Molecular Flexibility Demonstrated by Paramagnetic Enhancements of Nuclear Relaxation. Application to Alamethicin: A Voltage-Gated Peptide Channel

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ABSTRACT A nitroxide spin label attached to the C-terminus of the channel forming peptide alamethicin produces an enhancement of the nuclear spin-lattice relaxation rates of peptide protons as a result of both intermolecular and intramolecular magnetic dipole-dipole interactions. The intermolecular contribution provides evidence that alamethicin monomers collide preferentially in a C-terminal-to-N-terminal configuration in methanol. From the intramolecular paramagnetic enhancement of nuclear spin-lattice relaxation times, effective distances between the unpaired electron on the nitroxide at the C-terminus of alamethicin and protons along the peptide backbone were calculated. These distances are much shorter than distances based on the reported crystal structure of alamethicin, and cannot be accounted for by motion in the bonds that attach the nitroxide to the peptide. In addition, the differences between distances deduced from the nuclear spin relaxation and the distances seen in the crystal structure increase toward the N-terminal end of the peptide. The simplest explanation for these data is that the alamethicin backbone suffers large structural fluctuations that yield shorter effective distances between the C-terminus and positions along the backbone. This finding can be interpreted in terms of a molecular mechanism for the voltage-gating of the alamethicin channel. When the distances between a paramagnetic center and a nucleus fluctuate, paramagnetic enhancements are expected to yield distances that are weighted by r^{-6}, and distances calculated using the Solomon-Bloembergen equations may more nearly represent a distance of closest approach than a time average distance. Therefore, the use of paramagnetic centers such as spin labels or metal ions with long electron T_1 values provides a distance measurement that reflects a dynamically averaged structure where the averaging process heavily weights short distances. The results of such measurements, when combined with other structural information, may provide particularly clear evidence for the magnitude of structural fluctuations involving distances greater than 10 Å.

INTRODUCTION

The molecular dynamics of proteins and peptides provides important insight into their molecular function; however, there are relatively few methods to examine dynamics directly. Structure determination from high-resolution nuclear magnetic resonance (NMR) typically involves the measurement of a large number of distances <5 Å; as a result, such a structural determination is not very sensitive to the internal motions of the macromolecule (Bruschweiler et al., 1992; Post, 1992; Tropp, 1980). Although high-resolution NMR structures may yield information on internal dynamics, with difficulty, the magnetic field dependence of the NMR relaxation rates can yield the rates of tumbling and internal motions of a peptide or protein (Noack, 1986). For example, it is possible to distinguish regions of a structure that exhibit higher flexibility as shown in recent studies on calbindin and calmodulin (Barbato et al., 1992, Palmer et al., 1991). For small peptides in solution, the spin-lattice relaxation rate alone is not sensitive to the internal dynamics of the peptide if the rates of motion are slower than the isotropic tumbling rate of the peptide. Spin-spin (T_2) measurements may be helpful in this case but are subject to a number of interpretive difficulties and systematic errors. Clearly, additional methods that can provide information on dynamics would be highly desirable.

Alamethicin is a small 20-amino acid peptide from the fungus *Trichoderma viride* that forms voltage-gated channels in lipid membranes. It is of interest both as a model for voltage gating in larger intrinsic ion channels and as a model for peptide-membrane interactions (Cafiso, 1994). There are a limited number of mechanisms that can explain the voltage-gating of this peptide. Gating does not involve the movement of charge through the membrane, but instead must result from the interaction of a helix dipole with the membrane electric field. This interaction might occur through a change in orientation of the helix with respect to the bilayer normal or through a change in the conformation of the peptide that involves the formation of a dipole along the bilayer normal. In its crystal structure, alamethicin is predominantly helical with a slight bend about Pro 14 (Fox and Richards, 1982). In the center of the peptide as well as the C-terminal half there is some 3_{10} hydrogen-bonding character. Based on their crystal structure, Fox and Richards (1982) proposed a model for gating based on the reorientation of the C-terminus upon the application of an electric field. Other mechanisms have been proposed (Boheim et al., 1983, Mathew and Balaram, 1983) that involve the movement of alamethicin as a rigid helix within the membrane (see Fig. 1).

Definitive evidence for flexibility in the alamethicin backbone has been difficult to obtain. In solution, the magnetic field dependence of the NMR relaxation suggests that the

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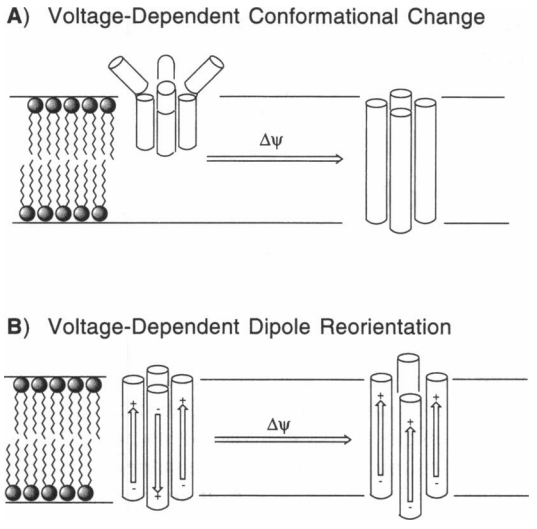

FIGURE 1 A number of mechanisms have been proposed to account for the voltage gating of alamethicin that require quite different properties of the peptide monomer. For example, (*A*) shows a model involving a voltage-dependent conformational change in alamethicin that leads to the membrane insertion of an aggregate (Fox and Richards, 1982); (*B*) shows a model that involves the voltage-dependent reorientation of the alamethicin helix, leading to electrostatic repulsion in the aggregate and an opening of the channel (Boheim et al., 1983; Mathew and Balaram, 1983).

structure is rigid in the sub-ns time scale; however, the absence of NOEs in the C-terminal portion of the molecule has been taken as evidence for some molecular flexibility in this portion of the peptide (Esposito et al., 1987, Kelsh et al., 1992). Experiments using ^{15}N-labeled alamethicin were not able to distinguish whether this peptide is flexible in solution (Yee and O'Neil, 1992); however, a recent NMR determination of the micelle structure of alamethicin provides evidence for flexibility revealing both linear and bent configurations of the peptide. This also suggests a voltage-dependent structural change similar in principle to that described by Fox and Richards (Franklin et al., 1994).

Paramagnetic enhancements of nuclear magnetic spin-lattice relaxation produced by protein-bound nitroxides were suggested more than 25 years ago as a method to determine distances in proteins (McConnell, 1967; Sternlicht and Wheeler, 1967), and reviews of this area including the details of the approach can be found elsewhere (Kosen, 1989; Krugh, 1976). However, it is not generally appreciated that these enhancements provide nearly a distance of closest approach in cases where there are large structural deformations. In this report, we describe the use of these paramagnetic enhancements to obtain information on molecular flexibility. We show that a nitroxide spin label attached to the C-terminus of alamethicin produces enhancements of proton spin-lattice relaxation rates along the alamethicin backbone that can be used to estimate motionally averaged distances between the nitroxide and protons on the peptide. The effect of the spin label is weighted by $1/\langle r^3\rangle^2$, which provides clear evidence that the solution structure is dramatically different from the crystal structure for this peptide. The data provide the strongest evidence to date for molecular flexibility along the peptide backbone of alamethicin.

MATERIALS AND METHODS

Materials

Alamethicin was obtained from Sigma Chemical Co. (St. Louis, MO) and was purified by high-performance liquid chromatography as described previously (Kelsh et al., 1992). The major fraction of alamethicin having the sequence Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-Val-MeA-Gly-Leu-MeA-Pro-Val-MeA-MeA-Gln-Gln-Phol, where MeA represents α-methylalanine, was used for the experiments described here. A C-terminal spin-labeled analog of alamethicin (CP-alam) was synthesized by forming an ester linkage between 3-carboxy proxyl and the C-terminal phenylalaninol using a procedure described previously (Archer et al., 1991). The solvent used for the NMR experiments, CD_3OD, was obtained from Cambridge Isotope Laboratories (Woburn, MA).

NMR measurements

NMR spectra were recorded on alamethicin suspended in 0.5–1 ml of CD_3OD at a temperature of 25°C. Spectra of unlabeled peptide were taken at a concentration of ~2 mM. Spectra of labeled alamethicin were taken at eight concentrations from 3.7–0.37 mM. Proton relaxation data were collected on a General Electric Omega 500 NMR spectrometer operating at 500.13 MHz for ^{1}H using the inversion recovery sequence, 180-τ-90 acquisition with presaturation of the H_2O peak. A typical T_1 measurement consisted of 13–14 data sets collected for τ values between 40 ms and 6 s. A 7-s relaxation delay was used between scans. For each data set, 128 or 256 scans of 16K points were collected over a 5000-Hz sweep width. Data sets were interleaved to randomize time-dependent signal changes over all τ values. The data were fitted with a three-parameter exponential function using the NMR1 software (New Methods Research, Inc., Syracuse, NY). The values used in the distance calculations were the average of two (labeled) or three (unlabeled) separate T_1 measurements.

Energy minimization on nitroxides appended to the crystal or NMR structures of alamethicin were performed using 100 iterations of steepest descent followed by conjugate-gradient minimization to convergence using version 2.9 of Discover (Biosym Technologies Inc., San Diego, CA).

Estimating ^{1}H-nitroxide distances

The paramagnetic contribution to the relaxation rate, R_{1para} (or T_{1para}^{-1}), was calculated from the T_1 relaxation rate for the unlabeled peptide (R_{1alam}) and the labeled peptide ($R_{1CPalam}$) according to:

$$R_{1para}^{-1} \equiv (R_{1CPalam} - R_{1alam})^{-1} \quad (1)$$

The distance between the nitroxide and the observed proton, r in Å, was estimated from $R_{1para(0)}$ (which is the value of R_{1para} extrapolated to 0 concentration) using a simplified form of the Solomon-Bloembergen equation,

$$r = C\left\{\left(\frac{3\tau_C}{1+\omega_I^2\tau_C^2} + \frac{7\tau_C}{1+\omega_S^2\tau_C^2}\right)R_{1para[0]}^{-1}\right\}^{1/6} \quad (2)$$

where C is a constant having a value of 540 Å at 500 MHz, and ω_I and ω_S are the nuclear and electron Larmor frequencies (Krugh, 1976). This expression is generally valid for spin labels where the electron relaxation times are long relative to the correlation time, τ_c, for the electron nuclear dipole-dipole coupling. A value of 0.7 ns is used for τ_c, which is the effective overall correlation time for peptide rotation in methanol (Esposito et al., 1987). This choice of τ_c presumes that high-frequency (ps) motions of the peptide are of limited amplitude, an assumption that appears to be reasonable for alamethicin based on a previous ^{13}C relaxation study (Kelsh et al., 1992). In any case, the distances that are obtained from Eq. 2 are remarkably insensitive to the value of τ_c, and as a result, the qualitative conclusions reached in the present study are not altered by this choice of τ_c.

RESULTS AND DISCUSSION

Both intermolecular and intramolecular interactions contribute to the paramagnetic effect on T_1

Nitroxides will enhance both the longitudinal and transverse relaxation rates of nearby nuclei. Shown in Fig. 2 are the results of the inversion-recovery experiment for the Cα protons of Leu 12, Pro 14, and Pro 2. As expected, the ^{1}H spectrum of the peptide with the proxyl nitroxide at the C-terminus exhibits larger linewidths and enhanced relaxation rates relative to unlabeled alamethicin. In this sample, an enhanced proton relaxation rate could result from both intramolecular and intermolecular contributions. The intermolecular contribution was determined by examining the concentration dependence of the paramagnetic enhancement of the relaxation rate, R_{1para}. Shown in Fig. 3 is a plot of $R_{1CPalam}$ as a function of peptide concentration for Cα protons associated with Pro 2, Gly 11, and Pro 14. As seen in this figure, the intermolecular contribution, which is proportional

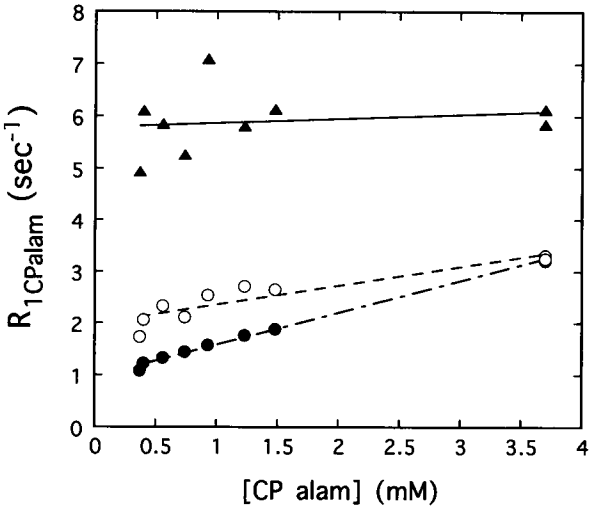

FIGURE 3 Plots of R_1, the paramagnetic enhancement in the proton relaxation rate, for the Pro2 (●), Gly11b (○) and Pro14 (▲) Cα protons as a function of peptide concentration. The extrapolation of the data to 0 concentration gives the intramolecular rate, which is used to calculate the nitroxide-proton distances.

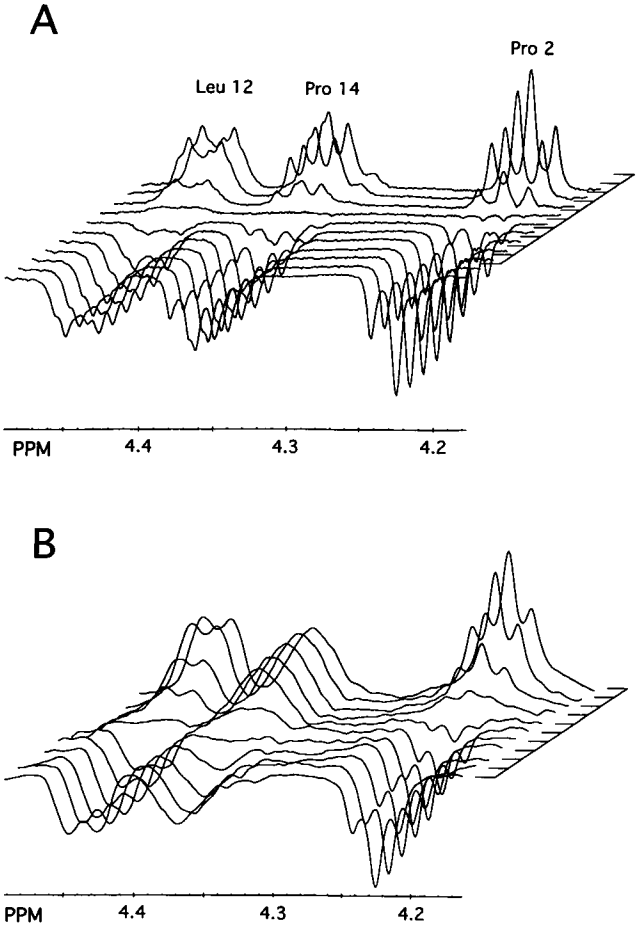

FIGURE 2 1H NMR spectra showing the time-dependent magnetization of the Cα protons of Leu12, Pro 14, and Pro 2 as measured by the inversion-recovery sequence 180-t-90-acquisition, as a function of τ, where τ = 1 μs, 1, 20, 50, 100, 200, 500, 800 ms, and 1.2, 3.0, 5.0 s. In (*A*) spectra for 2 mM native alamethicin in methanol; in (*B*) spectra for 2 mM alamethicin with a proxyl spin label attached to the C-terminus in methanol.

to the slopes of the lines, appears to be largest near the N-terminus. For alamethicin at a concentration of 3.7 mM, the intermolecular contribution increased the relaxation rates by up to a factor of two depending upon the Cα position along the peptide backbone. Fig. 4 shows the concentration dependence (from the lowest to the highest concentrations of peptide) for R_{1para} (dR_{1para}/dC) for Cα protons along the alamethicin backbone, and clearly reveals a trend of stronger concentration dependence towards the N-terminus. These data suggest that a close diffusional approach of the labeled

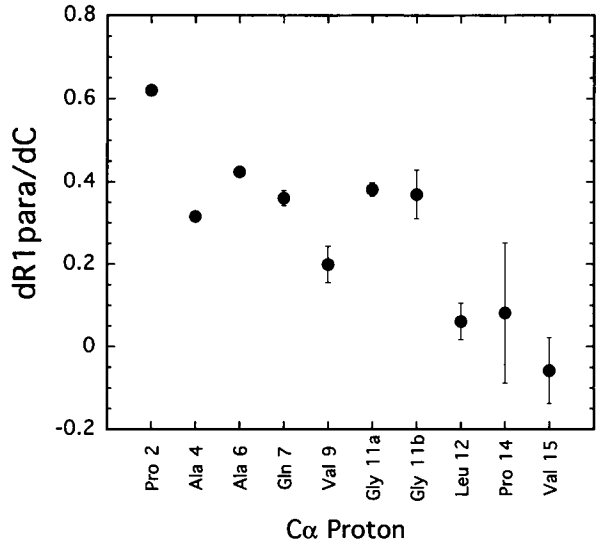

FIGURE 4 Plots of R_1/dC, the slope of the concentration dependence shown in Fig. 3, as a function of chain position. The slopes are greatest for positions at the N-terminus, which suggests that a close diffusional approach of the labeled C-terminus to a neighboring alamethicin molecule is not of equal probability along the backbone, but favors an interaction with the N-terminus.

C-terminus to a neighboring alamethicin molecule is not of equal probability along the backbone, but is more favorable toward the N-terminus. This observation is consistent with arguments based on electrostatic interactions due to the helix dipole of alamethicin.

The intramolecular contributions of R_{1para} are isolated by extrapolating to 0 concentration. These values of $R_{1para(0)}$ are summarized in Table 1 for the Cα protons of alamethicin along with their standard errors. These values were used to compute nitroxide-proton distances using Eq. 2. It should be noted that the errors associated with these rates are reduced by the sixth root during this computation, and the distances are quite well determined.

Distances obtained from the paramagnetic effect are shorter than those in the crystal structure

The effective distances calculated from the values of R_{1para} extrapolated to 0 concentration using Eq. 2 are shown in Fig. 5. In addition, Fig. 5 shows the expected distances for the energy-minimized crystal structure with a proxyl moiety appended onto the C-terminus. The differences between the experimental distances and distances from the crystal structure are largest at the N-terminal end of the peptide, where the distances obtained from the NMR relaxation data are seen to be as much as 15 Å shorter than those found in the crystal structure. At the C-terminal end of the peptide, distances in the crystal structure are much closer to those measured experimentally. As discussed below, the close match of the crystal structure toward the C-terminus, but the dramatically shorter distances near the N-terminus, is most easily explained if the peptide backbone suffers structural deformations resulting in shorter effective distances between the spin label and the observed proton.

Several atoms attach the spin label to the C-terminus and are likely to impart additional rotational degrees of motion to the N-oxide. However, motions alone about these additional bonds cannot account for the shorter distances that are seen in Fig. 5. When the orientation of the nitroxide appended to the crystal structure is manually varied, it can be shown that motions about these bonds can only account for a shortening of the distances in the crystal structure by a few Å.

TABLE 1 Relaxation rates and standard errors for alamethicin and carboxyproxyl alamethicin given in s^{-1}

Cα proton position	$R_{1CPalam(0)}$	R_{1alam}	$R_{1para\ (0)}$
Pro 2	0.960 ± 0.051	0.746 ± 0.020	0.214
Ala 4	0.780 ± 0.036	0.676 ± 0.020	0.104
Ala 6	1.027 ± 0.042	0.685 ± 0.020	0.342
Gln 7	1.504 ± 0.067	0.676 ± 0.020	0.828
Val 9	1.068 ± 0.166	0.775 ± 0.018	0.292
Gly 11a	1.808 ± 0.060	1.56 ± 0.018	0.246
Gly 11b	1.984 ± 0.222	1.56 ± 0.018	0.421
Leu 12	1.749 ± 0.166	0.80 ± 0.024	0.949
Pro 14	5.786 ± 0.636	0.758 ± 0.028	5.03
Val 15	4.675 ± 0.298	0.833 ± 0.010	3.84

R_{1alam} is the relaxation rate for unlabeled alamethicin, $R_{1CPalam(0)}$ represents the relaxation rate for carboxyproxyl alamethicin extrapolated to 0 concentration and $R_{1para\ (0)}$ is the paramagnetic contribution to the relaxation rate for carboxyproxyl alamethicin extrapolated to 0 concentration.

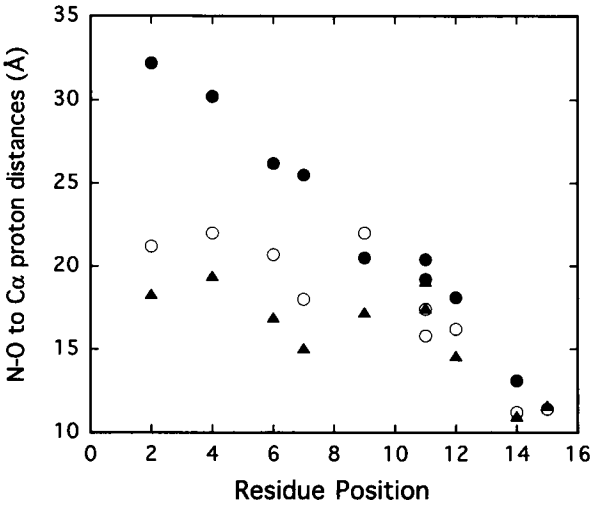

FIGURE 5 Nitroxide to α-carbon proton distances determined from the intramolecular contribution to R1 (▲), the crystal structure (●) shown in Fig. 6 *A*, and a bent structure obtained from a simulated annealing of NMR data for alamethicin in micelles (○) shown in Fig. 6 *B* (Franklin et al., 1994).

Effect of molecular flexibility on the magnitude of the paramagnetic effect

The systematic deviation from the expected crystal structure distances indicated in Fig. 5 could result from leveling of the relaxation rates caused by spin diffusion among the protons. However, both the spin diffusion and the electron-nuclear couplings are dipole-dipole couplings, and the proton-proton couplings important for the spin diffusion processes are proportional to γ_H^4, whereas the electron-nuclear couplings are proportional to $\gamma_H^2\gamma e^2$. Thus, the direct electron-nuclear contribution is 4.3×10^5 larger than the proton-proton coupling if the correlation times and distances are the same. If we assume that the correlation times for both types of dipole-dipole coupling are the same, the distance where the electron-nuclear coupling will be comparable to the proton-proton coupling will be 8.7 times longer than the proton-proton distance. If we assume a proton-proton distance of 2.2 Å, the coupling between protons at this distance is equivalent to that with an electron 19 Å away. Because multiple proton-proton transfers are required for the proton spin diffusion to reach 19 Å, its contribution in the present cases is negligible.

An alternative explanation for the shorter distances toward the N-terminus are structural fluctuations in the peptide that make the intermoment distances time dependent. An inspection of Fig. 5 shows that at shorter values of the intermoment separation, the differences between the crystal and NMR determinations of distance are small. This is reasonable, because low-amplitude, localized distance fluctuations have little effect on average intermoment distances, whereas the large electron magnetic moment produces effects detectable at longer distances, and distance fluctuations may be much

more significant. Because the dipole-dipole coupling is proportional to r^{-3}, which enters the relaxation equations as the square, the shorter distances contribute much more strongly to the relaxation rate than do longer distances. Thus, qualitatively, we expect that the relaxation rates will yield distances that are significantly shorter than expected if there is peptide chain flexibility. If one computes $\langle 1/r^3 \rangle^2$, assuming a constant probability distribution for the distance over the range from 10–30 Å, then one obtains 13 Å for r. This estimate suggests that the values derived from the alamethicin data are reasonable if there is conformational averaging of the peptide distances.

This type of NMR measurement may very clearly demonstrate the effects of dynamical averaging of intermoment distances over times that are much longer than other spectroscopic distance measurements such as fluorescence energy transfer. However, the measurement is simply a weighted average and by itself carries no recoverable information about the nature of the trajectories taken to achieve the average. We make no attempt to provide any detailed information about the trajectory or any distribution function for distance, because none we could show may fit the data uniquely. However, it is clear that the apparent shortening of the intermoment distances between the known crystal structure and the solution average obtained here is very substantial for the larger distances connecting the ends of the molecule. Thus, whatever the trajectories are, they must involve substantial bending of the structure, on a time scale short compared to the T_1 values measured, i.e., tenths of s, and must bring the ends of the molecule into much closer proximity than is implied by the essentially linear structure of the peptide deduced from the crystal data.

A recent study of the structure of alamethicin in micelles employing simulated annealing and almost 200 NMR restraints yielded both bent and linear conformations for alamethicin. These could be easily interconverted by rotations of the ψ and ϕ angles of MeA 10 and Gly11. As discussed, this result is also not unexpected given the location of proline at position 14 (Franklin et al., 1994). To determine whether highly bent structures such as those obtained from this NMR analysis are consistent with the intermoment distances seen here, one of the bent structures obtained from this analysis was appended with a spin label and the entire structure energy minimized using the steepest descent and conjugate gradient algorithms of Discover 2.9. The minimization was repeated several times with the proxyl residue moved manually through a range of starting positions. The model consistently found the same minimum conformation with the peptide backbone remaining nominally unperturbed. This structure is shown in Fig. 6 along with the crystal structure for alamethicin. The distances measured from the nitroxide to various Cα protons in this bent structure are shown in Fig. 5 along with distances obtained from R_{1para}. This bent structure produces a reasonably close match with the experimental data, and the positioning of the spin label away from the bend in the structure also produces a periodicity in the distances that is seen from the R_1 data.

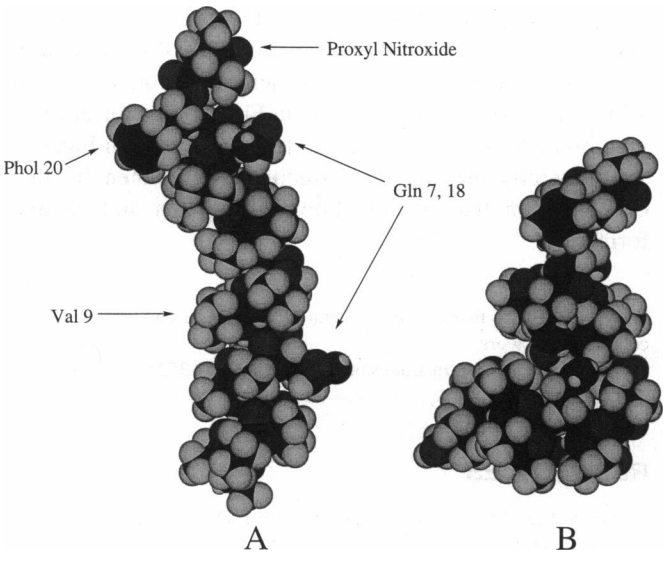

FIGURE 6 The structure shown in (*A*) was obtained by appending a proxyl moiety to the C-terminus of the crystal structure for alamethicin and energy minimizing the result. The structure shown below in (*B*) was one obtained by appending the proxyl nitroxide to a bent form obtained from simulated annealing of NMR data (Franklin et al., 1994), followed by energy minimization. This structural form is bent about MeA 10. MeA 10 would normally be hydrogen bonded to position 14 in an α-helical configuration, but cannot in alamethicin because of proline at this position.

Structural fluctuations in alamethicin and the mechanism for channel gating

It is important to recognize that the distances obtained from Fig. 6 *B* are taken from a static structure and not the trajectories of a dynamics simulation; as a result, quantitative comparisons between these distances and distances obtained from the relaxation rate data may not be justified. The comparison does demonstrate that alamethicin must spend a significant fraction of its time in a highly bent configuration, not unlike that shown in Fig. 6 *B*. This result dramatically contrasts with x-ray crystallographic and earlier NMR work suggesting that the peptide is on average linear (Esposito et al., 1987; Fox and Richards, 1982). It should be noted that the data obtained here could also be interpreted in terms of a static bent configuration for alamethicin; however, the available evidence suggests that this is not the case. Recent NMR data and dynamics simulations provide evidence for fluctuations in the central portion of the alamethicin helix (Franklin et al., 1994; Fraternali, 1990; Yee and O'Neil, 1992), and a static bent configuration should have revealed nuclear Overhauser effects, which are not seen (Franklin et al., 1994). Thus, the simplest interpretation of the data presented here is that alamethicin is in dynamic equilibrium between linear and bent configurations such as those shown in Fig. 6.

The presence of large structural fluctuations between an open versus a bent form for alamethicin suggests that this conversion may provide the conformational switch associated with the gating of the alamethicin channel. In this model, alamethicin would be membrane bound in a bent form resembling the structure in Fig. 6 *B* in the absence of voltage.

Interestingly, in this bent structure, the side chains of Gln 7 and Gln 18 appear to be in a position to hydrogen bond to each other. Application of a transmembrane potential would allow conversion of the structure in Fig. 6 *B* to a linear transmembrane form resembling the structure shown in Fig. 6 *A*. This laterally amphipathic structure would then be in a conformation that would allow aggregation and channel formation.

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