Collisions Between Helical Peptides in Membranes Monitored Using Electron Paramagnetic Resonance: Evidence that Alamethicin is Monomeric in the Absence of a Membrane Potential

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ABSTRACT Alamethicin is a 20-amino-acid peptide that produces a voltage-dependent conductance in membranes. We investigated the state of aggregation of alamethicin in egg phosphatidylcholine and dioleoylphosphatidylcholine membranes by examining the EPR spectra obtained from an active analog of this peptide that is spin-labeled at its C-terminus. The dependence of both the linewidth and signal intensity as a function of peptide concentration exhibit exchange broadening as the peptide concentration is increased; however, the exchange rates are linear with peptide concentration as is expected for the simple diffusion of monomers. In addition, the spin-exchange rates obtained from the linebroadening are consistent with collisional rates that are predicted from free Brownian diffusion. The results provide strong evidence that in the absence of a membrane potential, alamethicin is largely monomeric in these membranes.

INTRODUCTION

Alamethicin is a small 20-amino-acid peptide from the fungus *Trichoderma veride* that produces a voltage-dependent conductance in bilayer systems (Sansom, 1993). It is of interest both as a model for voltage-gating and as a model for the behavior of a membrane-associated helix. Alamethicin is relatively hydrophobic, and it appears to be largely helical in its N-terminal domain, with a less well defined structure towards its C-terminus (Esposito et al., 1987, Kelsh et al., 1992, Franklin et al., 1994). An analysis of recent NMR data obtained in micelles provides evidence that alamethicin has both bent and extended low energy configurations (Franklin et al., 1994). In a helical configuration alamethicin is weakly amphipathic, suggesting that the channel structure might consist of an aggregate of laterally amphipathic helices. Indeed, the single channel behavior and high concentration dependence to the conduction are consistent with an aggregated form for the channel (Hall et al., 1984).

Alamethicin strongly associates with model membranes and it exhibits a cooperative binding to membranes composed of fluid phase phosphatidylcholines as determined both by CD and by EPR methods (Archer et al., 1991, Stankowski and Schwarz, 1989). Given that the alamethicin channel is most likely an aggregate, a reasonable interpretation of the sigmoidal binding is that it reflects peptide aggregation. However, when the EPR spectra of a spin-labeled alamethicin analog are recorded in the presence of low and high concentrations of unlabeled peptide, no changes in the motion of the label can be detected (Archer et al., 1991). Under conditions where the peptide is predicted to be monomeric or aggregated (based on the binding curves) the EPR lineshapes are identical and consistent with a monomeric form of alamethicin. The EPR spectrum is highly anisotropic and is in a motional range which should render it sensitive to aggregation. Not surprisingly, the label exhibits a shorter rotational correlation time than do backbone protons as determined by NMR (Archer et al., 1991). It should be noted that this analog of alamethicin is active and is voltage-gated in both bilayers and vesicle systems (Archer et al., 1991, Wille et al., 1989).

Recently, CD data was obtained for the existence of two forms of membrane bound alamethicin that interconvert as a function of alamethicin concentration (Woolley and Wallace, 1993). This is not inconsistent with previous EPR data on oriented bilayers which provided evidence for two forms of alamethicin and previous work using oriented CD (Archer et al., 1991, Huang and Wu, 1991). However, in this case the result was interpreted in terms of aggregation, since it is easy to understand how both monomeric and aggregated forms of alamethicin might interconvert as a function of concentration. Further, the analysis was extended to provide information on the thermodynamics of helix-helix association in membranes. The earlier EPR data, which did not provide evidence for aggregation, was considered to be unreliable because of the possibility that the spin-label on the C-terminus was highly flexible and not sensitive to the motion of the peptide.

For aggregation states such as those depicted in Fig. 1 *A–C*, a flexible C-terminal proxyl label is expected to experience collisions with neighboring nitroxides in the aggregate. As a result, aggregation should be accompanied by Heisenberg exchange, a spin exchange process that occurs during collisions between nitroxides in different hyperfine manifolds (Wertz and Bolton, 1972). This Heisenberg exchange should also arise from molecular collisions between alamethicin monomers as a result of their Brownian diffusion in the plane of the bilayer.

Knowledge of the aggregation state of alamethicin is clearly critical to an accurate interpretation of spectroscopic studies on this peptide. It is also important to establish

Received for publication 14 February 1994 and in final form 27 April 1994.
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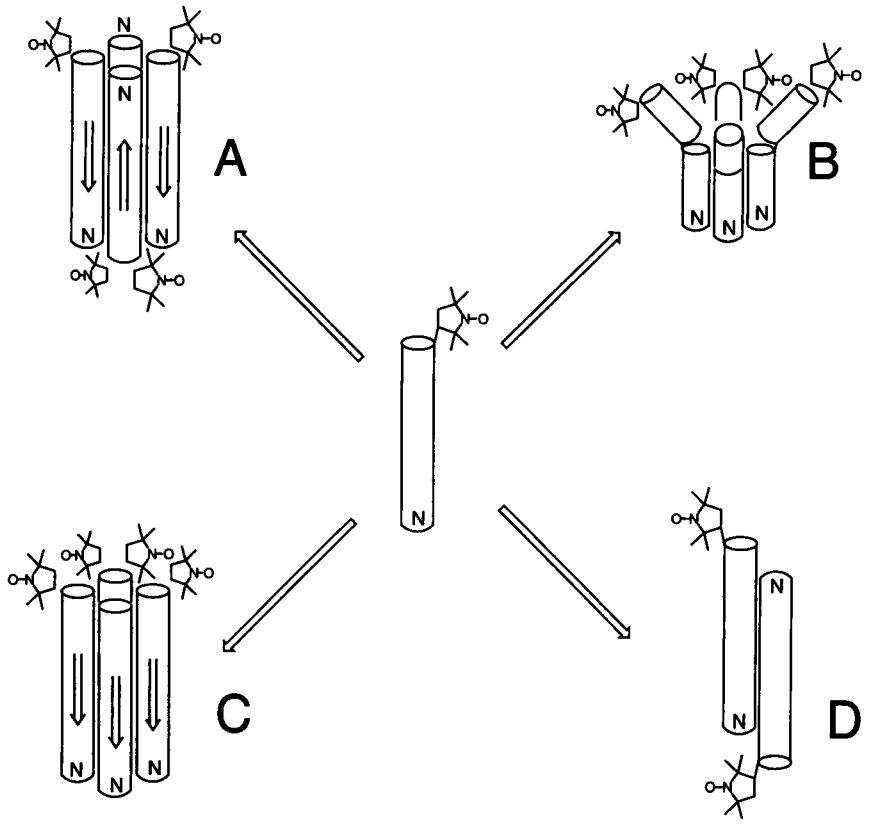

FIGURE 1 Several aggregated states that have been proposed or can be imagined for alamethicin. A tetrameric aggregate is shown here for clarity, although electrical data suggest that active channel aggregates could include up to 8–12 monomers. (*A*) An antiparallel aggregate; (*B*) an aggregate with a bent C-terminus; (*C*) a parallel aggregate; (*D*) a dimer of alamethicin monomers. In *A*, *B*, and *C*, the mobility of the spin-label and dynamics of the C-terminus is expected to lead to collisional exchange for these labels. If the spin label were rigidly fixed to the monomer and not mobile, the label might not exhibit strong spin-exchange, but in this case the rotational correlation time of the nitroxide should be sensitive to aggregation. Previously, changes in the rotational correlation time for the nitroxide were not seen over a wide range of alamethicin concentrations. Spin-exchange would not be expected for an antiparallel dimer such as that shown in *D*.

whether recent reports are indeed providing thermodynamic data on helix-helix association. In this report, we investigate the concentration dependence of the EPR linewidth and first derivative peak-to-peak amplitudes for the labeled alamethicin analog. We show that both the linewidth and resonance amplitudes are strongly modulated by spin-exchange and dipole-dipole exchange resulting from Brownian diffusion and that EPR provides a highly sensitive approach to monitor the frequency of collisions between labeled peptides in membranes. However, the exchange data provide no evidence for aggregation and are consistent with previous EPR results indicating that alamethicin is monomeric in fluid phase phosphatidylcholine over a wide concentration range.

MATERIALS AND METHODS

Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification, and EPC (egg yolk phosphatidylcholine) was isolated from fresh hens eggs and purified on alumina oxide as described elsewhere (Singleton et al., 1965). Spin-labeled carboxylproxyl alamethicin (CP-alamethicin) was synthesized as described previously (Archer et al., 1991) and purified on a reverse phase C_{18} HPLC column using a gradient of 40%A, 60%B to 10%A, 90%B where A is 0.05% trifluoroacetic acid (TFA) in water and B is 0.05% TFA in acetonitrile. A carboxylproxyl-labeled alamethicin having a deuterated and ^{15}N-labeled spin label was synthesized in an identical fashion except that 2,2,5,5-tetramethylpyrrolidin-d_{15}, 1-^{15}N-1-oxyl-3-carboxylic acid (Merck Isotopes, Montreal, Quebec, Canada) was used in the synthesis. The fraction of alamethicin containing alanine at position 6 was isolated and yielded an m/z of 2133 for the $M{\cdot}H^+$ ion by mass spectrometry. Alamethicin was added to phospholipid vesicles or dispersions using two methods. In one case, a stock solution of CP-alamethicin in methanol was used to deliver aliquots of alamethicin to lipid vesicles formed by extrusion in buffer as described previously (Archer et al., 1991). In the second method, aliquots of CP-alamethicin in methanol were co-dissolved with samples of lipid in chloroform, and the solvent was removed by argon and placed under a high vacuum for 3 h. The samples were then hydrated in buffer and freeze-thawed 5 times. The buffer contained 125 mM Na_2SO_4, and 10 mM morpholinoethanesulfonic acid, pH = 6.5. The samples were placed in a standard X-band cavity using a 100 μl quartz flat cell and recorded using a Varian E-line spectrometer with an E-102 bridge at nonsaturating microwave power of 10 mW and a modulation amplitude of 1 gauss p-p.

RESULTS

Shown in Fig. 2 is a series of EPR spectra for CP-alamethicin taken at several membrane concentrations of peptide. In these spectra, the concentration of lipid vesicles is sufficiently high so that virtually all the peptide is membrane associated. The spectra are similar to those shown previously, are highly anisotropic, and correspond to an intermediate rate motion of about 3 ns (Archer et al., 1991). In membranes, spin-spin relaxation has been used to examine lipid translational diffusion and interactions in membranes (Devaux et al., 1973; Sachse et al., 1987; Sackmann and Trauble, 1972). These spin-spin interactions can result from two processes: spin-spin exchange (Heisenberg exchange) and dipolar spin-spin broadening. Although the exchange process usually dominates in liquids, dipolar broadening can also be significant for membrane-bound nitroxides such as spin-labeled lipids (Sachse et al., 1987). To test for the membrane aggregation of alamethicin, both the peak-to-peak

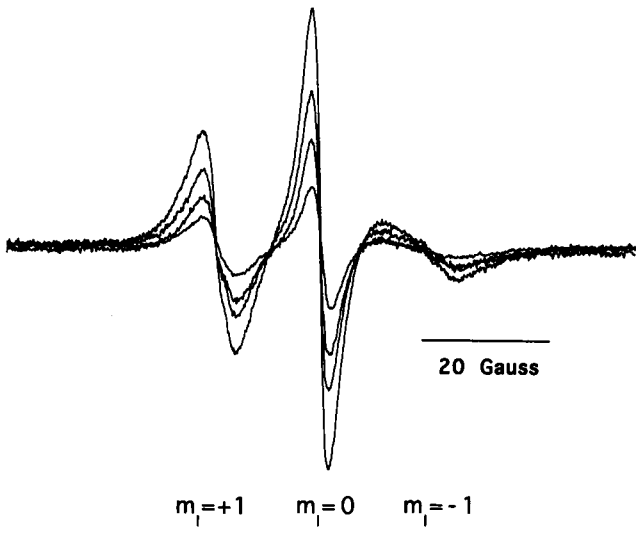

FIGURE 2 EPR spectra of the spin-labeled alamethicin analog, CP-alamethicin, dispersed with DOPC at a lipid concentration of 10 mg/ml. The EPR spectra from the lowest amplitude to the highest amplitude correspond to molar lipid:peptide ratios of 600:1, 300:1, 200:1, and 120:1, respectively.

amplitudes and linewidths were examined as a function of peptide concentration.

For spectra such as those shown in Fig. 2, the peak-to-peak linewidth of the first derivative Lorentzian line is directly related to the transverse relaxation time of the electron by $\Delta\omega = (2/\sqrt{3})T_2^{-1}$. Under the conditions used here, spin-spin exchange rates are slow, and interactions between nitroxides will lead to a Lorentzian broadening of the hyperfine lines in the EPR spectrum as a result of spin-spin exchange and dipole-dipole interactions. If $\Delta\omega_{ex}$ and $\Delta\omega_{dd}$ represent the line broadening produced by spin-spin exchange and dipole-dipole interactions, respectively, the linewidth of the first derivative EPR absorption, $\Delta\omega$, can be described by

$$\Delta\omega = \Delta\omega_0 + \Delta\omega_{ex} + \Delta\omega_{dd}, \quad (1)$$

where $\Delta\omega_0$ represents the linewidth in the absence of these interactions. Note that the magnitude of $\Delta\omega_{ex}$ is directly proportional to the frequency of collisions between spins. As a result, if collisions between nitroxides result exclusively from the diffusion of alamethicin monomers, $\Delta\omega_{ex}$ will increase linearly with the concentration of the peptide (Wertz and Bolton, 1972). In the case of simple diffusion, $\Delta\omega_{dd}$ is also expected to have a linear dependence on concentration of peptide (Sachse et al., 1987). As a result, in the absence of aggregation, $\Delta\omega$ is expected to increase linearly with the peptide concentration as given by

$$\Delta\omega = \Delta\omega_0 + c\{P\}, \quad (2)$$

where c is a constant that depends on the diffusion coefficient and the probability of successful spin-spin relaxation upon collision and $\{P\}$ represents the concentration of peptide in the membrane surface. If the peptide begins to aggregate in a manner that brings nitroxides in close proximity, $\Delta\omega$ should deviate from this behavior because of an enhanced frequency of spin-spin or dipolar exchange.

To test for aggregation, a series of spectra such as those shown in Fig. 2 were recorded over a range of lipid:peptide ratios from 600:1 to 7:1 in dispersions of DOPC, and Fig. 3 shows the linewidths of the $m_I = +1$ EPR resonance as a function of peptide concentration at 22 and 42°C. The solid lines in Fig. 3, *A* and *B* represent the best fit of Eq. 2 to the data, and clearly indicate that the linewidths increase linearly with concentration. The presence of aggregation such as that indicated in Fig. 1 would have been accompanied by a dramatic increase in linewidths as a function of concentration.

The peak-to-peak amplitudes of the EPR linewidths were also measured as a function of concentration, and these are shown in Fig. 4. The signal intensity increases nearly linearly at low concentrations but shows a strong nonlinear behavior at higher concentrations of peptide. If there were no interactions between spins, the amplitude of the EPR signal would continue to increase linearly with the concentration of spin; however, linebroadening caused by spin-spin exchange or dipole-dipole relaxation should result from Brownian diffusion and lead to a nonlinear dependence of amplitude on peptide concentration. For the first-derivative of a pure Lorentzian line, the peak-to-peak amplitude (I_{pp}) is proportional to the inverse of the square of $\Delta\omega$, and if the Brownian

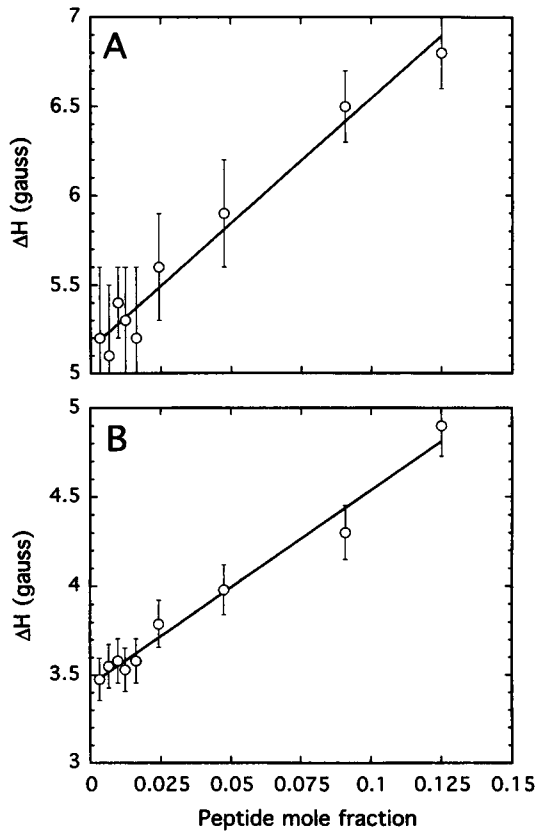

FIGURE 3 Plots of the peak-to-peak linewidths ($\Delta\omega$ or ΔH in gauss) of the first derivative $m_I = +1$ resonance of CP-alamethicin as a function of the peptide mole fraction in membranes composed of DOPC at (*A*) 22°C and (*B*) 42°C. The solid lines represent a best fit of these data to Eq. 2.

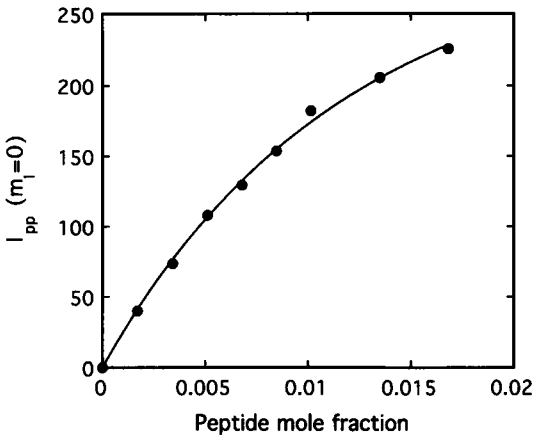

FIGURE 4 A plot of the peak-to-peak amplitude of the $m_I = 0$ resonance (I_{pp}) as a function of the mole fraction of the peptide. The solid line represents the best fit of these data to Eq. 3.

diffusion of monomers is the only process leading to exchange, it can be shown that the peak-to-peak amplitude of the Lorentzian line I_{pp} should vary with peptide concentration as given by

$$I_{PP} = \frac{a\{P\}}{(1 + b\{P\})^2} \quad (3)$$

where a and b are constants. The solid line in Fig. 4 represents a fit to data using Eq. 3. The fit with the experimental data is excellent, and similar data and fits were obtained to dispersions of DOPC at 22 and 42°C.

Equations 2 and 3 will be valid provided that the EPR lines are homogeneously broadened as the peptide concentration is increased. To ensure that a Gaussian narrowing of the unresolved proton hyperfine structure did not occur, the Lorentzian and Gaussian contributions to the linewidth were deconvoluted as described previously (Bales, 1982). When plotted as a function of peptide concentration, only the Lorentzian contribution to the linewidth was seen to change (data not shown). As an additional check that the behavior in Figs. 3 and 4 was not influenced by a Gaussian narrowing, these experiments were repeated with a deuterated, ^{15}N proxyl label attached to the C-terminus of alamethicin. Both the linewidths and amplitudes of the two line ^{15}N-labeled alamethicin show the same concentration dependence as that for the ^{14}N spin-label (Figs. 3 and 4).

From the data shown in Figs. 3 and 4, the dependence of the exchange rate on peptide concentration clearly follows a behavior expected for the random diffusion of monomers. In addition, the absolute spin-exchange rate obtained from EPR is consistent with the expected lateral diffusion rate for this peptide. From Fig. 3, the linewidth increase at a lipid:peptide ratio of 10 is approximately 1 gauss at 22°C. As shown elsewhere, the spin-spin exchange frequency for nitroxides is expected to be approximately one-third the collisional frequency under the conditions used here. If approximately 50% of the linebroadening is caused by spin-spin exchange at this temperature,[1] a linewidth increase of 1 gauss would correspond to a collisional frequency of about 2×10^7 s^{-1} (Sachse et al., 1987). Remarkably, this is almost exactly the collision frequency expected for simple diffusion. If 1×10^{-7} cm^2 s^{-1} is taken as the translational diffusion coefficient of monomeric membrane-bound alamethicin, a collision frequency near 3×10^7 s^{-1} would be expected (Sachse et al., 1987).

DISCUSSION

Recent work using CD has identified two forms of alamethicin that interconvert as a function of peptide concentration (Woolley and Wallace, 1993). Clearly, a logical explanation for these results and the sigmoidal binding behavior is that alamethicin forms an aggregate as the peptide concentration is increased. In this report, the interactions between peptides were monitored directly by measuring interactions between spins attached to the C-terminus of alamethicin. The data presented above cover the concentration range used in the CD work where the peptide is believed to convert from a monomeric to an aggregated form; however, the exchange rate that is measured as the membrane concentration of peptide is varied can be completely accounted for by a Brownian diffusion of monomers. No evidence for aggregation in EPC or DOPC membranes can be found, consistent with earlier work indicating that alamethicin remains monomeric over a wide concentration range (Archer et al., 1991). It should be noted that a simple antiparallel dimer of alamethicin monomer (Fig. 1 *D*) might not lead to enhanced spin-exchange, but more extensive aggregation of antiparallel monomers should have been detected in this experiment. Molecular dynamics simulations of CP-alamethicin indicate that the nitroxide on the C-terminus undergoes significant motion even when the peptide is restricted to a highly helical configuration (L. P. Kelsh and D. S. Cafiso, unpublished data). From the amplitudes of this motion, substantial collisional exchange is expected in aggregates having significant helix-helix contact.

It was argued previously that the EPR spectrum of CP-alamethicin (diluted into unlabeled alamethicin) might not be sensitive to the aggregation state of the peptide because its C-terminus is highly flexible (Woolley and Wallace, 1993). Although the C-terminal label exhibits a shorter correlation time than the peptide backbone, the EPR spectrum of CP-alamethicin is sensitive to lipid composition and the presence of cholesterol. In fact, evidence for aggregation can be seen

[1] The linewidth dependence on concentration, $d(\Delta\omega)/dc$, was investigated as a function of temperature, to determine the relative contributions made by $\Delta\omega_{ex}$ and $\Delta\omega_{dd}$ to the linewidth increase. The dipole-dipole interactions are expected to decrease as a function of temperature, whereas spin-spin exchange is expected to increase as a function of temperature. A plot of $d(\Delta\omega)/dc$ as a function of temperature goes through a minimum near 50°C and is similar to that seen previously for certain phospholipid spin labels (Sachse et al., 1987). At 40°C, the contributions of $\Delta\omega_{ex}$ and $\Delta\omega_{dd}$ appear to be roughly equivalent.

for this label in certain long-chain saturated lipids in the gel state (M. Barranger and D. S. Cafiso, unpublished data). Thus, the available evidence indicates that the EPR spectrum of CP alamethicin is capable of revealing aggregation. When taken together with the spin-exchange results presented above, these data provide strong evidence against the formation of stable aggregates in EPC or DOPC.

The implications of these results for spectroscopic studies are important because they indicate that the aggregated form of the channel can only be present in low concentrations in these membrane systems in the absence of a potential. It is possible, of course, that the application of a membrane voltage will lead to the aggregation of alamethicin as proposed previously (Sansom, 1993). Further, it is clear that the interpretation of previous spectroscopic measurements in terms of an aggregated state or helix-helix interactions might not be correct.

There are several explanations for the different conclusions reached in the present study compared with a recent CD study (Woolley and Wallace, 1993). Because the time scales for CD spectroscopy and EPR are quite different, it is not inconceivable that CD is responding to short-lived structural changes or aggregates not detectable by EPR. Because the EPR should be sensitive to aggregates that exist on the nanosecond time scale, any aggregation that the CD is detecting must be very short-lived. Without invoking aggregation, there might be other interpretations for the observation that alamethicin interconverts between two structural forms as the peptide concentration is increased. For example, alamethicin modulates the structure of the membrane interface, as observed using NMR, and it also produces changes in bilayer morphology (Banerjee et al., 1985; McIntosh et al., 1982). These changes in the structure of the interface or the instantaneous radius of curvature of the membrane interface could alter the free energy of conformers of the peptide without direct peptide-peptide interactions. If two conformeric forms were similar in energy in the bilayer, relatively minor differences in energy could change the populations of these conformers.

Spin-spin interactions represent a powerful method to monitor the interactions between macromolecules in membranes, and several approaches can be taken to optimize its sensitivity. For example, the ^{15}N-labeled and deuterated nitroxide used here reduces the intrinsic EPR linewidth and leads to greater sensitivity at low exchange rates. Methods such as electron-electron double resonance have been used to monitor collisional exchange between ^{14}N and ^{15}N spin-labels at extremely low spin concentrations (Shin and Hubbell, 1992). These approaches are now being employed to investigate conditions that might promote the aggregation of alamethicin in membranes and to examine the voltage-dependence of alamethicin helix-helix interactions.

We would like to thank Drs. Robert Bryant and Jeffrey Ellena for helpful discussions during the course of this study.
This work was supported by National Institutes of Health grant GM35215 to D. S. Cafiso.

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