueous TFA and B was CH_3CN with 0.085% TFA. The retention time for the Alam-V9C-SL was 14 min. The retention time for the Alam-V15C-SL was 19 min. These peptides were identified by EPR spectroscopy and confirmed by mass spectrometry which produced an *m/z* of 2167 for each. These peptides had a purity in excess of 97% as determined by HPLC.

The L1 analogs having the native sequence Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Gln-Gln-Phe were synthesized on a Gilson Automated Multiple Peptide System (AMS 422). This synthesis was accomplished using Fmoc protected amino acids which were activated with benzotriazollyloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP). The solid phase synthesis was carried out using a phenylalanine derivatized alkoxybenzyl alcohol resin, and the N-terminus of the peptide was acetylated as described above for alamethicin. The sequences produced included native L1, L1V9C, and L1V15C. These peptides were cleaved from the resin with 95/5 TFA/Et_3SiH, precipitated, washed, and deprotected. The amino methyl pyroxyl spin-label was attached to the C-terminus of L1 by adding equimolar quantities of (hydroxybenzyl)-triazole and diisopropylcarbodiimide to a 0.4 M solution of L1 in DMF. After stirring for 2 min, 3 equiv of spin-label were added and the solution was allowed to stir for at least

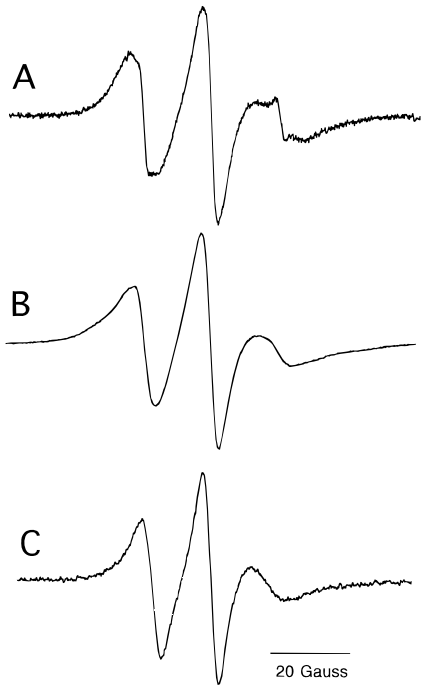

FIGURE 1: X-band EPR spectra of membrane bound spin-labeled alamethicin analogs in extruded vesicles of egg PC. The peptides were added from a stock methanol solution to the lipid vesicle suspension. The total lipid concentration is approximately 50 mM, and the peptides were at a concentration of approximately 50−100 μM. (A) Alam-Phol-SL, (B) Alam-V15C-SL, (C) Alam-V9C-SL.

3 h. L1-V9C was esterified at its C-terminus and spin-labeled with the MTSSL as described above. L1-V15C was also esterified and then spin-labeled with the BMSL by dissolving the peptide in acetone with 1 equiv of K_2CO_3 and one crystal of NaI. After the mixture was stirred for 5 min, 3 equiv of the spin-label were added and the mixture was stirred overnight. The purification of the L1 analogs was performed on the analytical column described above with a linear gradient of 80% A/20% B to 30% A/70% B over 30 min and a flow rate of 1 mL/min, where A was 60/40 ethanol/water with 0.05% TFA and B was 90/10 ethanol/water with 0.05% TFA. The retention time for the L1-Phe-SL was 23 min and yielded the appropriate value of *m/z* = 2356 by mass spectrometry. L1-V9C-SL eluted in 12 min and was also confirmed by mass spectrometry (*m/z* = 2391). The peptide L1-V15C-SL was purified on the semipreparative column using a gradient of 80% A/20% B to 100% B over 30 min and a flow rate of 3 mL/min. This peptide had a retention time of 5 min and yielded an *m/z* of 2280. All the above peptides had a purity in excess of 97%.

A spin-labeled analog of glutathione was produced by double acetylating glutathione with 2 equiv of acetic anhydride in water. The reaction took place in 2 h at room temperature, and completion was monitored with ninhydrin and DTNB. The thiol acyl group was selectively cleaved as described previously (Zervas et al., 1963), and the Ac-glutathione-SH was labeled as described above with the MTSSL. The product was purified by column chromatography on silica gel in a solvent of ethylacetate/pyridine/water/acetic acid, 10/5/3/1. The product had an R_f of 0.75 in this solvent and yielded the correct *m/z* of 414 by mass spectrometry.

Preparation of Lipid Vesicles. Vesicles were formed by extrusion of egg PC suspensions through 500 Å nucleopore filters in a buffer of 125 mM Na_2SO_4/10 mM MOPS, pH = 6.5, in a manner similar to that described previously (Archer et al., 1991). The vesicle suspensions were at egg PC concentrations of approximately 10 mg/mL, and spin-labeled peptides were added from concentrated stock solutions to final concentrations of between 20 and 200 μM. For measurements made in the presence of aqueous nickel, NiAA was added to the buffer before extrusion to a final concentration of 20 mM. In this case, NiAA is present on both surfaces of the bilayer.

EPR Spectroscopy. EPR spectra were recorded on an E-line Centuries series spectrometer using an X-band loop gap resonator with a standard two loop one gap configuration. Unless otherwise indicated, the microwave power was 2 mW and the peak-to-peak modulation amplitude was 1.25 G. Quartz capillary tubes, i.d. = 0.6 mm, o.d. = 0.84 mm (Vitro Dynamics, Inc., Rockaway, NJ), were used to hold nitroxide samples and were nominally filled with 5 μL of sample. For power saturation measurements, samples were placed in gas permeable TPX capillary tubes (Medical Advances, Milwaukee, WI) and EPR spectra were taken at regular power intervals ranging from 0.1 to 50 mW.

Power saturation measurements were carried out on spin-labeled alamethicin and alamethicin analogs that were totally membrane bound to lipid vesicles formed from egg PC. As the microwave power was increased, the peak to peak amplitude of the $m_I = 0$ resonance, *A*, was measured. These power saturation data were fit to the function:

$$A = IP^{1/2}\left[1 + (2^{1/\epsilon} - 1)\frac{P}{P_{1/2}}\right]^{-\epsilon} \qquad (1)$$

as described previously (Altenbach et al., 1994), where $P_{1/2}$, ϵ, and *I* were allowed to be adjustable parameters. Here, *P* represents the microwave power, *I* is a scaling factor, $P_{1/2}$ is the power required to reduce the resonance amplitude to half its unsaturated value, and ϵ is a measure of the homogeneity of saturation of the resonance. Power saturation data were obtained for each sample under three different sets of conditions: equilibrated with a nitrogen atmosphere, equilibrated with air, and equilibrated with nitrogen in the presence of 20 mM NiAA in the aqueous phase. Gasses were allowed to equilibrate for 30 min before power saturation measurements were made. Values for $\Delta P_{1/2}$ were obtained from differences in the $P_{1/2}$ value in the presence and absence of the relaxation reagents. The parameter Φ was then calculated using the following equation from the experimentally measured values of $\Delta P_{1/2}$ obtained in the presence of air and NiAA:

$$\Phi = \ln\left(\frac{\Delta P_{1/2}(O_2)}{\Delta P_{1/2}(\mathrm{NiAA})}\right) \qquad (2)$$

As described previously, this parameter is directly related to the difference in the standard state chemical potentials of O_2 and NiAA. These chemical potentials vary as a function of depth in the bilayer; thus, Φ provides an estimate of the nitroxide depth in the bilayer (Altenbach et al., 1994). The depth calibration obtained previously using spin-labeled bacteriorhodopsin and chain labeled nitroxides in egg PC was used here.

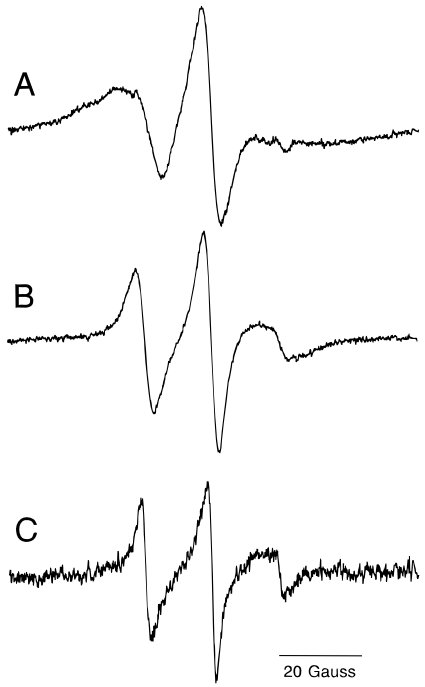

FIGURE 2: X-band EPR spectra of membrane bound spin-labeled L1 in extruded vesicles of egg PC. In this conductive analog of alamethicin, MeA residues are replaced by leucine. The peptides were either added from a stock methanol solution or dissolved into the lipid mixture before drying and addition of buffer. The total lipid concentration in these samples is approximately 50 mM, and the peptides are at a concentration of 20−150 μM. (A) L1-Phe-SL, (B) L1-V15C-SL, (C) L1-V9C-SL.

RESULTS

EPR Spectra of Membrane Bound Alamethicin Analogs. Shown in Figure 1 are EPR spectra of three spin-labeled alamethicin analogs bound to extruded vesicles formed from egg PC. The spectra in (A), (B), and (C) correspond to nitroxides attached to the C-terminus and to cysteines at positions 15 and 9, respectively. Under the conditions used here, the peptide is completely membrane associated. The motion of the nitroxide attached to alamethicin is characteristic of a nitroxide that is undergoing anisotropic motion and with an apparent correlation time of about 3 ns as reported previously (Archer et al., 1991).[2] All three spectra shown here are similar, indicating that these labels have a comparable degree of motion in the ns time scale. Under the conditions used here the C-terminal labeled alamethicin was previously shown to be monomeric (Archer et al., 1991; Barranger-Mathys & Cafiso, 1994). The fact that all three labels exhibit a similar motion is consistent with this result, as helix−helix contact should produce positionally dependent differences in nitroxide motion (Altenbach et al., 1990).

Shown in Figure 2 are spectra of three spin-labeled analogs of the leucine analog, L1, bound to extruded lipid vesicles formed from egg PC. The spectra shown in (A), (B), and (C), are from labels at the C-terminus and positions 15 and 9, respectively, the same three positions as shown in Figure 1. Spectra from positions 9 and 15 have a similar lineshapes, while the C-terminus is more restricted in its motion. The C-terminal proxyl nitroxide is linked to the peptide via an amide linkage, and this may account for the difference in the motion of this label. However, at the present time we cannot distinguish this from other explanations for the slight difference in the motion of this label.

[2] It should be noted that this estimate of rotational correlation time assumes an isotropic diffusional model and does not represent a rigorous analysis of the EPR lineshapes in terms of label dynamics (Keith et al., 1970; Nordio, 1976).

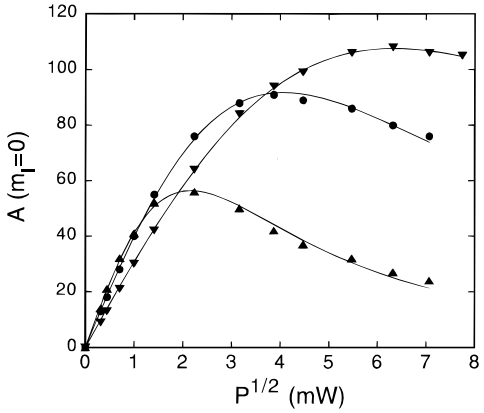

FIGURE 3: Power saturation curve for the $m_I = 0$ resonance of approximately 20 μM Alam-V15C-SL in the presence of N_2 (▲), air (●), and 20 mM NiAA (▼). The lipid concentration was approximately 10 mM. The solid lines represent fits of the data to eq 1.

Table 2: Power Saturation Parameters for Spin-Labeled Peptides[a]

peptide	$\Delta P_{1/2}$ (O_2)	$\Delta P_{1/2}$ (NiAA)	Φ	depth (Å)
Alam-V9C-SL	42.2	8.9	1.56	11.7
Alam-V15C-SL	14.0	41.96	−1.09	1.0
Alam-Phol-SL	5.38	47.0	−2.17	−3.4
L1-V9C-SL	15.7	1.4	2.39	15.0
L1-V15C-SL	14.6	18.8	−0.25	4.3
L1-Phe-SL	16.8	67.5	−1.39	−0.28
SL-glutathione	5.19	80.0	−2.74	aqueous

[a] Alam: native alamethicin spin-labeled at the indicated site. L1: analogs of alamethicin where the eight MeA residues are replaced by leucine (see Table 1). Glutathione-SL: a spin-labeled and acetylated analog of glutathione.

Power Saturation Provides a Depth Measurement for Spin-Labeled Alamethicin. When used in combination with paramagnetic relaxation agents, power saturation can provide a measure of the position of nitroxides within the membrane interior. This depth measurement exploits the preferential solubility of O_2 or NiAA in the hydrocarbon or aqueous phases, respectively (Altenbach et al., 1994). Shown in Figure 3 are three power saturation curves for alamethicin spin-labeled at position 15. In the presence of O_2 or NiAA the power required to saturate the nitroxide is increased, reflecting an increase in the T_1 relaxation time for the electron. Also shown in Figure 3 are fits to the data points using eq 1. The value of $P_{1/2}$ which is obtained from this fit is directly related to the value of T_1, and $P_{1/2}$ is then used to determine $\Delta P_{1/2}$ for either O_2 or NiAA. As described elsewhere, $\Delta P_{1/2}$ is related to the frequency of collisions between these paramagnetic species and the nitroxides and is proportional to the local concentrations of these agents (Altenbach et al., 1994). Using eq 2, these $\Delta P_{1/2}$ values then yield an estimate of Φ, a dimensionless parameter that is related to the nitroxide depth in the bilayer. Table 2 summarizes the Φ values obtained for the alamethicin and L1 analogs synthesized here. For native alamethicin, the Φ parameters increase from the C-terminus toward the N-

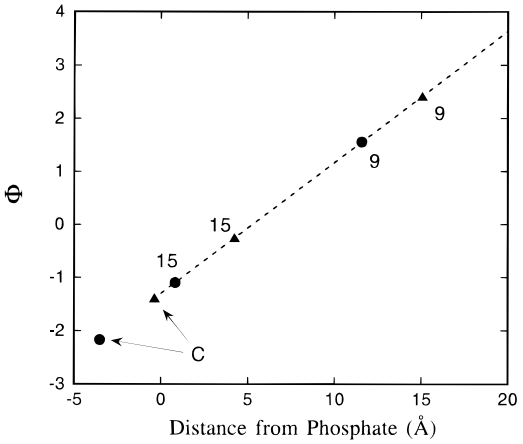

FIGURE 4: Plot of the power saturation parameter Φ defined by eq 1 versus distance for positions 9 and 15 and the C-terminus of spin-labeled alamethicin (●) and spin-labeled L1 (▲). The C-terminus of alamethicin lies outside the membrane−solution interface, while the C-terminus of L1 is at the interface. Each of the three labeled positions for L1 appears to be translated inward from the membrane interface relative to alamethicin. The distance calibration used was taken directly from Altenbach et al. (1994).

terminal end of the peptide. The same trend is seen for the leucine analog, except that the Φ values at each position are slightly more positive.

Shown in Figure 4 are the Φ values for the alamethicin and L1 spin-labels plotted on a calibration curve established previously for the dependence of Φ on bilayer depth in egg PC (Altenbach et al., 1994). From this calibration curve, distances for the nitroxide from the membrane interface can be estimated and are listed in Table 2. On this curve, the Φ value for the C-terminus of alamethicin places it outside the membrane solution interface at a distance of about 4 Å from the membrane surface.[3] Below, we show that these three distances are consistent with a linear form of alamethicin that was obtained by simulated annealing of high resolution NMR data in SDS micelles. For the leucine analog, spin-labels at equivalent positions lie approximately 4 Å deeper in the membrane than do labels on alamethicin. Thus, this analog appears to be in a similar configuration to native alamethicin, but translated approximately 4 Å deeper into the membrane hydrocarbon.

Distances Are Consistent with Linear Forms of Alamethicin. The depths shown in Figure 4 provide a measure of nitroxide distances to either membrane interface. In previous work utilizing high resolution NMR, several low energy structural forms of alamethicin were obtained by applying simulated annealing to the NMR restraints (Franklin et al., 1994). The structures that were obtained fell into two general classes, one linear and the other bent. The linear and bent forms could be converted primarily by changes in ϕ and ψ angles for MeA 10 and/or Gly 11. Shown in Figure 5 are the placement of three different structural forms of alamethicin within the lipid bilayer: a bent and linear NMR form and the crystal structure (Fox & Richards, 1982; Franklin et al., 1994). For the first or bent form, two orientations were examined, one where the N-terminus lies along the bilayer normal, and a second where the C-terminus lies along the bilayer normal, shown in Figure 5, panels A and B, respectively. The placement shown in Figure 5A is not consistent with the data shown in Figure 4 as it requires much shallower nitroxide depths at positions 9 and 15 than are measured. The structure shown in Figure 5B is consistent with the power saturation data; however, this structure can be discarded because recent solid state NMR data indicates that this segment of alamethicin lies along the bilayer normal (North et al., 1995). Panels C and D, Figure 5, show the placement of a linear NMR derived structure and the crystal structure through the lipid interface, respectively. Both these structures produce nitroxide positions from the interface that are consistent with the data, within experimental error. Given the placement of the C-terminus in the aqueous solution, the N-terminus of this peptide resides within the membrane hydrocarbon and fails to transit the membrane hydrocarbon (see Discussion).

The conclusions reached here regarding the zero voltage structure of alamethicin are consistent with the uncertainty inherent in these power saturation data. It should be noted that there is uncertainty associated with both the placement of the nitroxide relative to the membrane interface and the relative distances between nitroxide groups. The former error is associated with the calibration of Φ values as a function of depth and appears to be on the order of 2−3 Å. Measurements made with any one nitroxide are highly reproducible, and the relative distances between sites on the peptide are probably more accurate, with an uncertainty of less than 2 Å.

DISCUSSION

The EPR measurements described above provide strong evidence that alamethicin is in a linear form and inserted along the bilayer when membrane bound. The C-terminus of the peptide lies on the aqueous side of the membrane−solution interface and is estimated to be about 4 Å from the phosphate groups. This placement of the C-terminus when combined with depths for positions 15 and 9 is consistent with high resolution structures that are obtained from NMR and X-ray diffraction (Fox & Richards, 1982; Franklin et al., 1994). The leucine-MeA analog of alamethicin, L1, is placed approximately 3−4 Å deeper in the membrane hydrocarbon than is alamethicin.

Shown in Figure 6 are CPK structures for alamethicin and the leucine analog of alamethicin, L1, within the lipid bilayer. These structures are based on high resolution NMR data in SDS micelles for alamethicin and L1 and are positioned using the EPR data obtained here. Interestingly, this position for the C-terminus places the N-terminus of alamethicin within the membrane hydrocarbon. For the models shown in Figure 5C,D, the N-terminus is at a distance of 27 Å from the phosphate headgroup. Taking a phosphate to phosphate distance of 43 Å for egg PC (Caffrey & Feigenson, 1981),

[3] None of the nitroxide labels used for the calibration curve (dashed line in Figure 4) show Φ values as negative as that for the alamethicin C-terminus (Altenbach et al., 1994). The assignment of −4 Å for this label was made by extrapolating the linear dependence of the calibration curve. This is not unreasonable because gradients of O_2 and NiAA should extend outside as well as inside the membrane−solution interface. To test for the validity of this assumption, we measured the Φ value for an aqueous peptide. Listed in Table 2 are power saturation parameters and the Φ parameter for spin-labeled glutathione in aqueous solution. This labeled aqueous peptide provides a limiting value for Φ which is slightly more negative than that for the C-terminal alamethicin label. Thus, the C-terminus is not yet in a bulk aqueous environment. It should be noted that because the gradients of [O_2] or [NiAA] are diminished away from the interface, there will be a higher uncertainty for the placement of this position compared to other labeled positions.

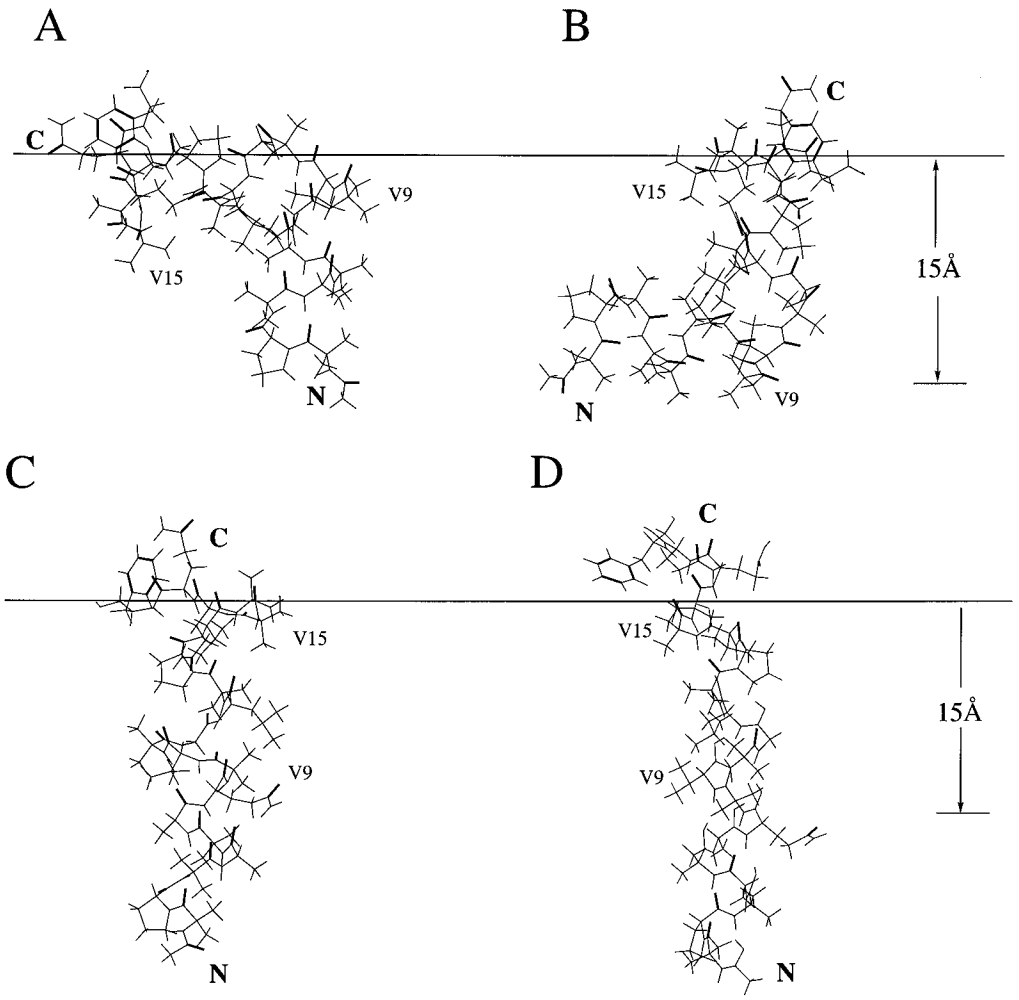

FIGURE 5: Models for different orientations and conformations of alamethicin that were considered in the analysis of the cw power saturation EPR data. (A) A bent structure derived from NMR data in SDS with the N-terminus aligned along the bilayer normal (Franklin et al., 1994). (B) Bent structure in (A) with its C-terminus aligned along the bilayer normal. (C) Linear structure derived from NMR data in SDS. (D) X-ray crystal structure of alamethicin (Fox & Richards, 1982). The structures and positions shown in (D) and (C) produce the best fit with the EPR data.

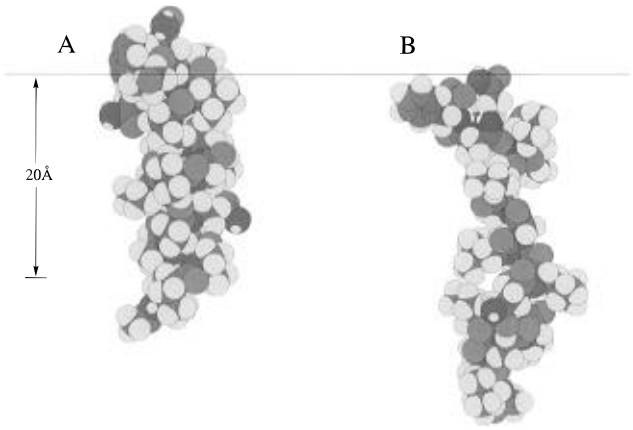

FIGURE 6: A comparison of the position of alamethicin with the alamethicin analog L1. The structure of L1 that is shown was obtained from the results of a simulated annealing of NMR restraints for L1 in SDS micelles (Jacobs and Cafiso, unpublished).

the N-terminus lies approximately 16 Å from the opposite interface, well within the hydrocarbon domain of the lipid bilayer. The exposure of a peptide terminus within the hydrocarbon would be expected to be quite unfavorable due to the presence of several unsatisfied hydrogen bonds. However, alamethicin may be well suited to bury its N-terminus. Proline is present at position 2, a feature that would eliminate one hydrogen bond donor, and the N-terminal end might also exist in a 3_{10} configuration, again reducing the hydrogen bonding requirement on this end of the structure. Acetylation of the N-terminus should also lower its energy within the membrane hydrocarbon.

The alamethicin analog where MeA residues are replaced by leucine lies deeper in the bilayer than native alamethicin. The most obvious explanation for this difference is an increased hydrophobic contact area at positions 16 and 17. These two MeA residues appear to have an aqueous exposure in alamethicin (Figure 6A), but are buried within the membrane hydrocarbon when replaced by leucine (Figure 6B). The replacement of these MeA residues for Leu should increase the free energy of partitioning by about 1 kcal/mol per residue.[4] Thus, an additional 2 kcal/mol in hydrophobic binding energy in the C-terminal region is sufficient to translate the peptide 3−4 Å deeper into the membrane interior. Electrical measurements on the L1 analog indicate that it exhibits a similar voltage-dependent activity to alamethicin, except that it has much shorter channel lifetimes

[4] The difference in the transfer free energies in an α-helical peptide between alanine and leucine is 1.2 kcal/mol (Engleman et al., 1986). Since MeA has an additional methyl group when compared to alanine, we expect that the difference between MeA and Leu will be ≤ 1 kcal/mol.

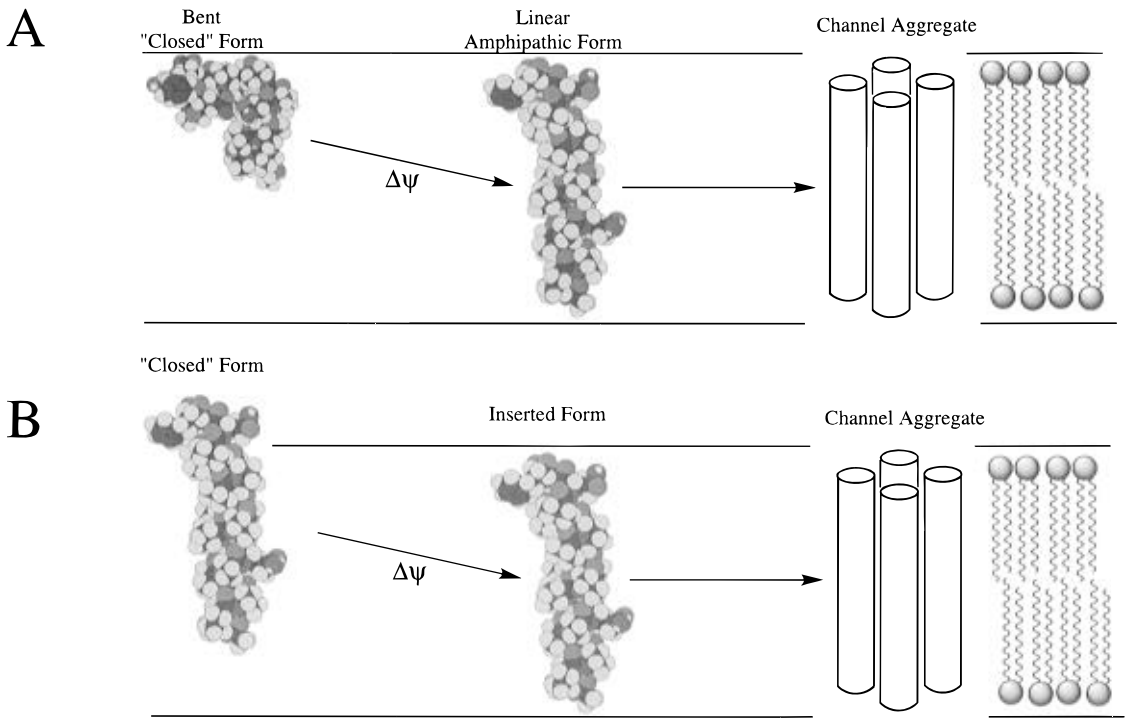

FIGURE 7: Two models for the gating of alamethicin. In (A), alamethicin is in a bent configuration in the absence of voltage. Application of a transmembrane electric field then linearizes the peptide and facilitates the formation of a laterally amphipathic helix that can aggregate. In (B), alamethicin is displaced out of the membrane interface in the absence of a potential. Application of a field drives peptide insertion and may promote a structural change that results in aggregation and channel formation.

(Molle et al., 1989). It has also been shown in this laboratory that while native alamethicin crosses vesicle bilayers quite slowly the leucine analog crosses with rates that are at least 1000 times faster (Jayasinghe and Cafiso, unpublished). The differences in translocation rates between these two peptides may be related to the difference in their equilibrium positions.

Previous work using oriented circular dichroism and X-ray diffraction provides evidence that alamethicin can undergo a transition between a surface and transmembrane orientation both as a function of peptide concentration and as a function of lipid hydration (Huang & Wu, 1991; Wu et al., 1995). We have not investigated these peptides using EPR at hydration levels less than 100%, or in membranes composed of diphytanoyl PC. In egg PC at full hydration we observe the peptide to be inserted over a range of peptide concentrations, and this result is consistent with the previous CD and X-ray work. In the same types of lipid vesicles formed from egg PC, the peptide is also observed to be voltage dependent (Archer & Cafiso, 1991). Thus, models for voltage-gating that involve a change in helix orientation (Baumann & Mueller, 1974) do not appear to be likely since the peptide is already aligned along the bilayer normal in the absence of a potential.

Recent work using high resolution NMR and paramagnetic enhancements of nuclear relaxation indicate that alamethicin is highly flexible in solution and micellar environments (North et al., 1994). This flexibility suggests a mechanism for gating which is shown in Figure 7A. In this model, the inactive form of alamethicin is based on an NMR derived structure where the hydrophilic face, formed from Gln 7, Gly 11, and Gln 18, is on the inner face of the bent structure. Application of a potential linearizes the peptide and promotes its insertion across the membrane to form a laterally amphipathic structure that can aggregate to form a conductive channel. This model suggests that the C and N helical domains of this peptide should lie in different orientations along the bilayer normal in the absence of voltage. The data shown here, however, provide strong evidence that the peptide takes on a roughly linear conformation when membrane bound, and there is no evidence for the presence of a highly bent form or for a model such as that shown in Figure 7A. This is also consistent with recent solid state NMR data on alamethicin, showing that the peptide is α-helical and is inserted along the bilayer normal (North et al., 1995). This NMR work provides an indication that the substitutions made here to attach nitroxides are not promoting major structural changes in the peptide.

The observation that the C-terminus is displaced out of the membrane interface suggests that further insertion and rearrangement of the peptide may be a process that leads to gating. Thus, a model like that shown in Figure 7B would be consistent with the EPR data presented here. This model is also not inconsistent with previous reports on spectroscopic changes that occur with voltage. One EPR study on the C-terminal nitroxide labeled alamethicin in chloroplasts showed that energization of the chloroplast membrane produced spectral changes that could be interpreted as a deeper insertion of the peptide (Wille et al., 1989). Work in model systems using charged polymers also indicated an increase in helical structure with the application of a potential (Brumfeld & Miller, 1990). This increase in helical content might be expected if the aqueous C-terminal domain were inserted deeper into a more hydrophobic environment with the application of a potential. Currently, the spin-labeled peptides and approach used here are being used to examine the structure of alamethicin in the presence of membrane electric fields.

In summary, site-directed spin-labeling and EPR have been used to investigate the position of labeled nitroxides attached to alamethicin and an alamethicin-like analog relative to the membrane−solution interface. This work demonstrates that alamethicin is inserted in a linear fashion along the bilayer normal with its C-terminus lying on the aqueous side of the membrane−solution interface. A linear SDS micelle NMR structure and the crystal structure are consistent with the depth measurements made here.

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