Dynamics and aggregation of the peptide ion channel alamethicin

Measurements using spin-labeled peptides

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ABSTRACT Two spin-labeled derivatives of the ion conductive peptide alamethicin were synthesized and used to examine its binding and state of aggregation. One derivative was spin labeled at the C-terminus and the other, a leucine analogue, was spin labeled at the N-terminus. In methanol, both the C and N terminal labeled peptides were monomeric. In aqueous solution, the C-terminal derivative was monomeric at low concentrations, but aggregated at higher concentrations with a critical concentration of 23 μM. In the membrane, the C-terminal label was localized to the membrane-aqueous interface using ^{13}C-NMR, and could assume more than one orientation. The membrane binding of the C-terminal derivative was examined using EPR, and it exhibited a cooperativity seen previously for native alamethicin. However, this cooperativity was not the result of an aggregation of the peptide in the membrane. When the spectra of either the C or N-terminal labeled peptide were examined over a wide range of membrane lipid to peptide ratios, no evidence for aggregation could be found and the peptides remained monomeric under all conditions examined. Because electrical measurements on this peptide provide strong evidence for an ion-conductive aggregate, the ion-conductive form of alamethicin likely represents a minor fraction of the total membrane bound peptide.

INTRODUCTION

Small peptide ion channels provide attractive, tractable systems with which to examine the molecular features of ion conduction. Among these model systems, alamethicin is a particularly intriguing peptide because it produces a highly voltage-dependent ion conductance in planar bilayers (Mueller and Rudin, 1968). Alamethicin is a 20 amino acid peptide from the fungus *Trichoderma viride*. It often is isolated as two components with the sequence Ac-Aib-Pro-Aib-Ala-Aib-Ala(Aib)-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol, where one component has Ala and the other Aib at position 6 (Pandey et al., 1977). The voltage-dependent ion conduction of alamethicin is accompanied by a strong concentration dependence and multiple conductance states (see, for example, Boheim and Kolb, 1978). These observations are most easily interpreted in terms of an aggregated state for the peptide ion channel, where changes in the size of the aggregate account for the multiple conductance states. Several models have been advanced to describe the voltage sensitivity and conduction of this peptide, and in each of these models the ion conductive state is an aggregate of alamethicin monomers.

The partitioning of alamethicin between the aqueous and membrane phases has been examined in phospholipid vesicles using the change in molar ellipticity at 224 nm that accompanies its binding (Schwarz et al., 1986; Rizzo et al., 1987; Stankowski and Schwarz, 1989). The concentration dependence of alamethicin binding indicates that the free energy of binding becomes more negative as the ratio of peptide to lipid increases. A logical interpretation of this cooperative behavior, consistent with the proposed structural models, is that the increase in membrane affinity is a result of the aggregation of the peptide within the membrane interior. A thermodynamic treatment, based on this interpretation of the binding data, was developed and used to study the state of aggregation of the membrane-bound peptide (Schwarz et al., 1986).

In this report, the dynamics and state of aggregation of two spin-labeled derivatives of alamethicin are described. One of these spin labels was previously synthesized and contains a proxyl label on the C-terminal phenylalaninol of alamethicin (Archer and Cafiso, 1989; Wille et al., 1989). The other is an N-terminally labeled derivative of a synthetic analogue of alamethicin where Aib residues are replaced by Leu (Molle et al., 1989). The spin-labeled alamethicins are used here to characterize the binding of this peptide to the membrane and to examine the peptide dynamics and state of aggregation. While the binding data is consistent with that previously obtained using optical techniques (Schwarz et al., 1986), the EPR spectra clearly indicate that aggregation of the membrane-bound peptide cannot account for the cooperativity seen in the binding data. The work carried out here has important implications for future

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work directed at elucidating the structure and mechanism of gating of this peptide ion channel. In particular, structural studies based on spectroscopic approaches (optical and magnetic resonance) may have difficulty probing the active form of this peptide.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (egg PC) was isolated from fresh hen eggs according to the procedure of Singleton et al. (1965), and was stored as a chloroform solution at −20°C under argon. Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Birmingham, AL). The spin-label precursor 3-carboxyproxyl was obtained from Eastman Kodak (Rochester, NY). Alamethicin was obtained from Sigma Chemical Co. (St. Louis, MO) and its chemical purity and sequence was characterized using HPLC and mass spectrometry. HPLC of alamethicin from Sigma Chemical Co. was carried out on a C-8 column in acetonitrile/water (0.1%TFA) and separated the peptide into two components, with component 1 being about twice as abundant as component 2. Components 1 and 2 had major ion peaks at an *m/z* of 1,964 and 1,978, a difference of one methyl unit. Sequencing of the peptide from the secondary ion fragments showed that position 6 was Ala in component 1 and Aib in component two. Residue 18 of alamethicin has been reported to contain either Glu or Gln, depending upon the source (Jung et al., 1981; Rizzo et al., 1987). The alamethicin used here from Sigma Chemical Co. has Gln at position 18. This was determined by measuring the masses after rigorous methylation and was also indicated by the lack of a reaction of residue 18 under conditions used to spin label the C-terminus. An analogue of alamethicin, I, was obtained from a commercial source (Peptide Technologies, Washington, DC.) having the sequence Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Cys-Gly-Leu-Leu-Pro-Val-Leu-Leu-Gln-Gln-Phe-NH_2. This sequence was chosen because it resembles a leucine analogue of alamethicin that is functionally and structurally similar to alamethicin (Molle et al., 1988, 1989), and because it has two possible sites for spin labeling. After receiving the peptide, the molecular weight of the peptide was confirmed by mass spectrometry, which yield primary ions at *m/e* 2,149 for MH^+ ion and 2,132 for MH^+-NH_3 ion.

Synthesis of a C-terminal spin labeled alamethicin analogue

The C-terminal spin labeled analogue of alamethicin, C-ala, was produced using a procedure similar to one described previously by coupling 3-carboxyproxyl to the C-terminal phenylalaninol of alamethicin using *N, N*-dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (Wille et al., 1989). The spin-labeled product, which was purified by flash chromatography in silica gel in chloroform/methanol (80:20), yielded a mass spectrum with major primary ions at *m/e* values of 2,133 and 2,147. These corresponded to the MH^+ ions for proxyl attached to component 1 and component 2, respectively. Fragment ions were used to further confirm the product, and the following C-terminal B ions from fragmentation at Pro14 were prominent in the spectrum: $B_{16}Y_7$ (282), $B_{17}Y_7$ (367), $B_{18}Y_7$ (495), $B_{19}Y_7$ (623) and Y_7-proxyl (942). This nomenclature follows that outlined previously for single and multipoint fragmentation of the peptide backbone where $B_{16}Y_7$ represents P-V-α, $B_{17}Y_7$ represents P-V-α-α, etc. (Hunt et al., 1986). The absence of the following ions: $B_{18}Y_7$-proxyl (663), $B_{19}Y_7$-proxyl (791) and Y_7 (774), indicates that the label is attached to the C-terminal Phol residue.

Synthesis of an N-terminal spin labeled leucine alamethicin analogue

An N-terminal spin-label of the leucine alamethicin analogue, I, was produced by first blocking the Cys10 residue with maleimide to produce a product referred to here as II. II was then reacted at the N-terminus with a symmetric spin-labeled anhydride to produce the spin-labeled product (this peptide label will be referred to as N-leu-ala). To produce II, Cys^{10} was blocked with maleimide by dissolving 10.5 mg of I in methanol followed by addition of a solution of *N*-ethylmaleimide (5.4 mg in 40 μL methanol plus 2 mL sodium acetate buffer pH 4.5). The reaction mixture was stirred at 40°C for 1.5 h and monitored by TLC on silica gel G plates (Analtech, Newark, DE) in $CHCl_3$/MeOH/H_2O (65:25:4). In this eluent, the R_f of the product II was 0.48. The reaction mixture was then subjected to rotary evaporation and lyophilized to remove solvent and buffer. A 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) test of the lyophilized product II indicated that there were no free sulfhydryls. II was purified on silica gel 60 (70-230 mesh ASTM; EM Science, Cherry Hill, NJ) eluted with $CHCl_3$/MeOH (75:25) and then $CHCl_3$/MeOH/H_2O (65:25:4). The fractions containing II were subjected to rotary evaporation and lyophilized to yield a white powder. The product was confirmed by mass spectrometry which yielded the primary ions MH^+ at 2,274 and MNa^+ at 2,297. The symmetric anhydride of 3-carboxyproxyl was produced by dissolving 11.8 mg of 3-carboxyproxyl in 0.1 mL DMF (peptide synthesis grade) along with 120 μL of a 0.275-M solution of DCC in dichloromethane. The mixture was stirred for 1 h and then filtered to remove insoluble dicyclohexyl urea. The symmetric anhydride solution was added to a solution of II (6.6 mg) dissolved in 1 mL DMF. The reaction mixture was stirred for 1 h and monitored by TLC on silica gel G plates in $CHCl_3$/MeOH/H_2O (65:25:4). In this eluent, the product had an R_f of 0.67. The DMF was removed by repeated additions of water followed by lyophilization. The product was purified on a column of silica gel (EM Science) run with $CHCl_3$/MeOH/H_2O (65:25:4), and then rotary evaporated and lyophilized from water to yield a white powder. The product, *N*-leu-ala, was confirmed by mass spectrometry which yielded major ions at an *m/e* of 2,444, 2,428, and 2,466 corresponding to the primary ions MH^+, MH^+-H_2O, and MNa^+.

Preparation of lipid vesicles

Sonicated lipid vesicles were prepared by drying aliquots of lipid under vacuum for periods of at least 15 h, hydrating the lipid in the appropriate buffer solution, and ultrasonically irradiating the sample at 0°C under argon as previously described (Castle and Hubbell, 1976). To prepare larger "extruded" vesicles, the vortexed lipid suspension was freeze-thawed five times, and then extruded 10 times through 0.05 μM polycarbonate filters using a commercially available unit (Lipex Biomembranes Inc., Vancouver, B.C.). After the final extrusion, the phospholipid vesicles were stored at 4°C under an argon atmosphere until use. Final lipid concentrations were determined using a modified Fiske-Subbarrow phosphate assay (Bartlett, 1959). Sonicated vesicles were sized using both ^{14}C sucrose (Cafiso and Hubbell, 1978) and a spin probe assay (Todd et al., 1989). Both sizing techniques indicated that the average diameter of the sonicated vesicles was 220 ± 30 Å. The extruded vesicles had an average diameter of 400 ± 50 Å as determined using the spin probe assay.

EPR spectroscopy

EPR spectroscopy was carried out using either a Varian (Sunnyvale, CA) E-line Century Series or a modified V-4500 series X-band spectrometer. A 100-μl quartz sample cell was used to hold the sample. EPR spectra were obtained with a modulation amplitude of

1.0 G and a power of 10 mW. Spectra used for the determination of rotational correlation times τ_c, were taken with modulation amplitudes in the range of 0.2–0.4 G. Processing and manipulation of the EPR spectra were carried out on an IBM compatible personal computer using the software package "EPR Data Acquisition System, Version 2.2" (Philip D. Morse, II and the University of Illinois College of Medicine, Urbana, IL). The klystron frequency was determined using a Hewlett Packard X532B frequency meter and the magnetic field strength was measured using an ^{1}H NMR gauss meter.

To measure the binding of spin-labeled alamethicin to phospholipid vesicles, a special quartz sample cell was used that allowed additions of peptide or lipid to the sample without removal of the cell from the EPR cavity. The binding of the peptide to the membrane was determined from the EPR spectrum by measuring the amplitude of the high field nitroxide resonance in a manner identical to that used previously for nitroxide probes (see Castle and Hubbell, 1976). The partitioning of the probe can be expressed as the ratio of the number of spins bound to the membrane to the number of spins in solution, N_b/N_f or λ. The binding data was then analyzed and expressed using two different approaches. In one, the measurable parameter λ was related to a partition coefficient β having units of length. In this case, $1/c_l = \beta A_l(1/\lambda) + V_l$, where c_l is the lipid concentration, A_l is the average lipid surface area (5.16×10^6 cm^2/g lipid for sonicated vesicles) and V_l is the specific volume of the lipid (see Flewelling and Hubbell, 1986). In another approach, the molar ratio, r, of lipid associated peptide to lipid ($r = c_b/c_l$) was plotted as a function of the free peptide concentration, a representation used previously to illustrate the cooperative binding of alamethicin (Schwarz et al., 1986).

Spectra of oriented bilayers were obtained from stacks of hydrated lipid bilayers on glass slides. To form these layers, spin-labeled peptide (0.05–0.10 mol%) and/or 5-doxylstearate (0.2 mol%) was added to a solution of lipid (3.2 mg egg PC in ~125 μL of chloroform). One drop of this mixture was applied to each glass slide (9.9 × 12 mm). The lipid-coated slides were dried for 2–3 h in a vacuum desiccator. The slides were then sealed in an argon/water saturated chamber for at least 24 h to allow the lipid layers to hydrate. After hydration, 10–12 lipid-coated slides were stacked and pressed together. The stack of slides was secured in a teflon holder that could be rotated in the EPR cavity. To ensure that the system used here produced oriented bilayers, spectra of 5-doxylstearate were obtained and compared with previous data on oriented nitroxides (see, for example, Gaffney and McConnell, 1974).

Determining the membrane location of peptide associated spin labels

The location of the nitroxide label attached to the C-terminus of alamethicin was determined by investigating the effect of the nitroxide on the ^{13}C spin-lattice relaxation rate of the membrane lipid. This was carried out in a manner similar to that used previously to localize paramagnetic hydrophobic ions (Ellena et al., 1988). NMR spectroscopy was performed on a General Electric GN 300 NMR spectrometer operating at a proton frequency of 300.53 MHz and a carbon-13 frequency of 75.57 MHz. A sample size of 0.5 mL was used in a 5-mm NMR tube, with a sample temperature of 28 ± 1°C. ^{13}C spin-lattice relaxation rates (T_1^{-1}) were measured using a fast inversion recovery experiment, and all 180° pulses were 90x, 180y, 90x composite pulses. ^{1}H decoupling was used throughout the experiment. The membrane samples contained 130 mM lipid sonicated in 25 mM phosphate buffer, pD 7.4. To these samples either 3 mol% spin-labeled alamethicin or native alamethicin (as a control) were added.

Determining rotational correlation times from EPR spectra

The rotational mobility of the nitroxide probes was quantitatively determined from the transverse relaxation rates measured from EPR spectra using procedures similar to those described previously (Keith et al., 1970; Nordio, 1976). Here, the rotational correlation time, τ_c, was expressed in terms of the following relationship:

$$\tau_c = \frac{1}{b}\left(\frac{b}{8} - \frac{4\Delta\gamma Ho}{15}\right)^{-1}\left[\frac{T_2(0)}{T_{2(-1)}} - 1\right] T_2(0)^{-1},$$

where Ho is the strength of the magnetic field, $T_2(0)$ and $T_2(-1)$ are the transverse relaxation times of the $m_I = 0$ and $m_I = -1$ nitroxide resonances, $b = 4\pi/3[A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})]$, and $\Delta\gamma = (\beta_e/\hbar)[g_z - \frac{1}{2}(g_x + g_y)]$. The transverse relaxation time $T_2(0)$ was taken from the linewidth of the EPR spectrum, and the ratio of the T_2 values was taken from the relative peak height intensities. Solution spectra were used to measure A_o, the isotropic hyperfine coupling constant. A_{max} was determined from the spectra of samples frozen in liquid nitrogen, 77°K. Under these conditions, A_{zz} is equal to A_{max}, and these values were then used to calculate A_{xx}, A_{yy} (which are assumed to be equal). The g values for 3-carboxyproxyl were taken from previously reported values (Bordeaux et al., 1973) and were corrected for differences in g_0 values after a method described previously (Snipes et al., 1974). The g-values used were $g_{xx} = 2.0089$, $g_{yy} = 2.0055$, g_{zz} 2.0030. It should be noted that this calculation of τ_c assumes isotropic motion of the spin label.

Determining the activity of alamethicin derivatives in vesicles

The activity of both the spin labeled alamethicin analogues used here was tested in vesicle systems using procedures that are described in detail elsewhere (Archer and Cafiso, 1991). In these measurements, current-voltage curves for the peptides were obtained by establishing voltages in vesicles and monitoring the current flow through the alamethicin channel with potential sensitive nitroxide probes. Voltages were established across extruded lipid vesicles by creating a proton or K^+ electrochemical equilibrium. The current flow through the alamethicin channel was monitored using a nitroxide probe sensitive to the proton gradient, while protons were maintained in an electrochemical equilibrium with a protonophore. The spin-labeled peptides did not interfere with the signal from the probe nitroxides. Under the conditions of these measurements, the spin-labeled peptides were membrane bound and the amplitude of the high-field resonance (which was used to monitor the pH probe) was dominated by the aqueous pH probe signal.

RESULTS

Dynamics and aggregation of spin-labeled alamethicin in aqueous solution

Shown in Table 1 are the spectral parameters obtained for the C-terminal proxyl derivative of alamethicin (C-ala) and 3-carboxyproxyl. These values were used along with an estimate of the transverse relaxation rate to estimate the correlation time, τ_c, for the probe attached to C-ala (see Methods). This calculation yielded

TABLE 1 **The buffer solution used in these experiments contained 125 mM K_2SO_4, 25 mM MOPS, pH = 7.0**

Label	A_0	A_{max}	A_{xx}, A_{yy}
3-carboxyproxyl in buffer	16.26 ± 0.01	36.13 ± 0.6	6.33 ± 0.04
C-ala in buffer	15.98 ± 0.03	36.27 ± 0.06	5.84 ± 0.05
3-carboxyproxyl in methanol	15.26 ± 0.02	36.19 ± 0.06	4.8 ± 0.04
C-ala in methanol	15.13 ± 0.02	36.28 ± 0.06	4.56 ± 0.04
C-ala in eggPC		35.64 ± 0.06	

Measurements in sonicated lipid vesicles were carried out at a lipid concentration of 85 mg/ml.

a correlation time of 0.22 ns in a buffered aqueous solution at pH = 7. This value agrees with the value obtained previously for this derivative (0.28 ns) in water using a similar procedure (Wille et al., 1989), but is shorter than the values obtained for the proline protons of the peptide in methanol (0.4–0.7 ns) using NMR spectroscopy (Banerjee et al., 1983; Esposito et al., 1987). Because the proline protons reflect backbone motion, this lower correlation time likely reflects an additional segmental motion of the proxyl moiety. Measurements in methanol for C-ala yielded a correlation time of 0.12 ns, and the ratio of correlation times (methanol/water) is 0.55, which agrees well with the ratio of viscosities for these solutions of 0.60. Because the peptide is monomeric in methanol (McMullen and Stirrup, 1971; Esposito et al., 1987), this agreement indicates that the peptide is also monomeric at an aqueous concentration of 15 μM.

In methanol, spin-labeled analogues of alamethicin yielded signal intensities that were completely linear with concentration (5–100 μM), and showed no indication of aggregation. However, in aqueous solution, aggregation at low peptide concentrations was apparent. Fig. 1 shows the dependence of the intensity of the center field resonance ($m_I = 0$) as a function of the concentration of the labeled peptide C-ala. At low concentrations of C-ala, the amplitude of the EPR spectrum in aqueous solution increases linearly with the concentration of peptide; however, above 20 μM peptide the signal amplitude reaches a maximum and becomes independent of the peptide concentration.[1] At very high concentrations of peptide, an EPR spectrum

[1]In the absence of lipid, the signal intensity from spin-labeled alamethicin increased linearly with the concentration of peptide up to ~20 μM, but did not intersect the concentration axis at zero. The simplest interpretation of this observation is that the hydrophobic peptide binds to some portion of our mixing apparatus or EPR cell. This was a small effect that corresponded to the absorption of few tens of pmoles of peptide. The absorption of alamethicin to teflon cells used for planar bilayer studies has also been observed (Stankowski et al., 1988).

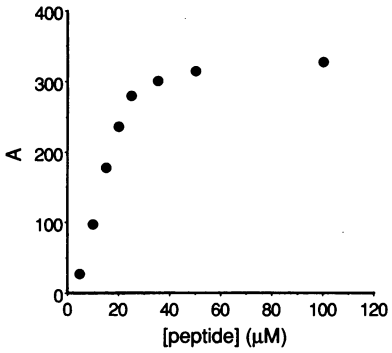

FIGURE 1 Signal intensity of the $m_I = 0$ resonance for the C-terminal spin-labeled alamethicin, C-ala, in aqueous solution as a function of the alamethicin concentration. For the linear portion of the curve alamethicin is monomeric, but undergoes a micellization at a critical concentration of ~23 μM. The aqueous sample contains 125 mM K_2SO_4, 25 mM MOPS, pH = 7.

taken at liquid nitrogen temperatures reveals clear evidence for spin exchange. These observations are consistent with an aggregation or micellization of the peptide, a process that was previously observed using hydrodynamic techniques (McMullen and Stirrup, 1971) or fluorescent fragments of alamethicin (Mathew et al., 1981). From the data shown in Fig. 1, a critical concentration of 23 μM is obtained for the aqueous micellization of C-ala.

The N-terminal label of the alamethicin derivative, *N*-leu-ala, is a much more hydrophobic peptide than native alamethicin and it was not soluble in aqueous solution at levels that would allow detection by EPR (greater than ~1 μM). However, this derivative was soluble in methanol and yielded a correlation time of 0.3 ns, which is about twice that obtained for the C-terminal proxyl nitroxide. This reduction in motional rates could reflect the additional double bound character in the peptide-label linkage, or the greater helical content of the N-terminal domain of the peptide (Esposito et al., 1987).

Both spin-labeled analogues yield nonlinear current voltage curves

Using procedures described elsewhere, the ion conductive properties of the spin-labeled peptides were tested in vesicles, under conditions where all the peptide was membrane bound. Shown in Fig. 2 are current voltage data for both C-ala and N-leu-ala. Because these experiments were carried out under two different sets of conditions, two different *y* axes are used. The activity obtained for C-ala and N-leu-ala are comparable to the activity obtained for native alamethicin under each of

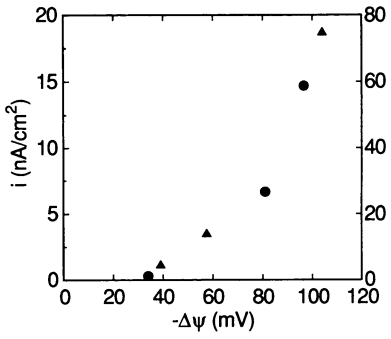

FIGURE 2 Current voltage-curves for C-ala (● and left *y*-axis) and *N*-leu-ala (▲ and right *y*-axis) in extruded 400 Å lipid vesicles. For the case of *N*-leu-ala, the membrane potentials were established using a pH gradient. In the case of C-ala, K^+ gradients were used to establish the membrane potentials. As a result, the absolute magnitude of the currents are not directly comparable. However, these currents are similar to those obtained for native alamethicin under each experimental arrangement. In each case, protons are maintained in an electrochemical equilibrium and the currents are monitored using a ΔpH-dependent paramagnetic probe as described elsewhere (for more details see Archer and Cafiso, 1991).

these conditions (see Archer and Cafiso, 1991). This data indicates that in spite of the attachment of the proxyl group to these derivatives, their ion conductive behavior has not been significantly altered.

Dynamics of spin-labeled alamethicin in lipid bilayers

Shown in Figs. 3, *a* and *b*, are the EPR spectra of C-ala and *N*-leu-ala, respectively, in lipid vesicles of egg PC. As expected, the C-ala derivative exhibits less mobility

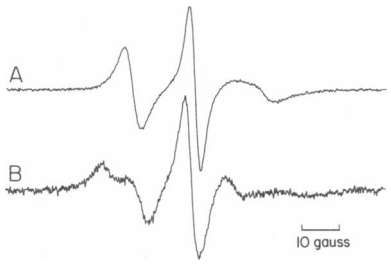

FIGURE 3 (*A*) EPR spectra for the C-terminal spin labeled alamethicin analogue, C-ala. This peptide is at a concentration of 15 μM in the presence of sonicated eggPC vesicles at a lipid concentration of 40 mg/ml. At this concentration of lipid the peptide is totally membrane associated. (*B*) EPR spectrum for the N-terminal spin labeled leucine alamethicin analogue, *N*-leu-ala. This labeled peptide is at a concentration of 20 μM in eggPC vesicles at a lipid concentration of 140 mg/ml. In both *A* and *B*, the aqueous buffer contains 125 mM K_2SO_4 and 25 mM MOPS, pH = 7.

than in aqueous solution. The apparent correlation time estimated from this spectrum is 3 ns. To provide some insight into the nature of the C-ala motion, EPR spectra for this spin-labeled derivative were obtained in oriented lipid bilayers. Shown in Figs 4, *a* and *b* are the $Ho \parallel z'$ and $Ho \perp z'$ spectra for C-ala in oriented eggPC bilayers, respectively. In each orientation, the spectrum exhibited at least two components which were most easily distinguished in the $Ho \parallel z$ spectrum. A control experiment was performed where the oriented sample was prepared in the same manner except that native alamethicin was incorporated along with 1 mol% 5-doxyl stearate. The spectra that were obtained from this control sample for the $Ho \parallel z'$ and $Ho \perp z'$ orientations indicate that the bilayers are highly oriented (Gaffney and McConnell, 1974) and that poor bilayer alignment is not an explanation for the spectra in Fig. 4.

The location of the nitroxide of C-ala in the membrane was determined by examining its effect on the ^{13}C relaxation rates of the membrane lipid (see Methods). The enhancement in the relaxation rate obtained for the lipid carbons in the presence of labeled vs. unlabeled alamethicin is shown in Fig. 5. The enhancement in the relaxation rate is dominated by proximity between the label and the lipid carbons (Ellena et al., 1988) and the data shown here is consistent with an interfacial location for the C-terminus of C-ala.

Phase partitioning and cooperative binding of spin-labeled alamethicin

Shown in Fig. 6 is a spectrum of C-ala in solution and in the presence of membrane vesicles at concentration of 0.15 mg/ml. In the presence of vesicles, the spectrum is a composite of two spectra, one that clearly arises from an aqueous population, and one that is identical to the membrane-bound spectrum. As described above (see

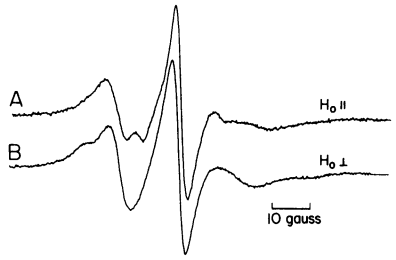

FIGURE 4 (*A*) An EPR spectrum of spin-labeled alamethicin in oriented eggPC bilayers at a concentration of 0.1 mol%. This spectrum was acquired with the magnetic field oriented parallel to the bilayer normal, *z'*. Two components (marked) are visible in this oriented spectrum (*B*) An EPR spectrum of the sample in *A*, except that the magnetic field is now oriented perpendicular to the magnetic field.

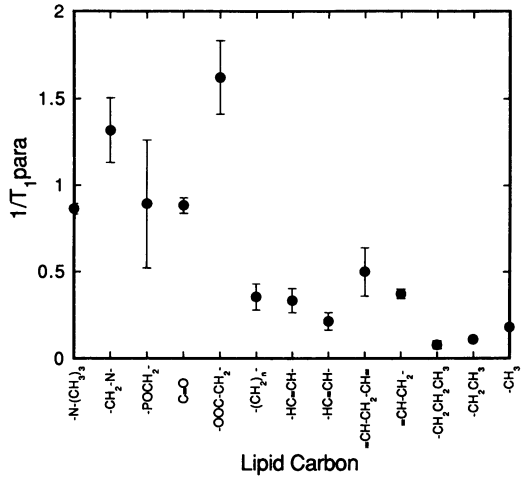

FIGURE 5 A plot showing the enhancement of the 13C relaxation rates for various membrane lipid resonances in the presence of the C-terminal labeled alamethicin analogue. The membrane lipid is eggPC and the values of $1/T_{1para}$ represent the difference in the relaxation rate between membranes containing 3 mol% spin-labeled alamethicin and membranes containing 3 mol% unlabeled alamethicin.

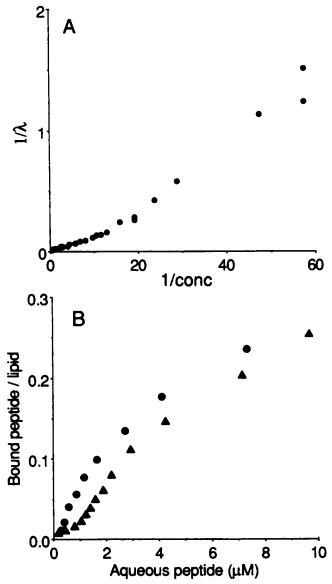

FIGURE 7 (*A*) Binding curve for the C-terminal labeled alamethicin in the presence of sonicated egg PC vesicles. The ratio of the aqueous to bound peptide populations (1/λ) is plotted as a function of the reciprocal of the lipid concentration in milliliters per milligrams. For the linear portion of this binding curve, the slope is fit well with a value for the partition coefficient β of 0.017 cm. In this experiment the total concentration of the C-terminal label is 11.4 μM and the buffer contains 125 mM K_2SO_4, 25 mM MOPS, pH = 7. (*B*) A binding curve for the C-terminal labeled alamethicin derivative (13.8 μM) in the presence of egg PC (●) and DOPC (▲). Here the bound peptide/lipid is plotted vs. the free peptide concentration in a fashion identical to that used previously (Schwarz et al., 1986). The cooperativity seen previously for native alamethicin is also apparent here for the C-terminal spin-labeled alamethicin derivative. The data for DOPC is made in 10 mM tris-HCL, pH = 7.3 for a comparison with data on unlabeled alamethicin.

Fig. 1) the signal intensity of the peptide resonance from solution is linear up to 15 μM, and the concentration of peptide in the partitioning experiments did not exceed this level. From the amplitude of the high-field resonance of spectra for C-ala, the membrane-aqueous phase partitioning for the peptide was determined (see Methods). Shown in Fig. 7 *a* is a binding curve for C-ala in sonicated eggPC vesicles. In this plot, the slope is constant up to ~300 peptides/vesicle (10 lipids/peptide) and then increases. This increase represents a decrease (or saturation) of the binding above this peptide concentration. From the linear portion of the curve, a partition coefficient, β, of 0.017 cm is obtained. If the peptide occupied the entire membrane volume, this partition coefficient would correspond to a free energy of binding of −6.2 Kcal/mol. The data for the binding of C-ala to eggPC and dioleoylphosphatidylcholine (DOPC), is plotted in Fig. 7 *b* as the ratio of peptide to lipid vs. the total peptide concentration. Plotted in this fashion, the saturation of binding appears as a decrease in slope above 2 μM aqueous peptide. The data for DOPC in Fig. 7 *b* is clearly different than that seen for egg PC, and shows a cooperativity in binding at low concentrations (<1 μM) that was seen previously for native alamethicin in lipid vesicles (Schwarz et al., 1986).

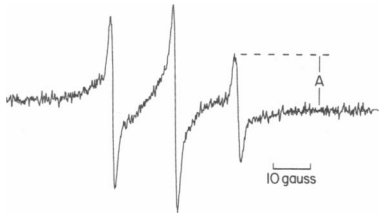

FIGURE 6 An EPR spectrum of the C-terminal labeled alamethicin at a concentration of 14 μM in the presence of sonicated eggPC vesicles (0.15 mg/ml lipid in 125 mM K_2SO_4, 25 mM MOPS, pH = 7). This spectrum is a composite of two spectra arising from both aqueous and a membrane associated peptide. It is qualitatively similar to spectra obtained previously for amphiphilic nitroxides. The phase partitioning of this peptide is quantitated from the amplitude of the high-field nitroxide resonance, A, using procedures described elsewhere (Castle and Hubbell, 1976).

The binding curves obtained previously for native alamethicin are similar to those shown here (Fig. 7 *b*). The cooperativity that appears in this binding curve was previously interpreted in terms of an aggregation of the membrane bound peptide. From the sharp upward turn in this curve at low concentrations, an apparent "critical"

concentration for the aggregation of alamethicin in the membrane can be determined. Above the critical concentration, a majority of the membrane-bound alamethicin is expected to exist in an aggregated state. For C-ala, this critical concentration is ~0.8 μM in DOPC. This is less than the 2.1 μM critical concentration reported for native alamethicin in this lipid; however, this could be a result of different experimental conditions. The critical concentration was shown previously to be sensitive to ionic strength and lipid saturation (Stankowski et al., 1988).

To test the idea that cooperativity in the binding is due to aggregation, EPR spectra were obtained for C-ala in lipid vesicles both below and above this critical alamethicin concentration. When the concentration of membrane bound C-ala was increased, changes in the spectrum at high peptide to lipid ratios were observed; however, these changes were not observed except at high concentrations where exchange broadening is normally seen (Scandella et al., 1972). The two spectra shown in Fig. 8 provide a more critical test for aggregation. Here the concentrations of C-ala are identical (i.e., the membrane concentration of spin are the same), but the ratio of lipid to peptide is varied from 100:1 to 5:1 by changing the level of unlabeled alamethicin. The spectral lineshapes for these two peptide concentrations (Fig. 8) are virtually identical; in addition, the intensities of the centerfield peak ($m_I = 0$) differed by only 4%, a difference that is at the level of our experimental uncertainty. Therefore there is no evidence for the aggregation of alamethicin even at concentrations well above the "critical" concentration. Although it was not possible to observe the partitioning of *N*-leu-ala, or determine its critical concentration, the spectra of *N*-leu-ala were examined at very high and low membrane peptide concentrations. Again the unlabeled analogue of *N*-leu-ala was used to vary the membrane concentration of peptide to avoid the effects of spin exchange. As was the case for C-ala, the spectra for *N*-leu-ala at dramatically different concentrations are virtually identical and provide no sign of aggregation (data not shown). From the Saffman-Delbruck theory of rotational diffusion in membranes, it can be shown that the ratio of expected rotational correlation times for alamethicin monomers, dimers, and tetramers is approximately 1:2:4 (Saffman and Delbruck, 1975). Because the EPR spectra will show a clear change in lineshapes for a twofold increase in correlation time, the above data indicates that the aggregation state of the alamethicin analogues examined here does not change over a concentration range where changes in the free energy of binding for alamethicin are seen. Thus, the data shown here do not support a model where binding "cooperativity" is due to peptide aggregation.

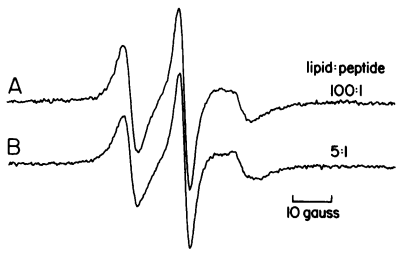

FIGURE 8 (*A*) An EPR spectrum of 10 μM C-terminal labeled alamethicin in sonicated eggPC vesicles at a lipid to peptide ratio 100. (*B*) An EPR spectrum of 10 μM C-terminal labeled alamethicin plus 190 μM unlabeled alamethicin to yield a total lipid to peptide ratio of 5. For each case, *A* and *B*, the lipid concentration is 0.8 mg/ml and the buffer is 125 mM K_2SO_4, 25 mM MOPS, pH = 7. If the cooperative binding of alamethicin is a result of the membrane aggregation of alamethicin, then the spectra in *A* and *B*, should represent primarily monomeric and highly aggregated peptide.

DISCUSSION

Two alamethicin spin labels were synthesized and used to examine the state of aggregation of the membrane bound peptide. The C-terminal proxyl derivative that was used here was synthesized previously (Archer and Cafiso, 1989; Wille et al., 1989) and was previously shown to be similar in its activity to native alamethicin in planar bilayer systems (Wille et al., 1989). The data presented in Fig. 2 demonstrates that this analogue is also active in extruded vesicle systems and is similar in its activity to that of native alamethicin in these same membrane systems (Archer and Cafiso, 1991). The EPR spectrum of this labeled peptide is sensitive to its state of aggregation in aqueous solution. Using EPR, a value of 23 μM for the critical micellization of alamethicin was obtained, which is in the range of values found for alamethicin fragments (Mathew et al., 1981). An N-terminal label of an alamethicin analogue, where the Aib residues are replaced by leucine, was also synthesized. This analogue was chosen because it was previously shown to be an active non Aib-containing analogue with a similar structure to native alamethicin (Molle et al., 1989). The data obtained here (Fig. 2) also demonstrates that this spin-labeled analogue has an ion channel activity in extruded vesicles. When the phase partitioning of C-ala is compared with that for native alamethicin using optical techniques (Stankowski and Schwarz, 1989), similar partition coefficients are obtained. Thus, in spite of the addition of a proxyl moiety to either the C- or N-terminus, the labeled peptides used here appear to behave in a similar manner to their unlabeled counterparts. Not surprisingly, the N-termi-

nal leucine analogue appears to be substantially more hydrophobic than native alamethicin and has a minimal solubility in water.

The binding data shown in Fig. 7 *b* shows a cooperativity for the binding of C-ala that was seen previously for alamethicin in DOPC (Schwarz et al., 1986). The binding of alamethicin increases as the level of membrane-associated peptide increases until a saturation is reached at very high peptide concentrations. Because the active form of alamethicin appears to be an aggregate, a logical explanation for the cooperativity is the conversion of monomeric alamethicin into an aggregated form. An elegant thermodynamic model based on this interpretation was developed and demonstrated that membrane aggregation at a critical concentration of 2.5 μM (in DOPC) could explain this binding behavior (Schwarz et al., 1986). A similar binding behavior was observed here for the proxyl labeled C-ala, and provided a unique opportunity to test this model. When EPR spectra for this probe were examined under conditions where the peptide should have been highly aggregated (according to this thermodynamic model), there was no evidence for the transition of alamethicin to an aggregated form. Because of its low aqueous solubility, a critical concentration for the N-terminal analogue could not be established; however, it also showed no evidence for conversion to an aggregated state when the lipid to peptide ratios were varied over a wide range. Thus, the membrane aggregation of alamethicin cannot account for the cooperative binding of alamethicin to membranes.

The membrane-bound form of the spin-labeled alamethicin analogues giving rise to the spectra shown here is almost certainly monomeric. Conceivably, the C- or N-terminal derivatives could exist in an aggregated form independent of the concentration of membrane bound peptide. This is not likely for at least two reasons. First, dilution of the labeled peptides with unlabeled analogues should have changed the line shape of the spectrum. Aggregation of the labeled peptide would have produced strong spin-exchange effects that would be dramatically reduced by dilution of the labeled peptide with the unlabeled analogue. Second, the line shapes that were obtained are not consistent with those expected for large aggregates. Thus, the membrane bound form of either of the spin-labeled analogues of alamethicin appears to remain monomeric under a wide range of concentrations examined here.

This surprising result raises several important questions. First, there is strong evidence from electrical measurements that the active form of alamethicin is a membrane-bound aggregate having as many as 10 monomers. However, the experiments carried out here failed to detect evidence for an aggregated form of the peptide. Assuming that the active form of alamethicin is indeed an aggregate, this observation indicates that the active form is only a minor fraction of the total membrane-associated peptide. This conclusion has important consequences for spectroscopic measurements (optical or magnetic resonance) aimed at studying the active form of alamethicin. For example, without procedures to enhance the population of the aggregated state, it will be extremely difficult to gain structural information on active state of alamethicin using spectroscopy. Presently, electrical measurements appear to be the only way to monitor the active state of this peptide.

Another intriguing question concerns the physical interactions that lead to the cooperative binding of alamethicin. The binding free energy of alamethicin to DOPC varies from −5.2 to −6 Kcal/mol below and above the inflection at 1 μM peptide (see Fig. 7 *b*). This modest energy difference of 0.8 Kcal/mol could be the result of any number of steric, electrostatic, or enthalpic peptide-lipid interactions. Indeed, structural changes in the lipid headgroup appear to accompany the binding of alamethicin (Banerjee et al., 1985). Such structural changes could alter the free energy of the membrane-bound peptide and lead to the observed cooperativity. Currently, experiments are underway to determine the source of the cooperativity in the binding.

Several observations regarding the behavior of the membrane-bound label deserve comment. The N-terminal label has a longer apparent correlation time than the C-terminal label. This could be a result of an additional motional restriction in the amide segment which joins the N-terminal label, or it could reflect the higher helical content believed to be present in the N-terminal portion of alamethicin (see, for example, Esposito et al., 1987). However, since the orientational dependence of this label is not yet characterized, the apparent difference in correlation time could simply reflect a difference in the orientation of the principal hyperfine tensor components relative to the axes of motional averaging for this molecule. Further work on this and other alamethicin derivatives is addressing these questions. The fact that there is a clear difference between the oriented spectra for C-ala (Figs. 4, *a* and *b*) indicates that a major fraction (and perhaps all) of the labeled peptide undergoes anisotropic motion. The presence of at least two components in the oriented spectra indicate that two or more distinct average orientations exist for the nitroxide label on this peptide. Because segmental motion about the ester linkage that attaches the label to the C-terminus should be very rapid on the EPR time scale, conformations about this segment are not expected to produce distinct populations in the spectra. Hence, the populations seen in Figs. 4, *a* and *b* are likely the result of different orientations for the peptide (or portions of the peptide) and not simply different label orientations. The

Ho ⊥ *z'* spectrum exhibits the larger outer splitting; therefore, the population giving rise to these outer extrema must have the average position of the nitroxide *z* axis aligned closer to the plane of the bilayer rather than to the bilayer director axis.

The use of ^{13}C-NMR to localize the nitroxide group in bilayers has been previously reported (Brulet and McConnell, 1975; Godici and Landsberger, 1974; Ellena et al., 1988). The measurements made here indicate that the C-terminus of alamethicin is near the membrane interface and demonstrate the feasibility of using this approach to obtain structural information on membrane-bound peptides. The use of this method is now being employed to provide further information on the structure and orientation of membrane-bound alamethicin.

In conclusion, two spin-labeled analogues of alamethicin were synthesized and EPR spectroscopy was used to examine the aggregation state and binding of alamethicin to membranes. The experiments carried out here indicate that the cooperative binding seen for alamethicin to lipid vesicles is not the result of a membrane aggregation of the peptide. No evidence for the aggregation of this peptide in the membrane could be found, and alamethicin appears to remain monomeric even at very high protein to lipid ratios. This observation indicates that the active, aggregated form of the peptide represents a very minor fraction of the total alamethicin.

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