Transmembrane Four-Helix Bundle of Influenza A M2 Protein Channel: Structural Implications from Helix Tilt and Orientation

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ABSTRACT The transmembrane portion of the M2 protein from the Influenza A virus has been studied in hydrated dimyristroylphosphotidylcholine lipid bilayers with solid-state NMR. Orientational constraints were obtained from isotopically labeled peptide samples mechanically aligned between thin glass plates. ^{15}N chemical shifts from single site labeled samples constrain the molecular frame with respect to the magnetic field. When these constraints are applied to the peptide, modeled as a uniform α-helix, the tilt of the helix with respect to the bilayer normal was determined to be 33° ± 3°. Furthermore, the orientation about the helix axis was also determined within an error of ±30°. These results imply that the packing of this tetrameric protein is in a left-handed four-helix bundle. Only with such a large tilt angle are the hydrophilic residues aligned to the channel axis.

INTRODUCTION

The structural characterization of membrane proteins remains one of the greatest challenges in structural biology today. Here we present preliminary results, using solid-state NMR-derived orientational constraints from the transmembrane α-helix of the influenza A viral M2 protein. Solid-state NMR is maturing as an important technique for protein structure determination in membrane environments (Arumugam et al., 1996; Bechinger et al., 1996; Cross, 1994; Cross and Opella, 1994; Ketchem et al., 1993, 1996; McDonnel et al., 1993; North et al., 1995; Prosser et al., 1994; Smith et al., 1994). Although 30% of cellular proteins are membrane associated, relatively few structures have been achieved, despite recent successes by x-ray diffraction, electron cryomicroscopy, and solution NMR that have been used to solve some very important structures, such as cytochrome *c* oxidase (Iwata et al., 1995; Tsukihara et al., 1996), cytochrome bc_1 complex (Akiba et al., 1996), the light-harvesting complex (Kühlbrandt et al., 1994), and the phospholamban ion channel complex (Mortishire-Smith et al., 1995). Here, continued progress in the development of solid-state NMR as a structural approach in this field is reported.

The M2 protein from the influenza A virus has been shown to function as an ion channel (Holsinger et al., 1995; Pinto et al., 1992; Tosteson et al., 1994; Wang et al., 1993). The protein as well as a peptide containing the predicted transmembrane region, M2-TMP (Duff and Ashley, 1992), have been shown to be capable of proton conductance. After the virion particle has been endocytosed into the target cell, the M2 protein allows protons from the low-pH environment of the endosome to pass into the virion particle, thereby aiding in the uncoating of the virion particle (Hay, 1992; Sugrue and Hay, 1991). The antiviral drug amantadine reversibly blocks the channel and prevents infection (Pinto et al., 1992; Tosteson et al., 1994; Wang et al., 1993).

The primary amino acid sequence of the M2 protein has been predicted from the Udorn/72 strain mRNA sequence (Lamb et al., 1985). A single highly hydrophobic stretch of 19 amino acids is observed in this sequence between residues 25 and 43. The entire protein is observed to have a type III membrane protein topology with an uncleaved signal anchor sequence and a $N_{exo}C_{cyt}$ orientation (Parks and Lamb, 1991; von Heijne, 1988). From electrophoresis studies, it has been shown that the M2 protein forms disulfide bonds, which stabilize the formation of dimers (30 kDa) and tetramers (60 kDa) (Holsinger and Lamb, 1991), and recently it has been clearly demonstrated that the active oligomeric state is tetrameric (Sakaguchi et al., 1997). Furthermore, it is known that this channel is formed in mutants without cysteine and hence without cross-links (Holsinger et al., 1995). In this study a peptide (M2-TMP) containing the predicted transmembrane region was synthesized: NH_2-Ser^{22}-Ser^{23}-Asp^{24}-Pro^{25}-Leu^{26}-Val^{27}-Val^{28}-Ala^{29}-Ala^{30}-Ser^{31}-Ile^{32}-Ile^{33}-Gly^{34}-Ile^{35}-Leu^{36}-His^{37}-Leu^{38}-Ile^{39}-Leu^{40}-Trp^{41}-Ile^{42}-Leu^{43}-Asp^{44}-Arg^{45}-Leu^{46}-CO_2H. Based on current knowledge, the transmembrane channel formed by this peptide, which has functional properties similar to those of the channel formed by the protein, is thought to be a four-helix bundle, with one helix contributed by each of four polypeptide monomers.

In NMR spectroscopy, the chemical shift for a nucleus is a function of the electron density about the nucleus and can be described by a second-rank tensor. This is represented by three orthogonal vectors, σ_{11}, σ_{22}, and σ_{33}, that have a specific orientation with respect to the molecular frame, which for ^{15}N is described by the Euler angles α_D and β_D (Fig. 1). The tensor element magnitudes are frequencies that characterize the static powder pattern spectrum (a solid-

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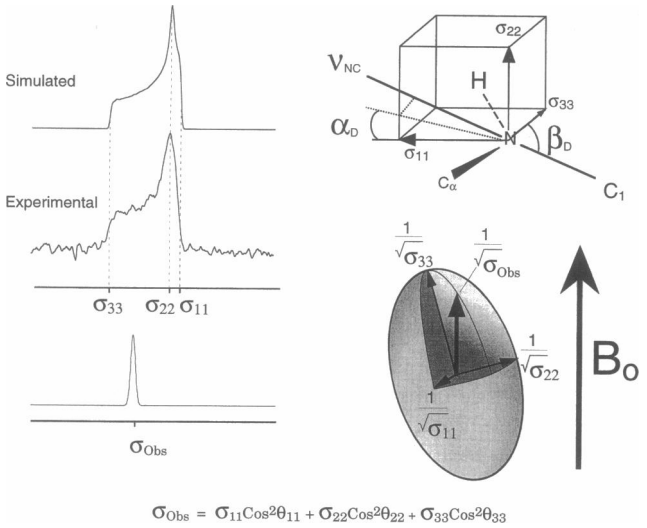

FIGURE 1 The ^{15}N chemical shift tensor element magnitudes (in ppm) are determined from an unoriented powder sample. A simulated powder spectrum, based on tensor element magnitudes (σ_{11}, σ_{22}, and σ_{33}), is compared to the experimental data. The tensor orientation is characterized by the angles, θ_{ii}, between the individual elements, σ_{ii}, and B_0. Observed ^{15}N chemical shifts (in ppm) from oriented peptide samples constrain the possible orientations of the chemical shift tensor and the molecular frame (by way of α_D and β_D) with respect to B_o.

state NMR spectrum of an unoriented sample). The chemical shift tensor can be represented as an ellipsoid with semiaxis magnitudes proportional to the σ_{ii} values (Sherwood, 1995). When this ellipsoid has a specific orientation with respect to the magnetic field (B_o), a single frequency (σ_{obs}) will be observed in the NMR spectrum corresponding to the magnitude of a vector that intersects the ellipsoid surface in the direction of B_o. This orientationally dependent chemical shift provides a constraint on how the molecular frame is oriented with respect to B_o. In combination with other such constraints, these become structural constraints, and a complete 3D structure can be achieved (Ketchem et al., 1996). Recently the structure of gramicidin in fully hydrated lipid bilayers, solved by this approach, has been deposited in the Brookhaven Protein Data Bank (#1MAG).

MATERIALS AND METHODS

[^{15}N]Isoleucine was purchased from Cambridge Isotope Laboratories (Cambridge, MA), from which the Fmoc derivative was prepared in our laboratory as previously described (Fields et al., 1988, 1989). Solid-phase 0.25 mmol syntheses of M2-TMP were performed on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) utilizing Fmoc (9-fluorenylmethoxycarbonyl) chemistry. The Fastmoc protocol (as described in the Applied Biosystems Model 431A Peptide Synthesizer User's Manual, Rev. B, version 2.0) was employed. This protocol uses HBTU (2-(^{1}H-benzptriazol-1-yl)1,1,3,-tetramethyluronium hexaflurorophosphate) activation and HMP (*p*-hydroxymethylphenoxyacetic acid)-modified polystyrene resin with the first amino acid (Fmoc-Leu) attached. The resin was treated with acetic anhydride after each amino acid coupling, to acetylate any peptides that did not successfully couple, thereby reducing the possibility of deletions in the peptide.

Cleavage of the peptide was performed with 25 ml of ice-cooled trifluoroacetic acid, 800 mg indole, 600 μl thioanisole, and 600 μl ethanedithiol. Synthetic yields were estimated to be between 80% and 90%. The cleaved peptides were reprecipitated by dissolving the peptide in a small amount (0.5–1.0 ml) of 95% tetrahydrofuran in HPLC-grade H_2O. This solution was then slowly added dropwise into a flask containing 200 ml of H_2O. This solution was centrifuged and the supernatant discarded. The precipitated peptide was then dried in a vacuum and stored in the freezer.

Amino acid sequencing was performed to determine the five N-terminal amino acids of the peptide on an Applied Biosystems 477 Amino Acid Sequencer. This technique does not provide a measure of the sample purity, because it does not detect acetylated peptides, but it does provide evidence that synthesis of the full-length peptide occurred. Samples were further characterized by solution NMR. M2-TMP was dissolved in 1:1 (v/v) chloroform-d:methanol-d_3 (Cambridge Isotope Laboratories) at a concentration of 10 mM. The gradient correlation spectroscopy (GCOSY) solution spectrum was recorded on a Varian UnityPlus 720 MHz spectrometer, equipped with three axis pulse field gradients, at 30°C with four scans and presented in absolute mode. A gradient level of 0.32 gauss/cm and a duration time of 1.9 ms were used for coherence selection. Along the F1 and F2 axes, 256 and 4096 data points, respectively, were collected. The residual chloroform peak was referenced to 7.27 ppm.

To characterize the secondary structure, circular dichroism (CD) spectra of M2-TMP in dimyristoylphosphatidylcholine (DMPC) (Sigma, St. Louis, MO) vesicles were observed. These vesicles were prepared by taking a sample prepared as for solid-state NMR and drying it under vacuum. High-performance liquid chromatography (HPLC)-grade H_2O was added to dilute the solution to 6 μg/ml of peptide. The solution was sonicated for 15 min to minimize light scattering. The CD spectra were recorded on a J-500 Jasco CD Polarimeter (Tokyo, Japan).

To prepare oriented samples, M2-TMP and DMPC at a 1:8 molar ratio were cosolubilized in methanol. The solution was spread on 75-μm-thick glass plates (5.8 × 15 mm) and allowed to air dry for 8–10 h before vacuum drying for another 12 h. The dry plates were then stacked in a borosilicate sample tube (6 × 6 mm i.d.) that was flame sealed at one end. Sufficient HPLC-grade H_2O was added to achieve 50% by weight hydration. The sample was sealed with a square glass cover and epoxy before incubating at 45°C for 1–2 weeks. Powder samples of the peptide without lipid were prepared by packing a sample tube with dry peptide.

^{31}P NMR spectra were used to characterize oriented samples. Such spectra were obtained on a narrow-bore IBM/Bruker 200SY spectrometer with a home-built solids package. A double-resonance $^{31}P/^1H$ probe was used for proton decoupling. All of the ^{31}P experiments were performed at a resonance frequency of 80.99 MHz, with a recycle delay of 4 s and a 90° pulse width of 11 μs. The ^{15}N NMR spectra were obtained on a home-built 400 MHz spectrometer, using a Chemagnetics data acquisition system and a wide-bore Oxford Instruments 400/89 magnet. A double-frequency probe permitted proton decoupling at a field strength of 70 kHz and cross-polarization. Spectra were obtained at a resonance frequency of 40.6 MHz, with a contact time of 1 ms, a recycle delay of 7 s, and a 90° pulse width of 4.5 μs.

The spectral data were processed with Felix (Biosym Technologies, San Diego, CA). Molecular modeling was performed with Insight II (Biosym Technologies). All computational work was performed on a Silicon Graphics Indigo 2 Extreme.

RESULTS

The identity of the peptide sample was checked by amino acid sequencing of the first five N-terminal residues and by solution NMR of the peptide. The amino acid analysis gave a positive sequence identification for all of the peptides used in this study. Fig. 2 shows the ^1H NMR GCOSY spectrum for the NH proton fingerprint region of [^{15}N]Ile^{35}-M2-TMP in 1:1 (v/v) chloroform-d:methanol-d_3. This spectrum indi-

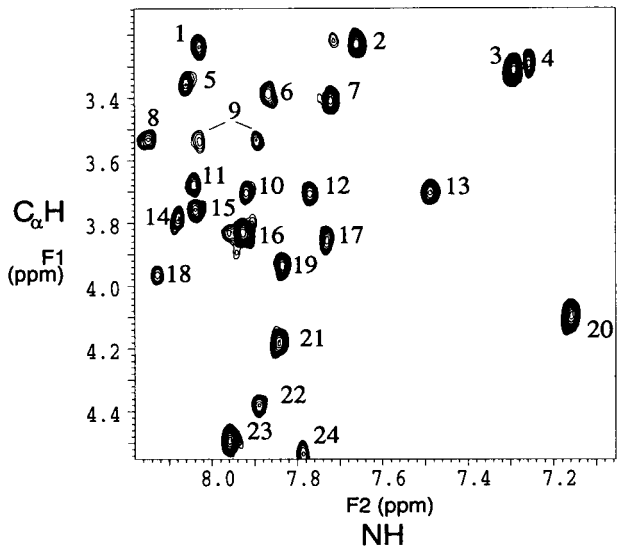

FIGURE 2 Fingerprint region of the GCOSY spectrum for ^{15}N-Ile^{35}-M2-TMP (10 mM) in 1:1 (v/v) chloroform-d:methanol-d$_3$. Peak 9 shows ^{15}N-^{1}H J coupling and is assigned to Ile^{35}. Peak 16 is believed to result from the coupling of the C$_\alpha$H to NH$_2$ in Ser^{22}. The 720-MHz spectrum was recorded at 30°C with four scans per increment. The lack of multiple resonances for each site is evidence of chemical and conformational purity.

cates the dominance of 24 cross-peaks with one ^{15}N *J*-coupled (~100 Hz) resonance assigned to Ile^{35}. A couple of much weaker resonances in this region may be due to conformational heterogeneity of the terminal residues, but the dominance of the primary signals documents the chemical purity of the peptide.

Circular dichroism (CD) studies of the M2-TMP in DMPC vesicles demonstrated the dominance of α-helical secondary structure in this lipid environment (Fig. 3), characterized by a double minimum at 222 and 208–210 nm and a maximum at 190–193 nm. Based on the molar ellipticity at these critical wavelengths, it is estimated that the peptide is 85–90% α-helix in this lipid environment (Yang et al., 1986).

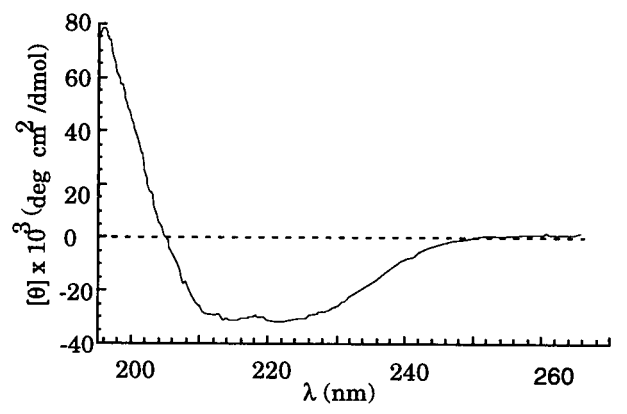

FIGURE 3 The CD spectrum for M2-TMP (6 μg/ml) in DMPC vesicles.

^{15}N Chemical shift powder pattern spectra were observed, using unoriented samples of each single site labeled peptide (Fig. 1). These tensor element magnitudes, σ_{11}, σ_{22}, and σ_{33}, were determined by simulating the spectra (Table 1).

Five individually [^{15}N]Ile-labeled peptides were oriented in fully hydrated DMPC bilayers. The orientation of the lipid bilayers was characterized by using samples with the glass plates oriented perpendicular to B_o by ^{31}P NMR spectra (Fig. 4). This single sharp resonance at $\sigma_\parallel$ in the spectrum of an aligned sample is indicative of lipid molecules in a bilayer phase where their rotational axis is parallel to the direction of the magnetic field. Based on this spectrum and similar spectra for other labeled M2-TMP peptides, the bilayers appear to be very well oriented (Moll and Cross, 1990). Anisotropic ^{15}N chemical shifts were observed for each of these oriented samples (Fig. 5). The chemical shifts, referenced to a saturated solution of $^{15}NH_4NO_3$, range from a low of 116 ppm for Ile^{35} to 172 ppm for Ile^{33} (Table 1). The line width at half-height for these resonances varies from 17 ppm for Ile^{35} to 25 ppm for Ile^{33} and is large compared with similar gramicidin A resonances. In part, this may be due to the relatively high concentration of peptide in these samples, but preliminary data (not shown) up to molar ratios of 1:100 do not appear to influence either the observed chemical shift or the line width. Alternatively, a small amount of conformational or orientational heterogeneity could account for the line widths.

A uniform α-helical model of the M2-TMP was generated by using Insight II, with $\phi = -65°$ and $\psi = -40°$ and a periodicity of 3.6 residues per turn. The peptide was oriented so that the helix axis was parallel to *Z*, equivalent to the bilayer normal and B_o. For the five Ile sites, chemical shift tensors were fixed to the helix (Fig. 6), using the experimentally observed tensor element magnitudes in Table 1 and $\alpha_D = 0°$ and $\beta_D = 105°$. These Euler angles represent typical values for ^{15}N amide tensors in peptides (Mai et al., 1993; Teng and Cross, 1989). Using this α-helical model of the peptide, calculations were made for the set of ^{15}N chemical shifts over a range of helix orientations described by two Euler angles: ρ, rotation about the helix-axis, and τ, tilt of the helical axis with respect to the bilayer normal and B_o. The calculated chemical shifts were then compared to the experimentally observed values for each site over the entire range of ρ and τ. This was done by calculating over all sites the RMSD between the calculated and observed chemical shifts. These values are plotted as a function of ρ and τ (Fig. 7), and a unique minimum on this

TABLE 1 ^{15}N chemical shift values

Peptide	σ_{11}	σ_{22}	σ_{33}	σ_{Obs}
Ile^{32}	34	59	210	124
Ile^{33}	31	54	202	172
Ile^{35}	31	56	200	116
Ile^{39}	28	56	201	127
Ile^{42}	36	55	209	130

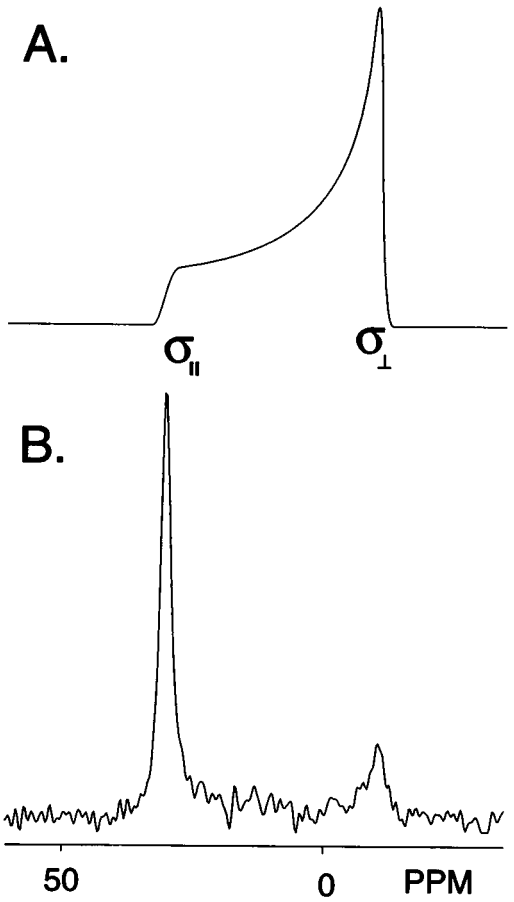

FIGURE 4 Simulated ^{31}P spectrum (*A*) for a hydrated powder of lipids in the lamellar phase is compared to an experimental ^{31}P spectrum (*B*) of an oriented sample of ^{15}N-Ile_{35} M2-TMP. The ^{31}P spectrum was obtained at 80.9 MHz and a temperature of 30°C, above the phase transition temperature for this sample. The parallel and perpendicular components of the motionally averaged chemical shift tensor are shown. The sharp resonance in the experimental spectrum coinciding with the parallel component indicates that the motional axis is aligned parallel to the magnetic field direction. Similar spectra were obtained for the other oriented samples.

RMSD surface corresponding to $\tau = 33°$ and $\rho = 300°$ is found.

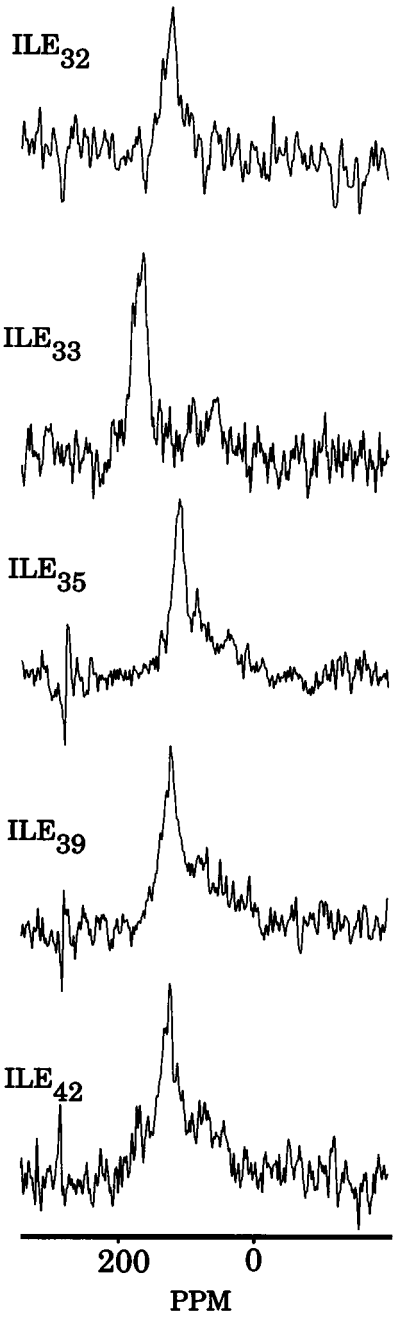

FIGURE 5 Five ^{15}N-Ile-labeled M2-TMP samples were synthesized by Fmoc chemistry. Oriented samples of these peptides were prepared by using DMPC at a lipid-to-peptide ratio of 8:1. These solid-state NMR spectra were obtained at 40.6 MHz with a contact time of 1 ms, a recycle delay of 7 s, and a 90° pulse width of 4.5 μs at 30°C in the liquid crystalline phase.

DISCUSSION

The rigid helix model is a crude approximation of a helix that is unlikely to be straight and will not be perfectly uniform. However, the initial observation that there is a very broad range of chemical shift values is in itself strong evidence for a significantly tilted helix. Furthermore, the analysis shown in Fig. 7 fits remarkably well to a uniform helical structure. Additional approximations have also been made in this preliminary analysis. Dry powders of the polypeptides have been used to define magnitudes of the chemical shift tensor elements. However, it has been shown previously that the local dynamic averaging in a dry powder at room temperature is similar to the local dynamic amplitudes in hydrated bilayers, in that the use of tensors from dry powders led to an interpretation of chemical shift orientational constraints that was consistent with extant structural models (Mai et al., 1993). It is important to note that global motions about the bilayer normal do not affect the interpretation of the chemical shifts. It is only the motion about axes that are not parallel to the magnetic field direction that affect the interpretation and hence the concern about small-

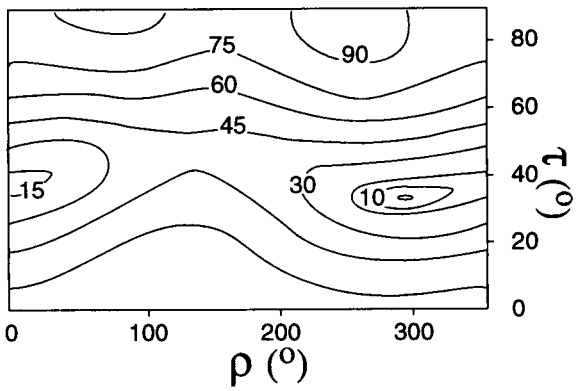

FIGURE 6 Chemical shifts were calculated for ρ from 0° to 360° and for τ from 0° to 90°. The RMSD between the experimental and calculated chemical shifts was calculated for each ρ and τ pair and plotted here. Contours at 90, 75, 60, 45, 30, 15, 10, and 5.5 ppm are shown. This plot has a single minimum at $\rho = 300°$ and $\tau = 33°$.

amplitude librational motions. In addition, the ^{15}N amide tensor orientation with respect to the molecular frame has been assumed, but this appears to be quite independent of the amino acid sequence, unlike the tensor element magnitudes (Mai et al., 1993). Although we have reproduced the CD results of Duff and co-workers (Duff et al., 1992) in our own samples, showing that the M2-TMP is largely α-helical, it is certainly not evidence for an ideal, rigid, perfectly straight helix. As we collect additional orientational constraints, model fitting the data will give way to defining a unique structure in much the same way as the structure of gramicidin has been solved.

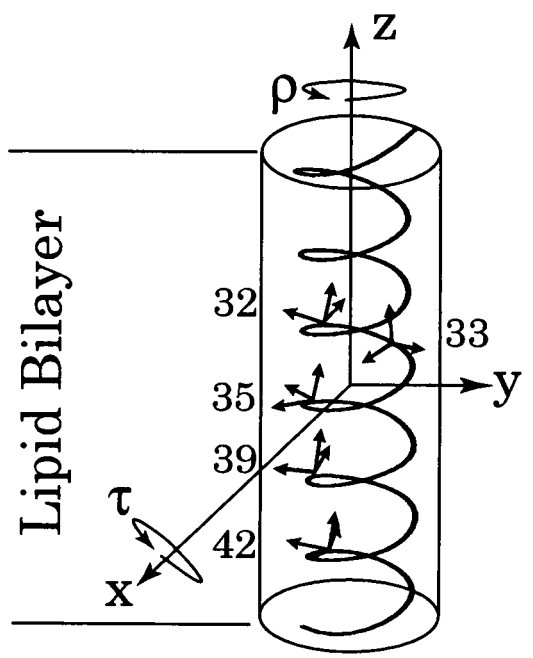

FIGURE 7 M2-TMP was modeled as an α-helix with $\phi = -65°$ and $\psi = -40°$. The ^{15}N chemical shift tensors were oriented with respect to the molecular frame, using $\alpha_D = 0°$ and $\beta_D = 105°$. The Euler angles, ρ and τ, describe the orientation of the helix with respect to the lipid bilayer, which has been oriented with the bilayer normal parallel to B_0.

The initial low-resolution results provide data for significant insights. Most membrane proteins are modeled with helices that have no tilt or only a small tilt with respect to the bilayer normal. The rotation and tilt angles are defined by the analysis in Fig. 6 for M2-TMP, and the error in these determinations can be estimated by calculating the change in ρ and τ for a given change in chemical shift. The chemical shift data presented in Fig. 5 have a significant error that is estimated to be less than ± 7 ppm. However, such a variation in chemical shift for the Ile^{33} site causes only a $\pm 3°$ change in τ while keeping ρ fixed at 300°, and a $\pm 15°$ change in ρ while keeping τ fixed at 33°. For the full set of data, Fig. 6 shows the RMS deviation in units of chemical shift. An RMS deviation of 10 ppm defines an orientational space that is approximately $\pm 3°$ in τ and $\pm 30°$ in ρ. Therefore, the error in tilt and rotation described for Ile^{33} is supported by the full data set, with the exception that ρ is less well defined. This angle of the helix is not as well defined by the orientational constraints (Fig. 6), because the unique tensor element, σ_{33}, of the amide ^{15}N chemical shift tensor, is aligned almost parallel to the helix axis and is therefore not significantly affected by the rotation angle. Instead, σ_{11} and σ_{22}, which have similar values, are modulated by the rotation angle; this leaves the ρ value defined with a larger error bar. The observed line widths appear to span a greater range of chemical shift than would be expected based on the ^{31}P NMR results. This line width could reflect an inherent variation in ρ and τ, but this would suggest nonspecific interactions between the helices. There are few high-resolution examples of helix-helix interactions in a membrane environment, but in glycophorin the interactions show an almost lock-and-key type of interaction that rigidly fixes the relative orientation of the two helices (Mackenzie et al., 1997). However, there is mutagenesis evidence that some membrane proteins may display considerable flexibility between transmembrane helices. In the M2 four-helix bundle, it will not be possible to change the tilt of the helices with respect to the bilayer normal without changing the relative orientation of one helix with respect to another.

However, even a relatively small change in the tilt angle and a somewhat greater range in ρ can completely account for the observed line width. Despite these broad lines, the solid-state data constrains the tilt angle quite precisely and limits heterogeneity. The precision of the tilt angle suggests that all monomers are identical (i.e., the structure has fourfold symmetry) or nearly identical (i.e., pseudo-fourfold symmetry) (Fig. 8). Clearly the structure is not a dimers of dimers, in which two monomers have very different orientations with respect to the bilayer normal, because a single resonance is observed for each single site label.

For this fourfold or pseudo-fourfold packing motif there are two possible arrangements, a right- or left-handed four-helix bundle. There are three hydrophilic residues in the hydrophobic portion of this peptide, Ser^{31}, His^{37}, and Trp^{41}.

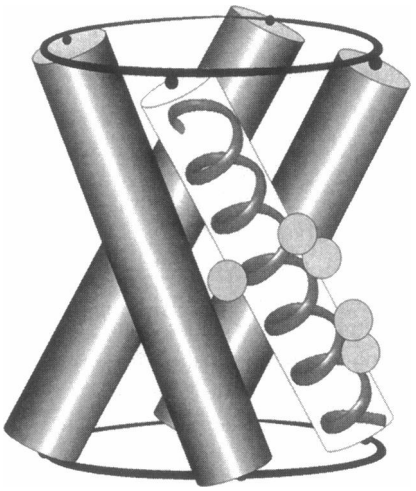

FIGURE 8 The C_β carbons for the five Ile sites are shown on a three-dimensional representation of the tilted four-helix bundle. No effort was made to model the interhelical spacing, but only to show the helical packing arrangement.

The two motifs result in a very different distribution of these residues (Fig. 9). Only the left-handed model places the hydrophilic residues in the interior of the structure, away from the hydrophobic lipid domain and toward the putative central location of the channel. Evidence has been presented that the His residue is important for the pH gating of this channel (Wang et al., 1995) and should therefore be exposed to the channel pore. Moreover, only with this very significant tilt angle are the hydrophilic residues aligned with the channel axis.

In this initial solid-state NMR effort with a transmembrane peptide from the influenza A M2 protein, we have strong evidence for the orientation and packing of the helices in a four-helix bundle. Relatively few orientational constraints have been required to achieve this result. Once again, solid-state NMR of aligned samples is shown to be a technique that can solve structural questions for membrane polypeptides in hydrated lipid bilayers. Work will continue in this laboratory toward a high-resolution structure of this protein.

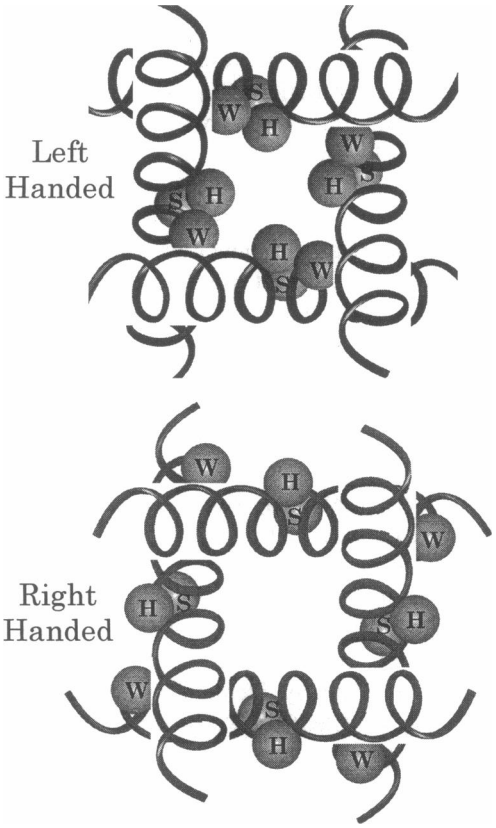

FIGURE 9 The determined ρ and τ values were applied to each monomer of a four-helix bundle. The monomers were then arranged in left- and right-handed motifs to determine which conformation places the polar residues in closest proximity to the adumbrated channel.

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