Membrane Orientation of the N-terminal Segment of Alamethicin Determined by Solid-State ^{15}N NMR

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ABSTRACT Alamethicin was synthesized with ^{15}N incorporated into alanine at position 6 in the peptide sequence. In dispersions of hydrated dimyristoylphosphatidylcholine, solid-state ^{15}N NMR yields an axially symmetric powder pattern indicating that the peptide is reorienting with a single axis of symmetry when associated with lamellar lipids. When incorporated into bilayers that are uniformly oriented with the bilayer normal parallel to the B_0 field, the position of the observed ^{15}N chemical shift is 171 ppm. This is coincident with the $\sigma_{\parallel}$ edge of the axially symmetric powder pattern for non-oriented hydrated samples. Thus the axis of motional averaging lies along the bilayer normal. Two-dimensional separated local field spectra were obtained that provide a measure of the N-H dipolar coupling in one dimension and the ^{15}N chemical shift in the other. These data yield a dipolar coupling of 17 kHz corresponding to an average angle of 24° for the N-H bond with respect to the B_0 field axis. An analysis of the possible structures and orientations that could produce the observed spectral parameters show that these values are consistent with an α-helical conformation inserted along the bilayer normal.

INTRODUCTION

Electrostatic interactions between proteins and membranes are fundamental to a wide range of biological processes. For example, conformational changes in membrane proteins that involve the movement of charges or dipole moments lead to the gating of ion channels. Electrostatic interactions also drive the membrane association and activation of certain proteins (Kim et al., 1991; Mosior and McLaughlin, 1992) and appear to be important in the folding of membrane proteins (von Heijne and Gavel, 1988). Alamethicin is a small 20-amino acid peptide that produces a voltage-dependent conductance in lipid bilayers. Because of its strong voltage dependence and elaborate channel behavior (Hall et al., 1984), it has been of interest as a model for voltage-dependent gating, and it has served as a model for peptide-membrane interactions (Cafiso, 1994).

Shown in Fig. 1 *A* is the crystal structure of alamethicin (Fox and Richards, 1982). The peptide is α-helical in its N-terminal domain but has some 3_{10} character toward its C-terminus. This helix is laterally amphipathic and is believed to form a conductive aggregate. A number of models have been proposed for the gating of alamethicin, and these are discussed in detail in recent reviews (Cafiso, 1994; Sansom, 1993; Wooley and Wallace, 1992). In the absence of a gating voltage, some models place the alamethicin helix at the membrane interface, but most models place the peptide across the lipid bilayer. Because of its importance for understanding gating, a number of investigations have been carried out to determine the orientation of alamethicin in lipid bilayers. Work using ^{31}P NMR and ^{2}H NMR on lipids provided an indirect indication that alamethicin was surface oriented (Banerjee et al., 1985). However, more recent studies using optical techniques indicate that alamethicin is incorporated into the membrane and oriented along the bilayer normal (Huang and Wu, 1991; Vogel, 1987). These studies also provide evidence that alamethicin changes its orientation as a function of hydration and the peptide:lipid ratio. At least one early measurement based on IR ATR spectroscopy reported that at full hydration, the peptide was extended (non-helical) and aggregated in the membrane (Fringeli and Fringeli, 1979). However, neither this aggregation nor an extended structure is supported by more recent work (Archer et al., 1991; Barranger-Mathys and Cafiso, 1994; Franklin et al., 1994). The most recent high-resolution NMR studies in micelles indicate that this peptide is approximately helical in its N- and C-terminal domains but may be flexible about the central region of the helix (Franklin et al., 1994). In addition, a solution study in methanol using paramagnetic enhancements of NMR relaxation demonstrated that alamethicin is flexible about residues MeA_{10} and Gly_{11} (North et al., 1994). A time-averaged structure that is consistent with both the NOESY and paramagnetic enhancement NMR data is shown in Fig. 1 *B*. In this bent structure, the more hydrophilic face of alamethicin is buried within the helix bend.

Given these recent structural studies on alamethicin, several important questions can be raised regarding its membrane conformation. Is the peptide bent or linear when bound to the membrane? If the peptide is bent in the membrane, as it is in Fig. 1 *B*, how are the C- and N-terminal segments of alamethicin positioned? Alamethicin contains a number of α-methylalanine (MeA) groups, and these are thought to promote 3_{10} helix formation (Toniolo and Benedetti, 1991). Indeed, alamethicin has some 3_{10} character in its crystal structure (Fox and Richards, 1982); however, there is not yet strong evidence to distinguish between an α or 3_{10} helical con-

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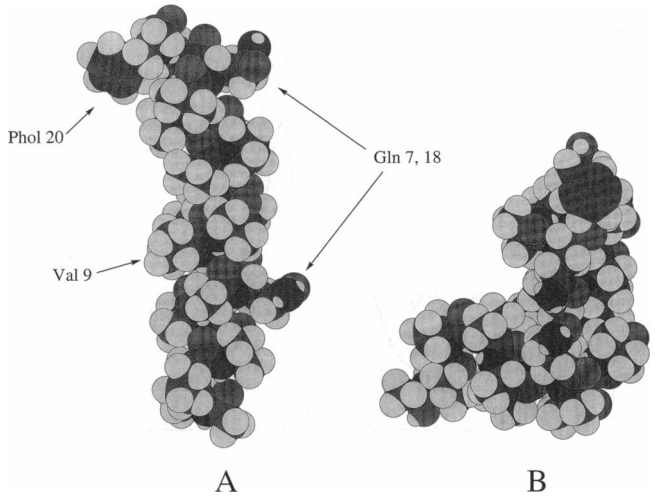

FIGURE 1 The crystal (Fox and Richards, 1982) (*A*) and time-averaged solution (North et al., 1994) (*B*) structures for alamethicin. In *A* Gln has been substituted for Glu. The sequence of alamethicin is Ac-MeA-Pro-MeA-Ala-MeA-Ala-Gln-MeA-Val-MeA-Gly-Leu-MeA-Pro-Val-MeA-MeA-Gln-Gln-Phol, where MeA represents α-methylalanine. The major alamethicin fraction in samples from Upjohn Pharmaceuticals had Glu at position 18, but more recent alamethicin preparations from Sigma Chemical Co. (St. Louis, MO) are dominated by a fraction that has Gln at position 18.

formation for membrane-bound alamethicin. These structural details may be critical for elucidating the molecular mechanisms of gating and are not easily addressed by optical and IR experiments that have focused primarily on global secondary structure.

An extremely powerful approach to defining the high-resolution structure of smaller membrane-bound proteins and peptides is solid-state NMR. In solid-state NMR, the observed system is anisotropic on the time scale of the experiment, and as a result, the ^{15}N chemical shift and ^{15}N-H dipolar splitting measurements are dependent on the orientation of the peptide plane and N-H vector in a polypeptide with respect to the B_0 magnetic field axis of the spectrometer. Thus, spectra retain information that can be used to determine the orientation and/or structure of macromolecules (Cross, 1986).

In this report, solid-state ^{15}N NMR was used to characterize the orientation of the alamethicin helix at the Ala_6 residue when the peptide is associated with dimyristoylphosphatidylcholine (DMPC) bilayers. NMR spectra of ^{15}N-Ala_6 alamethicin 1:8 with hydrated DMPC lipid were measured on random and uniformly oriented samples, and both chemical shift and dipolar interaction parameters were used to characterize the orientation and structure of alamethicin in the absence of a gating potential. This work demonstrates that the N-terminal segment of alamethicin is oriented along the bilayer normal and appears to be in an α-helical configuration. The results of this study combined with other recent independent findings have implications for the mechanisms of alamethicin gating.

MATERIALS AND METHODS

Synthesis and purification of ^{15}N-labeled alamethicin

Alamethicin containing ^{15}N-labeled alanine at position 6 (^{15}N-Ala_6 alamethicin) was made via solid phase peptide synthesis using standard FMOC chemistry. Amino acids used in the synthesis (Fmoc-Phe-alkoxybenzylalcohol resin, Fmoc-Gln(MTT)-OH, Fmoc-MeA-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Ala-OH) were purchased from Bachem Bioscience, Inc. (King of Prussia, PA). The Fmoc-^{15}N-Ala-OH was purchased from Cambridge Isotope Laboratories (Woburn, MA). To simplify the synthesis, this labeled alamethicin was made with a free C-terminus, which was then esterified to produce an acetylated C-terminus, Phe-O-Me, rather than the reduced phenylalaninol. Previous measurements indicate that this modification does not significantly affect the activity of the peptide (Archer and Cafiso, unpublished observations). Diisopropylcarbodiimide and 1-hydroxy-1,3-benzyotriazole hydrate were used as activators, and bromophenol blue (Krchnak et al., 1990) was used to monitor peptide coupling. The average coupling time for non-MeA residues was 1.5 h, but MeA was allowed to couple for 24–48 h, and double coupling was used for positions 9, 7, 5, 2, 1. After the synthesis was complete the resin was dried and cleaved using 95% trifluoroacetic acid (TFA)/5% triethylsilane. This reagent was cooled to −10°C, and 1.5 ml was added per 100 mg of resin. The TFA was immediately filtered into 10–30 times excess volume of cold ether, and the precipitated peptide was collected by centrifugation. The peptide was treated this way because of the highly acid labile MeA_{13}-Pro_{14} bond (Pandey et al., 1977). To form the methyl ester at the C-terminus the peptide was dissolved in methanol at a concentration of approximately 25 mg/ml, and 10 μl of TFA was added and the mixture stirred overnight at room temperature. The peptide was purified by high-pressure liquid chromatography (HPLC) on a Vydac reverse-phase C18 210TP1010 10 mm I.D. × 25 cm L HPLC column using an isocratic elution of 55/45 0.085% TFA in acetonitrile/0.1% TFA in water. The column was run at 3 ml/min, and the product had a retention time of 17 min. As judged by HPLC the peptide purity was greater than 97%. The identity of the ^{15}N-Ala_6-labeled alamethicin was confirmed by mass spectrometry, which yielded an *m/z* value (peptide OMe + Na^+) of 2015, and by high-resolution proton NMR.

Solid-state NMR: theory

The ^{15}N chemical shift tensor of a peptide plane is asymmetric, and the observed chemical shift in a static system is a function of the orientation of the peptide plane with respect to the B_0 field. The orientation of the tensor with respect to bonds of the amide ^{15}N has been measured (Teng and Cross, 1989). The σ_{33} principal axis is inside the H-N-C_1 angle, approximately 105° from the N-C_1 bond in the peptide plane (β = 105°), the σ_{22} principle axis is perpendicular to the peptide plane (α = 0°), and the σ_{11} axis is in the H-N-C_α angle orthogonal to the other two. In the case of a rod-shaped molecule constrained to rotating about its long axis, the chemical shift tensor averages to an axially symmetric tensor with principal components parallel and perpendicular to the axis of rotation ($\sigma_\parallel$ and $\sigma_\perp$, respectively). The observed chemical shift is then a function of the angle between the axis of rotation of the molecule and the magnetic field. Orientational information can also be obtained from the N-H dipolar interaction. This interaction is symmetric about the N-H bond and is a function of the angle between the internuclear vector and the B_0 field as given by

$$\Delta\nu_{obs} = \frac{\Delta\nu_\parallel}{2}[3\cos^2(\theta) - 1]. \quad (1)$$

Here, the value of $\Delta\nu_\parallel$, the maximum dipolar splitting, is a function of the internuclear distance and is 22.6 kHz, assuming an N-H internuclear distance of 1.024 Å (Lograsso et al., 1989).

When peptide-lipid mixtures are formed into uniaxially oriented bilayers by hydrating between glass plates, the samples and hence the peptide

may be oriented in the spectrometer with respect to the B_0 axis. Helical peptides that are bound to membranes are sterically restricted to motion about the long axis on the time scale of the NMR experiment. As a result, peptides that insert across the bilayer have chemical shifts near $\sigma_{\parallel}$ when the membrane plane is perpendicular to B_0 (Cross and Opella, 1994). Peptides that associate with the interface region of the lipids and lie perpendicular to the bilayer normal show a chemical shift near $\sigma_{\perp}$ (Bechinger et al., 1991, 1993). In addition, for peptides that are uniformly oriented about a single axis, the orientational dependence of the dipolar interaction may be used to determine the structure of the membrane peptide.

Orientation of the peptide plane

The possible orientations of the peptide plane that could produce the observed ^{15}N chemical shift were determined by applying a series of unitary transformations to the chemical shift tensor. The tensor is assumed to be oriented with respect to the bonds surrounding the amide ^{15}N as described above ($\beta = 105°$, $\alpha = 0°$). Beginning from an arbitrary starting position with the σ_{11}, σ_{22}, and σ_{33} along X_{lab}, Y_{lab}, and Z_{lab}, the tensor is transformed to reflect a convenient orientation of the peptide linkage plane. In this case, the $C_\alpha C_\alpha$ virtual bond vector is placed along $-X$, and the H-N-C_α plane is in the XY plane, so that the NH vector is close to $-Y$. Then unitary rotational transformations about X and Y represent rotation about the $C_\alpha C_\alpha$ axis and tilt of the $C_\alpha C_\alpha$ axis. Contour plots of observed chemical shift as a function of tilt and rotation were generated based on the limits of uncertainty of the measurements, and the union of the solution set areas is reported.

Solid-state NMR: experimental

Solid-state ^{15}N NMR experiments were performed on dry powdered ^{15}N-Ala_6 alamethicin, ^{15}N-Ala_6 alamethicin in randomly oriented hydrated DMPC, and ^{15}N-Ala_6 alamethicin in uniformly oriented hydrated DMPC bilayers. ^{15}N-Ala_6 alamethicin was incorporated into DMPC by codissolving peptide and lipid at a 1:8 molar ratio in 95% MeOH. For a randomly oriented sample, this mixture was then dried under vacuum in the presence of P_2O_5 overnight and packed into a sample chamber. H_2O was then added to make up 50% of sample weight, and the sample was allowed to equilibrate at ~50°C for 72 h. An oriented sample was prepared in a manner similar to that described previously (North and Cross, 1993). Briefly, the peptide lipid mixture in MeOH was spread onto glass coverslips (Fischer Scientific no. 1) cut to 5 × 11 mm. This was air-dried and then vacuum dried overnight in the presence of P_2O_5. The coverslips were stacked in a 6 × 6 × 16 mm i.d. rectangular sample chamber. Water was added to make up 50% of sample weight and the chamber was sealed. The sample was allowed to equilibrate for 72 h at ~50°C.

Nitrogen-15 NMR spectra were obtained on a highly modified NT-360 spectrometer fitted with a Tecmag data system, a Doty wide-band 0.5 kW amplifier on ^{15}N, a Henry 2002A for ^{1}H, and a multinuclear Doty Scientific goniometer probe. Spectra were recorded using cross-polarization followed by a Hahn echo to eliminate probe ringing. The temperature was 30°C. Times for 90° pulses were 6.4 μs, with a 1 ms contact time and a Hahn echo delay of 48 μs. A separated local field spectrum using cross-polarization was obtained on the oriented sample by incrementing in 20-μs steps the first portion of a 340-μs echo delay, during which the decoupler was turned off through 16 iterations. When transformed in the second dimension, peaks are observed at the chemical shift split by the magnitude of the dipolar interaction. All chemical shifts are relative to a saturated $^{15}NH_4NO_3$ solution.

RESULTS

The N-terminus of alamethicin is oriented along the bilayer normal

Shown in Fig. 2 are spectra for dry ^{15}N-Ala_6 alamethicin, ^{15}N-Ala_6 alamethicin in DMPC hydrated but with random

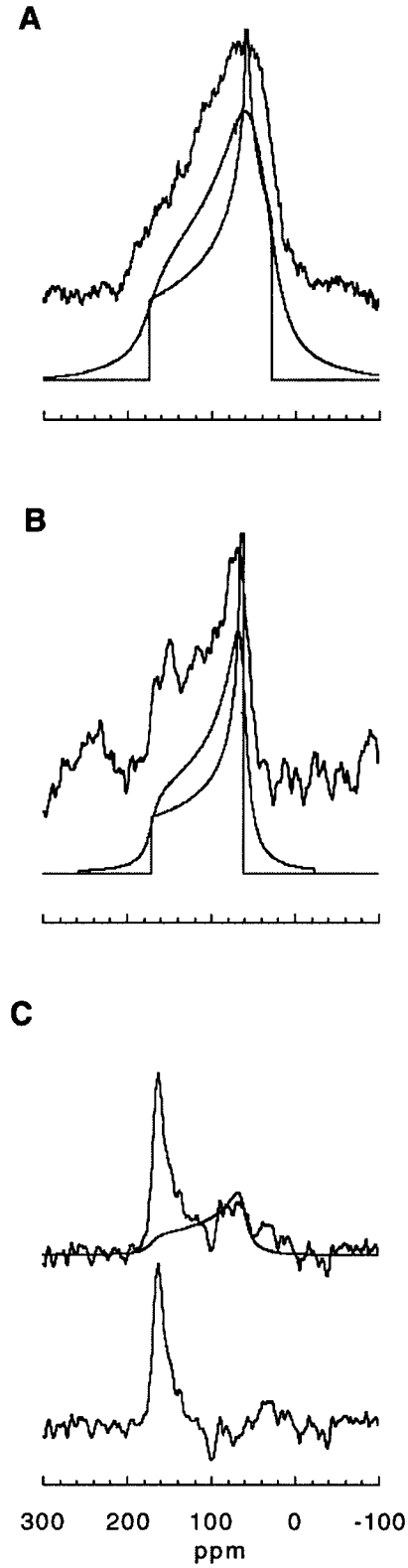

FIGURE 2 ^{15}N Solid-state NMR spectra of ^{15}N-Ala_6 alamethicin as a dry powder (*A*), with non-oriented hydrated DMPC (*B*), and in oriented hydrated DMPC bilayers (*C*). In *A* and *B*, observed spectra are shown above best-fit broadened and unbroadened calculated spectra. In *C*, the underlying powder pattern is fit and subtracted using the parameters determined from *B*. Spectra were obtained at 32°C using 1 ms cross-polarization followed by a 48 μs Hahn echo delay. 90° pulses were 6.4 μs.

orientation, and ^{15}N-Ala$_6$ alamethicin in hydrated oriented DMPC bilayers. The dry powder spectrum of ^{15}N-Ala$_6$ alamethicin (Fig. 2 *A*) is typical of a randomly oriented sample and can be fit by a simulated powder pattern, as shown in Fig. 2 *B*, yielding principal axis values of 22, 58, and 188 (±2) ppm relative to saturated $^{15}NH_4^+NO_3^-$. The spectra of ^{15}N-Ala$_6$ alamethicin in randomly oriented hydrated lipids show a reduced anisotropy due to motional averaging. This spectrum has the characteristic shape of an axially symmetric molecule reorienting about a single axis. Discontinuities in the spectrum that represent $\sigma_{\parallel}$ and $\sigma_{\perp}$ of the axially symmetric tensor occur at 171 and 62 (±3) ppm, respectively. The σ_{iso} was 89 ppm in dry powder and 98 ppm in the hydrated sample; the shift is due to the presence of water. Chemical shift tensor elements relative to σ_{iso} were used in the calculations of peptide plane orientations.

The spectrum of uniformly oriented ^{15}N-Ala$_6$ alamethicin (Fig. 2 *C*) is dominated by a single peak appearing at 171 ppm which corresponds to the $\sigma_{\parallel}$ value of the randomly oriented alamethicin in lipid bilayers. The half-width at half-height for the peak is 7 ppm. The peak is broadened asymmetrically downfield, suggesting that the polypeptide is not completely oriented. This appears to be the result of incomplete orientation of the membrane sample as a similar degree of non-orientation is also seen in ^{31}P NMR (data not shown). There is also some broad intensity downfield from the sharp peak, which is most likely the result of a portion of randomly oriented sample that has leaked from between the glass coverslips. This leakage results in a powder pattern underlying the oriented signal, which can be fit and subtracted as illustrated in Fig. 2 *D*. The single resonance peak observed near $\sigma_{\parallel}$ indicates that the peptide is inserted into the bilayer, so that the axis of molecular reorientation is aligned with the bilayer normal. If the peptide were lying on the membrane surface, a resonance near $\sigma_{\perp}$ would have been expected, as demonstrated for the highly charged peptide magainin 2 (Bechinger et al., 1993).

The orientation of Ala$_6$ is consistent with an α-helical configuration

The separated local field spectroscopy (SLOCF) experiment separates the CSA and NH-dipolar interactions onto orthogonal axes. Fig. 3 shows the SLOCF spectrum for ^{15}N-Ala$_6$ alamethicin in oriented hydrated DMPC bilayers. The angle between the dipolar interaction vector along the NH bond and the magnetic field is determined from the dipolar splitting according to Eq. 1. The SLOCF spectrum shows a dipolar interaction of 17 kHz for the resonance at 171 ppm, which corresponds to an average angle of 24° for the N-H bond with respect to the B_0 field axis.

Fig. 4 shows the orientations of the peptide plane relative to the magnetic field axis that could produce the 171 ppm chemical shift (*shaded ovals*) and the 24° angle (*unfilled ovals*) between the N-H bond and the Z_{LAB} axis measured in the SLOCF experiment on the oriented sample. The thickness of the ovals reflects the uncertainty in the measurements of the powder pattern discontinuities and the chemical shift of the oriented sample, as well as a conservatively estimated error of the dipolar splitting measurement as given above. The results of permutations of maximum errors were superimposed and the outside boundaries of the ovals were retained in Fig. 4. The calculations assumed a static structure; however, peptide plane librations of up to ±20° produce results contained by the error limits represented by the oval thickness. The orientation is defined by the tilt of the $C_\alpha C_\alpha$ axis and rotation of the peptide plane about the virtual $C_\alpha C_\alpha$ bond relative to a horizontal starting position as shown in Fig. 5. The resulting curves, including error bounds, intersect at four regions. These indicate a ±15–21° tilt for the $C_\alpha C_\alpha$ axis and a rotation that places the peptide plane approximately 24° from the *z* axis. The peptide plane positions relative to the LAB axis that could produce the oriented spectra are also the positions relative to a molecular symmetry axis (i.e., the long axis of a helix) that would produce the symmetrically averaged position seen in the randomly oriented ^{15}N-Ala$_6$ alamethicin in hydrated DMPC sample.

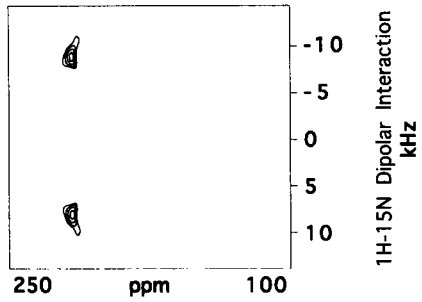

FIGURE 3 SLOCF spectrum of ^{15}N-Ala$_6$ alamethicin in oriented hydrated DMPC bilayers showing ^{15}N chemical shift on one axis and ^{1}H-^{15}N dipolar coupling on the other. The 17 kHz dipolar splitting is consistent with a 24° angle between the N-H bond and the applied magnetic field axis.

A 24° angle between the NH vector and a *Z*-oriented helical axis is consistent with an α-helix in the membrane. An α-helix

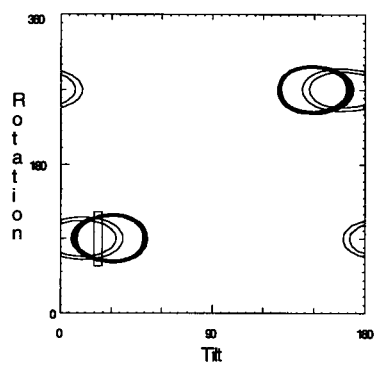

FIGURE 4 Solution sets of positions of the peptide plane that would produce the observed chemical shift (*shaded oval*) and dipolar splittings (*white oval*). The oval thicknesses reflect the outer limits of the measurement uncertainty. The rectangle represents the approximate range of tilt and rotation parameters for an oriented regular α-helix.

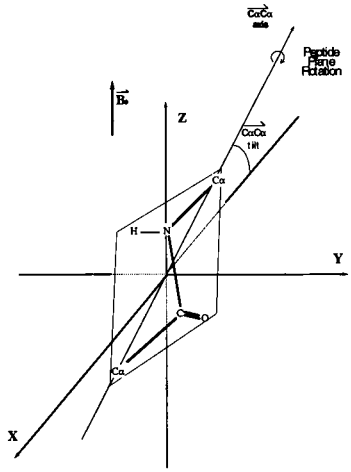

FIGURE 5 Illustration of tilt and rotation parameters. The tilt and rotation parameters can be related to the local helicity and peptide torsional angles of Peticolas and Kurtz (1980). The tilt of a peptide linkage plane in an oriented helix gives a direct measure of local helicity, whereas the rotation relates to the peptide torsional angle and measures deflection of the plane from the helix axis.

in the plane of the bilayer would show either an averaged 90° angle or a fixed angle between 60° and 90° with dipolar splittings of 3 to 11.6 kHz. Likewise, the apparent tilt of the $C_\alpha C_\alpha$ axis is also consistent with an α-helix oriented in the membrane. The peptide does not appear to be in a 3_{10} configuration, as a regular 3_{10} structure would be expected to have a tilt of the $C_\alpha C_\alpha$ axis of 30° or more.

DISCUSSION

The data shown here provide a strong indication that alamethicin orients about a single axis of symmetry when membrane bound. The chemical shift and the N-H dipolar coupling of the peptide obtained in oriented samples indicate that the N-terminus of the peptide is α-helical and oriented along the bilayer normal. This is consistent with previous spectroscopic studies indicating that alamethicin is inserted into the bilayer (Huang and Wu, 1991; Vogel, 1987), and it argues against models for gating that involve peptide transitions from a surface to a transmembrane orientation (Baumann and Mueller, 1974). The presence of MeA residues in peptides is thought to enhance their tendency to form helical structures, and polypeptides containing MeA residues have been shown to have a tendency to form structures with 3_{10} character (Toniolo and Benedetti, 1991). This does not appear to be the case with the N-terminal segment of alamethicin, as a much larger tilt angle of the $C_\alpha C_\alpha$ axis would have been expected for a regular 3_{10} structure.

Shown in Fig. 6 is a model for the gating of alamethicin that was proposed based on recent structural data in solution (North et al., 1994). In this model alamethicin is in a bent configuration in the absence of a membrane field, and the polar face of the peptide is positioned toward the interior bend of the peptide (the two Gln residues at 7 and 18 are in close proximity and may hydrogen bond). The peptide is depicted to be monomeric, which is consistent with EPR measurements indicating that alamethicin is predominantly monomeric in the absence of a membrane potential (Archer et al., 1991; Barranger-Mathys and Cafiso, 1994). Application of a transmembrane electric field linearizes the peptide and creates a laterally amphipathic helix. This conformational transition is driven primarily by reorientation of the alamethicin dipole, which is substantial even for the bent form (Lewis et al., 1995). In its linear voltage-activated form, peptide aggregation and channel formation then occur. The ^{15}N NMR data presented here in the absence of a membrane potential do not appear to support this model. If alamethicin were present in the membrane in a bent configuration, axial motion should not occur exclusively about a helical N-terminal segment. Although the N-terminal segment is inserted along the bilayer normal in the bent structure, the C-terminal segment would be expected to have a significant influence on the overall motion of the peptide and the data should not place the N-terminus exactly along the bilayer normal.

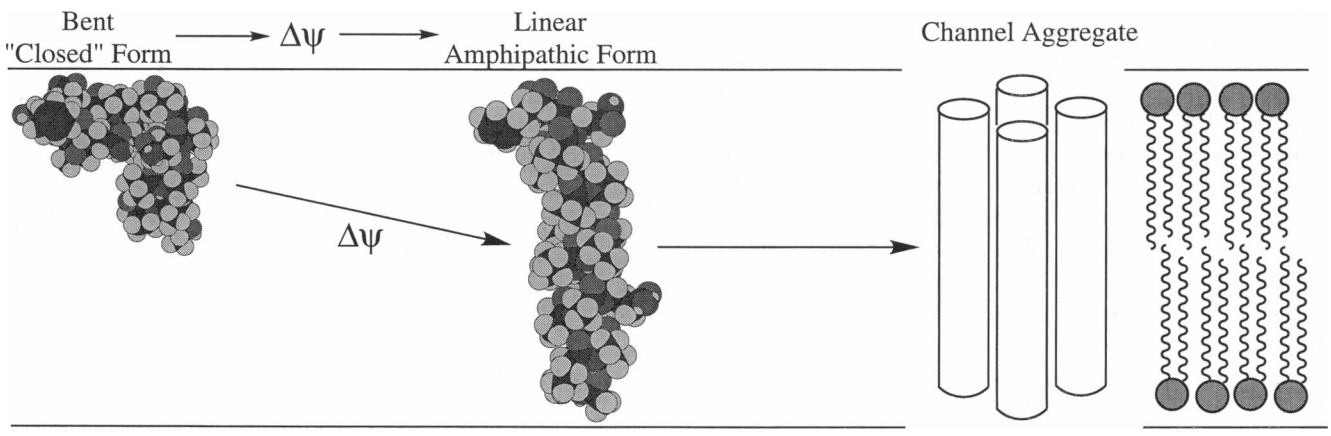

FIGURE 6 A model for the voltage gating of alamethicin based on recent structural and dynamic information obtained for alamethicin in detergent micelles and in solution.

It should be noted that the present experimental conditions may limit the possible conformations that alamethicin can assume. The conformational forms shown in Fig. 6 are close in energy, and the high peptide:lipid ratios that are used may limit conformations that do not pack efficiently into the bilayer interface. Experiments are currently under way to obtain NMR spectra for alamethicin under a wider range of concentrations and to obtain structural data for alamethicin that has been isotopically labeled in the C-terminal domain.

In summary, the solid-state NMR spectroscopy of oriented ^{15}N-Ala$_6$ alamethicin indicates that this molecule has an axis of motional averaging that lies along the bilayer normal in fully hydrated DMPC bilayers in the absence of a membrane potential. An analysis of the chemical shift and N-H dipolar splitting show that these data are consistent with this segment of alamethicin being inserted along the bilayer normal in an α-helical conformation. The data shown here represent the first solid-state NMR data that have been obtained on specifically labeled alamethicin and on MeA-containing peptides. The synthesis of MeA-containing peptides using solid-phase techniques is typically slow; however, new coupling methods are improving the efficiency of peptides containing this residue (Wenschuh et al., 1994), and the synthesis of additional specifically labeled alamethicin analogs should permit a detailed, high-resolution structure determination for this voltage-gated peptide in membranes.

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