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Orientational constraints as three-dimensional structural constraints from chemical shift anisotropy: The polypeptide backbone of gramicidin A in a lipid bilayer

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Abstract

Chemical shifts observed from samples that are uniformly aligned with respect to the magnetic field can be used as very high-resolution structural constraints. This constraint takes the form of an orientational constraint rather than the more familiar distance constraint. The accuracy of these constraints is dependent upon the quality of the tensor characterization. Both tensor element magnitudes and tensor orientations with respect to the molecular frame need to be considered. Here these constraints have been used to evaluate models for the channel conformation of gramicidin A. Of the three models used, the one experimentally derived model of gramicidin in sodium dodecyl sulfate micelles fits the data least well.

Keywords: gramicidin A; oriented bilayers; ^{15}N solid-state NMR; structural constraints; structure determination

Orientational constraints from solid-state NMR can provide the data necessary to determine the three-dimensional structure of a protein in much the same way that distance constraints can be used to achieve three-dimensional structures. Here, orientational constraints from ^{15}N chemical shift anisotropy of polypeptide backbone sites in gramicidin A, solubilized in uniformly oriented lipid bilayers, are used to evaluate several model structures. Only with the addition of numerous other orientational constraints will it be possible to determine the structure a priori; such studies for gramicidin are ongoing (Teng et al., 1991; Cross et al., 1992). Unlike the distance constraints from solution NMR, these orientational constraints are precise quantitative constraints that can lead to very high-resolution structural detail, such as the determination of the ω torsion angles in the polypeptide backbone (Ketchem & Cross, unpubl.). However, in order to achieve this structural detail it is necessary to characterize very accurately the nuclear spin interaction tensors with respect to the molecular frame. While the approximate orientation for the ^{15}N chemical shift tensor can be taken from the single crystal study of glycylglycine (Harbison et al., 1984), it is necessary to determine the chemical shift tensor element magnitudes and their orientation with respect to the molecular frame for the specific sites of interest to minimize the error associated with each orientational constraint (Teng & Cross, 1989).

Gramicidin is a polypeptide of 15 amino acid residues that as a dimer forms a monovalent cation selective channel across lipid bilayers and membranes. A single-stranded β-helical structure was first proposed by Urry in 1971 for the channel conformation. This conformation is a strand of β-sheet that is wrapped into a helical structure with all of the side chains arranged on the outside interacting with the surrounding lipid environment. This is made possible by the alternating D and L stereochemistry of the amino acid residues. Consequently, the pore is lined with the peptide linkages and for the 6.3-residue-per-turn conformer, a 4-Å-diameter pore contains a single file of water molecules. One of the first significant applications of ^{15}N chemical shift constraints was to show that the gramicidin channel conformation in a lipid bilayer is a right-handed helix (Nicholson & Cross, 1989) instead of the widely held belief, at that time, that the structure was left-handed. Three crystal structures of gramicidin have been achieved, but none of them resemble the channel conformation (Langs, 1988; Wallace & Ravikumar, 1988; Langs

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et al., 1991). Instead, these crystal structures are intertwined helical dimers typical of the structures observed in organic solvents. Cocrystals of gramicidin and lipid have been extant for many years, but the structure of the channel conformation remains unsolved by X-ray diffraction (Kimball & Wallace, 1981; Wallace & Janes, 1991). Despite this lack of a three-dimensional crystal structure a well-refined solution NMR structure of the channel-like conformation of gramicidin has been achieved by studies of gramicidin in sodium dodecyl sulfate (SDS) micelles (Arseniev et al., 1986; Lomize et al., 1992). Furthermore, there have been numerous computational efforts to refine the structure of the channel conformation of gramicidin (e.g., Venkatachalam & Urry, 1983; MacKay et al., 1984; Roux & Karplus, 1988, 1991; Chiu et al., 1989, 1991). These computational efforts not only provide a structural model and rationale for conductance properties, but they also generate models for the local motion of the polypeptide backbone. Recently, it has been shown experimentally that, at least in the absence of cations, these motional amplitudes are quite small (±12°; Nicholson et al., 1991; North & Cross, 1992) and that their effect on chemical shift tensor averaging is small (Lazo et al., 1992). Three of the structural models mentioned above are used in this study to predict chemical shifts for the polypeptide backbone nitrogen sites for comparison with the observed chemical shifts.

Until recently, it has not been possible to take full advantage of the chemical shift as an orientational constraint, because it had been suspected that crystal packing forces could distort tensor orientations in the model compounds used for determining the tensor orientations. Therefore, the tensors from single crystals of model compounds could provide only an approximate (±5–10°) orientation of the tensor with respect to the molecular frame. With the advent of relating the chemical shift tensor to a dipolar interaction that has a unique axis fixed in the molecular frame (Linder et al., 1980) it is possible to consider a determination of the chemical shift tensor orientation for specific sites in the molecule of interest. This was first achieved with ^{15}N backbone sites in gramicidin in 1989 (Teng & Cross, 1989), and other tensors have been determined since (Teng et al., 1992; Wang et al., 1992). One of the questions addressed in this study is a description of how variable the tensor element magnitudes and orientations are in a macromolecule.

Once a satisfactory description of the tensor is achieved relative to the molecular frame, significant orientational constraints can be achieved by observing the sharp line spectra of an anisotropic molecule in an oriented matrix. Here gramicidin is oriented in hydrated lipid bilayers between glass plates. When these samples are arranged so that the bilayer normal and gramicidin channel axis are parallel with respect to the magnetic field the alignment of the channels is enhanced. In fact, it has been recently shown that the mosaic spread for such preparations can be less than ±0.2° (Cross et al., 1992). As a result, the misalignment of the sample does not contribute significantly to the error in the resultant orientational constraint. Once a chemical shift frequency is achieved from the oriented preparations it can be compared with chemical shifts calculated from various model structures. In this report each of the amino acid backbone nitrogen sites has been so studied via single-site isotopically labeled gramicidin A. Preliminary results for a subset of the backbone ^{15}N sites have been previously reported (Chiu et al., 1991).

Results

The static powder pattern spectra of ^{15}N-labeled gramicidin were recorded from randomly dispersed samples of a dry mixture of specific-site ^{15}N-labeled gramicidin and dimyristoylphosphatidylcholine (DMPC). While these samples are a mixture of gramicidin and lipid, the gramicidin is probably in a nonchannel conformation (Zhang et al., 1992). The three sites studied—[^{15}N]Val_1, [^{15}N]Gly_2, and [^{15}N]Trp_{11}—have been chosen for two reasons. First, Gly_2 and Trp_{11} have chemical shifts that deviate substantially from the model-predicted chemical shifts, as described below, when assumed values of β_D and α_D are used for the predictions. Secondly, the chemical environment for Val_1 and Gly_2 is unusual. Val_1 is blocked at the amino-terminus with a formyl group that may result in a significantly different electronic environment for this site. Glycine is the only amino acid with a hydrogen for a side chain, and in the literature the orientation of this chemical shift tensor has typically been different from other amino acid residues (Munowitz et al., 1982; Harbison et al., 1984; Oas et al., 1987).

Shown in Figure 1 are the ^{15}N powder pattern spectra for these three isotopically labeled sites, and in Table 1 the magnitudes of the tensor elements (see Materials and methods and Fig. 2 for definitions) are presented for these and all of the other amide nitrogen sites in gramicidin with the sole exception of the ethanolamine site. An efficient mechanism for isotopically labeling this latter site has yet to be developed. It is immediately clear from a comparison of these tensor element magnitudes that these numbers vary substantially from site to site. The variation in $\sigma_{11} = \sigma_{bb}$ is from 22 to 40 ppm; in $\sigma_{22} = \sigma_{cc}$ from 51 to 68 ppm; and in $\sigma_{33} = \sigma_{aa}$ from 194 to 213 ppm. There is a variation of almost 20 ppm for each of these tensor elements over these 15 sites in gramicidin A. The variations among the valine sites occur in Val_1 with a σ_{33} value of 213 ppm and in Val_8 with a σ_{11} value of 28 ppm. Both of these values differ by approximately 10 from the other valine tensor element values. For leucine, σ_{11} varies from 33 to 38 ppm, σ_{22} from 61 to 68 ppm, and σ_{33} from 196 to 204. For tryptophan the variation in σ_{11} is from 35 to 37, σ_{22} from 60 to 65, and σ_{33} from 194 to 204. Consequently, even among the sites for the same amino acid, substantial variation occurs in the tensor elements. The

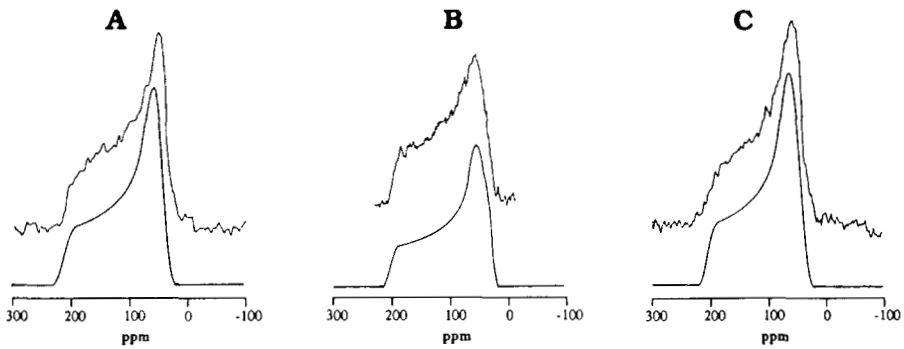

Fig. 1. ^{15}N powder pattern spectra obtained from dry powder pattern samples of gramicidin. **A:** [^{15}N]Val_1 gramicidin A. **B:** [^{15}N]Gly_2 gramicidin A. **C:** [^{15}N]Trp_{11} gramicidin A. Spectral simulations were performed as described in Teng and Cross (1989) on a Sun Sparc workstation. The numerical parameters determined from the simulation are given in Table 1.

Table 1. *Chemical shift tensor element magnitudes*[a]

Site	$\sigma_{11} = \sigma_{bb}$	$\sigma_{22} = \sigma_{cc}$	$\sigma_{33} = \sigma_{aa}$
Val_1	39	63	213
Gly_2	22	51	197
Ala_3	40	66	209
D-Leu_4	33	64	199
Ala_5	38	67	207
D-Val_6	37	62	202
Val_7	37	60	203
D-Val_8	28	55	201
Trp_9	37	65	204
D-Leu_{10}	38	68	204
Trp_{11}	36	63	194
D-Leu_{12}	38	66	196
Trp_{13}	37	60	195
D-Leu_{14}	35	61	198
Trp_{15}	35	64	196

[a] All tensor element magnitudes are reported here with an error of ±1.5 ppm. All chemical shifts are reported relative to the frequency of a saturated solution of $^{15}NH_4NO_3$.

variation appears to be greatest for the σ_{33} tensor element, the element that is in the vicinity of the N–H bond. This comparison of tensor elements can be taken a step further in comparing the tensors for identical dipeptides. There are two valyl-valine peptides; Val_6-[^{15}N]Val_7 and Val_7-[^{15}N]Val_8 gramicidin. These two tensors are very similar in σ_{33}, but they differ by 5 ppm in σ_{22} and 9 ppm in σ_{11}. There are three leucyl-tryptophan peptides: Leu_{10}-[^{15}N]Trp_{11}, Leu_{12}-[^{15}N]Trp_{13}, and Leu_{14}-[^{15}N]Trp_{15} gramicidin. These tensors are indeed very similar with the exception of a 4-ppm variation in σ_{22}. There are also three tryptophanyl-leucine peptides: Trp_9-[^{15}N]Leu_{10}, Trp_{11}-[^{15}N]Leu_{12}, and Trp_{13}-[^{15}N]Leu_{14}. Contrary to the leucyl-tryptophan results, these tensors show considerable variation in σ_{22} (7 ppm) and σ_{33} (8 ppm).

In Figure 3 the ^{13}C dipolar-coupled powder patterns of the same sites shown in Figure 1 are presented. For Val_1 gramicidin it was only possible to achieve a reasonable simulation of the spectrum by using a large value of α_D. This is the first time that a significantly nonzero value of α_D has been observed for an amide ^{15}N site. The sensitivity of the spectral simulations to α_D and β_D is shown in Figure 4. Based on these sets of spectral simulations and an error bar of ±1.5 ppm for the determination of the chemical shift discontinuities, error bars of ±5° for α_D and ±2° for β_D have been chosen. Also shown in Figure 3 are the best-fit spectral simulations for the Gly_2 and Trp_{11} sites. The polar angles obtained from these and other spectral simulations are given in Table 3 (note only the data that are underlined represent experimentally determined values; all other values represent predictions based on the experimental data of other sites). The glycine site shows a very substantial change in the value of β_D from an assumed value based on Leu_4 gramicidin of 105° to 98°. A less dramatic change in the tensor orientation for Trp_{11} is observed; from the assumed β_D value of 104° to the experimental value of 106°. Excluding those sites with unusual chemistry (Val_1 and Gly_2) all of the α_D values determined are 0°, and the β_D values are 105 ± 1°. The experimentally determined val-

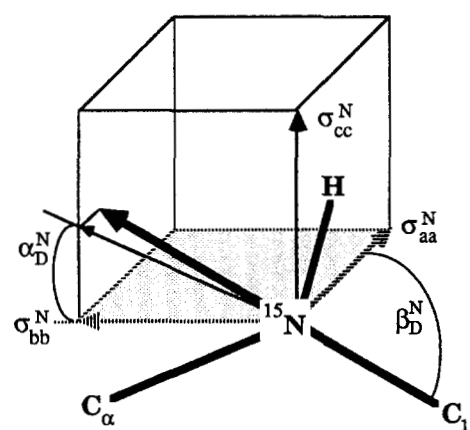

Fig. 2. ^{15}N chemical shift tensor orientation relative to the molecular frame: σ_{cc} is approximately perpendicular to the peptide plane, σ_{aa} lies in the C_1NH bond angle, and σ_{bb} completes the orthogonal coordinate system. The orientation shown is drawn to emphasize the unusually large value of α_D observed for the Val_1 site in gramicidin A.

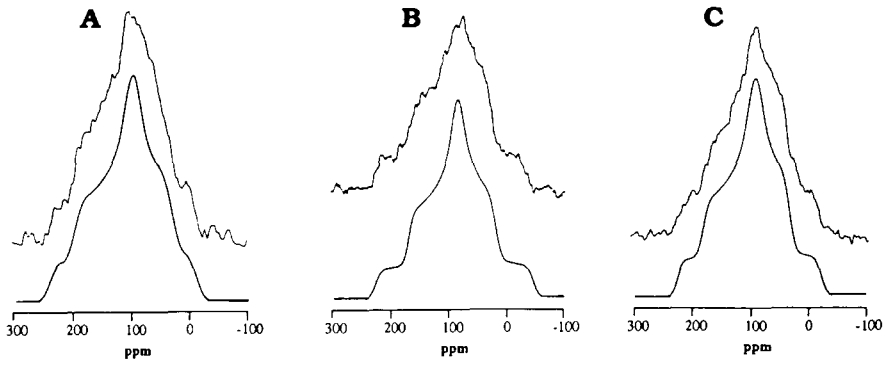

Fig. 3. ^{15}N chemical shift powder pattern spectra of ^{13}C dipolar-coupled sites in gramicidin A. **A:** [$^{13}C_1$]formyl-[^{15}N]Val$_1$ gramicidin A; α_D was determined to be 28° while $\beta_D = 106°$. **B:** [$^{13}C_1$]Val$_1$-[^{15}N]Gly$_2$ gramicidin A; α_D was determined to be 0° while $\beta_D = 98°$. **C:** [$^{13}C_1$]Leu$_{10}$-[^{15}N]Trp$_{11}$ gramicidin A; α_D was determined to be 0° while $\beta_D = 106°$.

ues therefore appear to be within the experimental error (±2° for β_D) and suggest that polar angles could be assumed for the remainder of the structure and possibly for other proteins.

Figure 5 shows the ^{15}N spectra at room temperature of uniformly oriented samples obtained with the channel

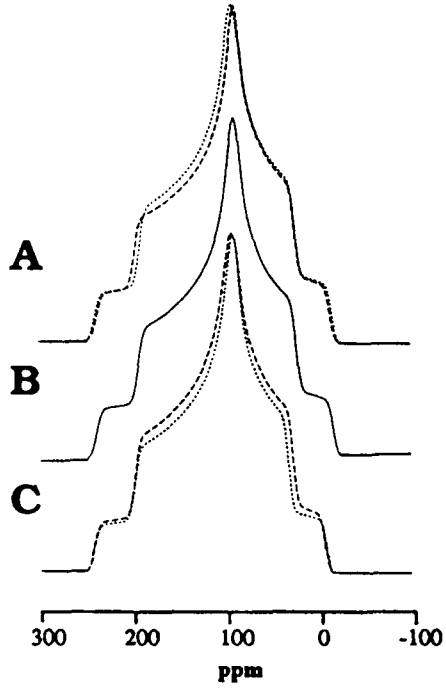

Fig. 4. Sensitivity of the powder pattern line shape to α_D and β_D is shown in a series of simulations for [$^{13}C_1$]formyl-[^{15}N]Val$_1$ gramicidin A. **A:** Dashed line is $\alpha_D = 28°$, $\beta_D = 108°$; dotted line $\alpha_D = 28°$, $\beta_D = 104°$. $\Delta\nu_{33}$, the dipolar splitting about the σ_{33} tensor element, which is dominated by β_D, affects changes by 7 ppm with this ±2° change in β_D. The frequency of the highest intensity peak changes by 4 ppm. **B:** Best fit from Figure 3A, $\alpha_D = 28°$, $\beta_D = 106°$. **C:** Dashed line is $\alpha_D = 33°$, $\beta_D = 106°$; dotted line $\alpha_D = 23°$, $\beta_D = 106°$. $\Delta\nu_{22}$, the dipolar splitting about the σ_{22} tensor element, which is dominated by α_D, affects changes by 7 ppm with this ±5° change in α_D. The frequency of the highest intensity peak changes by 2 ppm. Based on these results we claim that β_D is determined with an error of less than ±2° and α_D with an error of less than ±5°.

axis (and the lipid bilayer normal) parallel to the magnetic field. It has been shown that at this temperature global rotational motions about the bilayer normal are rapid on the NMR time scale (Smith & Cornell, 1986; Nicholson et al., 1987). Because all of these samples are single-site ^{15}N labeled, only one peak is observed, which corresponds to the amino acid residue where an isotopic label has been incorporated. The single narrow resonance for each site (widths at half height vary between 3 and 6 ppm) indicates that all of the isotopically labeled sites throughout the sample have the same orientation (i.e., conformation). This conformation has been identified as that of the channel state. Although the samples used were prepared using benzene/ethanol to cosolubilize the lipid and peptide, the observed chemical shifts in the final preparations are the same as those prepared using organic solvents, where it has been thoroughly documented through CD and ^{23}Na NMR that the gramicidin channel state is formed (Killian et al., 1988; LoGrasso et al., 1988; Moll & Cross, 1990). It can also be seen from these spectra that the observed chemical shifts show an alternating pattern with the odd sites close to σ_{33} and the even sites shifted considerably toward the isotropic value (approximately 100 ppm). This alternating pattern has been previously shown to be consistent only with a right-handed helical sense (Nicholson & Cross, 1989). Early structural models presented very regular helical conformations (Urry, 1971; Venkatachalam & Urry, 1983; Koeppe & Kimura, 1984). It is clear from a qualitative inspection of the chemical shifts that there are significant differences at the bilayer surface relative to the structure at the bilayer center. The observed chemical shifts for odd sites Val$_1$, Ala$_3$, Ala$_5$, and Trp$_9$ are 198 ppm; Val$_7$, Trp$_{11}$, Trp$_{13}$, and Trp$_{15}$ are 196, 185, 182, and 181 ppm, respectively. The even sites Leu$_4$, Val$_6$, and Val$_8$ yield chemical shifts at 145 ppm and Leu$_{10}$, Leu$_{12}$, and Leu$_{14}$ at 144, 132, and 131 ppm, respectively. These σ_{obs} values are reported in Tables 2 and 3.

From a knowledge of the chemical shift tensor magnitudes and orientations for gramicidin, it is possible to predict the chemical shifts for various models. Arseniev's structure is the only experimentally derived structure

Table 2. *Predicted chemical shifts using literature tensor orientations (underlined values represent experimentally determined values)*

Site	α_D (deg)	β_D (deg)	ν_J	ν_R	ν_A	ν_{obs}
Val_1	0	104	196	210	184	198 ± 1
Gly_2	0	105	136	132	121	113 ± 1
Ala_3	0	104	195	198	195	198 ± 1
D-Leu_4	0	105	141	154	135	145 ± 2
Ala_5	0	104	198	194	193	198 ± 1
D-Val_6	0	105	149	139	146	145 ± 2
Val_7	0	104	192	202	179	196 ± 1
D-Val_8	0	105	138	131	134	145 ± 1
Trp_9	0	104	190	189	198	198 ± 1
D-Leu_{10}	0	105	125	143	141	144 ± 1
Trp_{11}	0	104	176	179	180	185 ± 1
D-Leu_{12}	0	105	129	137	142	132 ± 2
Trp_{13}	0	104	181	188	177	182 ± 1
D-Leu_{14}	0	105	133	122	129	131 ± 1
Trp_{15}	0	104	178	176	176	181 ± 1

available; it was achieved from solution NMR of an SDS micellar-solubilized preparation of gramicidin (Arseniev et al., 1986; Lomize et al., 1992). Jakobsson's model is a mean structure produced by molecular dynamics computations beginning from the idealized Koeppe and Kimura (1984) right-handed structure (Chiu et al., 1991). The computations include water molecules, and the effect of the lipid is taken into account as artificial restraints on the polypeptide motion. Roux's model is derived from the Arseniev structure equilibrated with waters in the channel and refined by molecular dynamics. These three models all have the same folding motif—they are all single-stranded β-helical structures with a right-handed helical sense and approximately 6.3 residues per turn.

Table 2 presents both the experimental chemical shifts for all 15 amino acid residues in the gramicidin channel conformation, as well as the model-based predictions founded on the previously published tensor orientations (Teng & Cross, 1989; Teng et al., 1992). Based on the near equivalence of the experimentally determined α_D and β_D values it was initially considered a good assumption to choose an alternating pattern of β_D values throughout the peptide and a uniform value for α_D. As mentioned above the discrepancy between experimental and predicted chemical shifts for Gly_2 and Trp_{11} sites and the unique chemistry of the Val_1 and Gly_2 sites suggested these sites for tensor orientation studies. The new values of α_D and β_D result in a very significant change in the prediction of the chemical shift values for Gly_2 as shown in Figure 6 and subtle changes for the Val_1 and Trp_{11} sites. This result emphasizes both the sensitivity of these

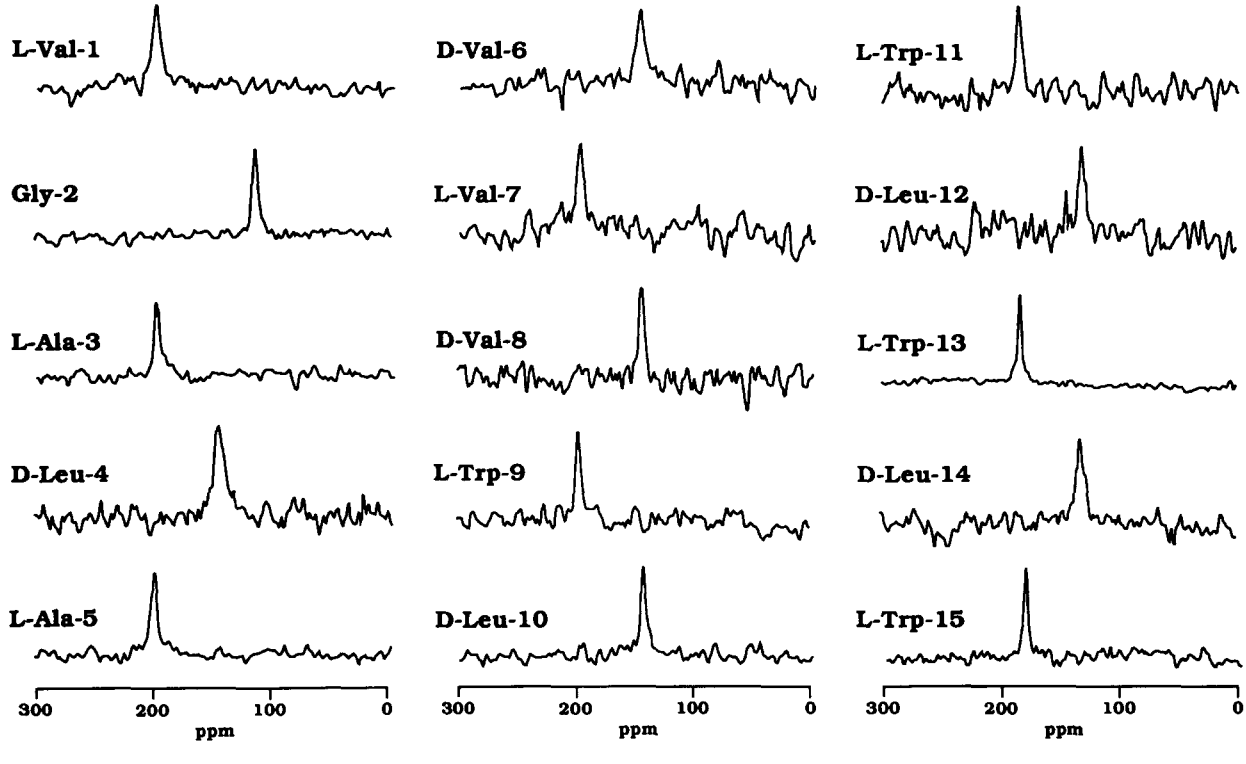

Fig. 5. ^{15}N chemical shift spectra of single-site ^{15}N-labeled gramicidin in fully hydrated lipid bilayers that have been aligned between glass plates. The samples have all been oriented so that the vector normal to the glass plate is parallel with respect to the magnetic field. Here the chemical shift is directly related to the orientation of the nitrogen site with respect to the magnetic field and hence to the channel axis.

Table 3. *Predicted chemical shifts using new tensor orientations (underlined values represent experimentally determined values)*

Site	α_D (deg)	β_D (deg)	ν_J	$\Delta\nu_J$	ν_R	$\Delta\nu_R$	ν_A	$\Delta\nu_A$	ν_{obs}
Val_1	28 ± 5	106 ± 2	199	+1	211	+13	179	−19	198 ± 1
Gly_2	0 ± 5	98 ± 2	115	+2	113	0	103	−10	113 ± 1
Ala_3	0 ± 5	104 ± 2	195	−3	198	0	195	−3	198 ± 1
D-Leu_4	0 ± 5	105 ± 2	141	−4	154	+9	135	−10	145 ± 1
Ala_5	0	104	198	0	194	−4	193	−5	198 ± 1
D-Val_6	0 ± 5	105 ± 2	149	+4	139	−6	146	+1	145 ± 1
Val_7	0	104	192	−4	202	+6	179	−17	196 ± 1
D-Val_8	0	105	138	−7	131	−14	134	−11	145 ± 1
Trp_9	0	106	193	−5	192	−6	198	0	198 ± 1
D-Leu_{10}	0	105	125	−19	143	−1	141	−3	144 ± 1
Trp_{11}	0 ± 5	106 ± 2	180	−5	182	−3	182	−3	185 ± 1
D-Leu_{12}	0	105	129	−3	137	+5	142	+10	132 ± 2
Trp_{13}	0	106	183	+1	190	+8	180	−2	182 ± 1
D-Leu_{14}	0	105	133	+2	122	−9	129	−2	131 ± 1
Trp_{15}	0	106	181	0	179	−2	178	−3	181 ± 1
		$RMSD_t$[a]		6.0		7.1		8.7	
		$RMSD_l$[a]		3.4		7.0		9.8	

[a] RMSD, root mean square deviation. $RMSD_t$ is for all sites, and $RMSD_l$ is for only those sites where the chemical shift tensor has been experimentally determined rather than assumed.

chemical shift measurements to slight changes in orientation and the nonlinear relationship between orientation and chemical shift. For Gly_2 a 21-ppm change in the predicted chemical shift results from a 7° change in orientation. Most of the observed frequencies are accurate to ±1 ppm, suggesting an orientational sensitivity of ±0.3°. Because the chemistry is unique for Val_1 and Gly_2 these tensor orientations have not been generally applied to the other sites, but the Trp_{11} orientation has been used for all of the tryptophans in the predictions presented in Table 3.

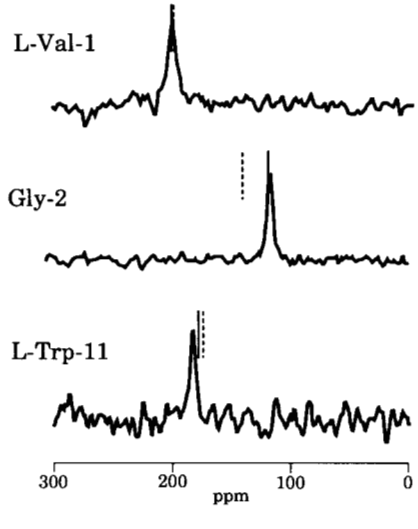

Fig. 6. Experimental ^{15}N chemical shift spectra as in Figure 5. Also shown is the chemical shift predictions from Jakobsson's model (Chiu et al., 1991) based on assumed tensor element orientations (dashed lines) and the experimentally determined orientations from Figure 3 (solid lines). For Val_1 a large change in the α_D angle from 0° to 28° results only in a modest change in frequency, 3 ppm, but a more modest change in β_D for Gly_2, 105° to 98°, results in a 21-ppm change in the predicted value. For Trp_{11} both angle (β_D = 104–106°) and frequency (4 ppm) changes are modest.

Discussion

For the first time we present here the entire set of static ^{15}N chemical shift tensor elements for a polypeptide. The tensor element magnitudes vary substantially irrespective (Fig. 7) of the surrounding covalent structure, i.e., the amino acid sequence. This suggests that if chemical shift is to be used as an orientational constraint that it will continue to be necessary to determine the magnitude of each set of tensor elements. There appears to be little hope that standard tensor elements based on amino acid residues or even dipeptide pairs could be assumed for other proteins. This finding is further supported by the crystalline tripeptide study of Hiyama et al. (1988) in which they found that the tensor element values differed dramatically for the same peptide in two different crystal forms. Consequently, the tensor elements are sensitive to noncovalent interactions including hydrogen bonding and van der Waal contacts. Furthermore, the tensors can be expected to vary with changes in the values of the backbone torsion angles. Correlations of torsional angles with isotropic chemical shifts have been developed, in particular, for ^{31}P in nucleic acids (Un & Klein, 1989), but efforts have also been made to correlate values for the ^{15}N chemical shift tensor elements with hydrogen-bond

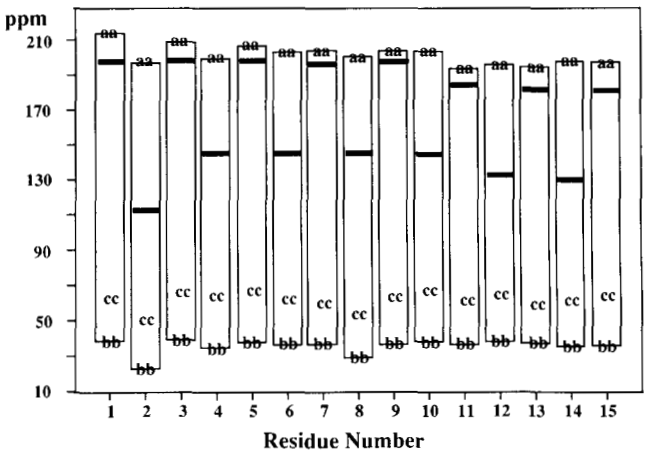

Fig. 7. ^{15}N chemical shift tensor element magnitudes as a function of residue number. Each element is identified by "aa," "bb," or "cc," and the chemical shift anisotropy is delineated by a rectangle for each site. While there is considerable variability among the tensor element magnitudes, there are few trends that are identifiable, with the exception that σ_{aa} appears to decrease from the amino-terminus toward the carboxy-terminus. The thick black line represents the observed chemical shifts in oriented preparations.

lengths (Kuroki et al., 1991). Here it is interesting to note that the value of σ_{33} is substantially different for the five residues of the carboxy-terminus relative to the other sites in the polypeptide. It is uncertain as to what is responsible for this trend toward the carboxy-terminus. If it was known that these were the tensor element magnitudes for gramicidin in the channel conformation then it might be easier to draw substantive conclusions. It is unfortunate, but the determination of the static chemical shift anisotropy is difficult to achieve for the channel state that forms once water is added to the samples, because of local and global motions (Smith & Cornell, 1986; Nicholson et al., 1989, 1991). Although the characterization is not for the conformation of interest, it is for the site of interest and consequently represents a significant advance over the use of crystalline model peptides. Further refinements of the tensors are anticipated in the future.

While it is clear that the magnitudes of the tensor elements must be determined for individual sites, it is less clear whether the tensor orientations have a significant variation from site to site with the exception of those sites where the chemistry is unique. It is however clear from model peptide studies that the β_D angle has a significant variance (Hartzell et al., 1987; Oas et al., 1987). This variability may not be observed in gramicidin where the conformation is quite regular (see Fig. 9 described below), i.e., all of the backbone torsion angles represent β-sheet-type angles.

The three structural models used in this study have been aligned (Fig. 8) by minimizing the backbone root mean square deviation (RMSD) distances. The value of this backbone RMSD (0.51 Å) was determined by calculating the RMSD between each of three pairs of model structures and then averaging the RMSD for these three pairwise measurements. The RMSD is even smaller (0.36 Å) when the Roux and Jakobsson models alone are compared. These RMSDs are such that if the models were to be considered as a set of structures from a solution NMR refinement, the structure would be considered well refined. Yet substantial variation in backbone torsion angles among the models is clearly shown in Figure 9. There are several interesting features that are apparent from this figure. First, the only experimentally derived model, the Arseniev structure, was constrained to have planar peptide linkages. Secondly, its values of phi and psi are much more variable than either the Roux or Jakobsson structure. In fact, the psi angles for the even-numbered residues (Roux: $\Delta\psi = 12°$; Jakobsson: $\Delta\psi = 7°$) and the odd-numbered residues (Roux: $\Delta\psi = 9°$; Jakobsson: $\Delta\psi = 13°$) are remarkably uniform for these two structures even though the mean values of ψ for the even sites differ significantly (Roux: $\psi_{avg} = -125°$; Jakobsson: $\psi_{avg} = -113°$). Furthermore, the phi angles for the odd sites are also remarkably uniform for these two models (Roux: $\Delta\phi = 10°$; Jakobsson: $\Delta\phi = 10°$, excluding site 15). However, the Jakobsson model shows a linear change in the phi angle of the even sites as a function of residue number, while the Roux model shows a nonlinear function for the variation in the omega angle of the even sites with residue number. In fact, this variation in the omega torsion angle suggests that the gramicidin channel has a distorted structure at approximately the location where cations are thought to bind. In other words, Roux's model may suggest that the channel has an inherent binding site for cations. The omega torsion angle is important because it causes the carboxyl oxygen to rotate in toward the channel axis, making the partial negative charge available for solvating the cations that have been largely stripped of their waters of hydration. Recently, it has been shown that the omega torsion angle can be determined from orientational constraints by solid-state NMR (Ketchem & Cross, unpubl.).

Figure 10 displays the data of Table 3 and shows that with only three exceptions the range of predicted chemical shifts from the three models spans the observed chemical shift model. This suggests that the channel conformation lies within the variation of the model structures presented here. The qualitative agreement between observed and predicted chemical shifts is remarkably good, as summarized (Table 3) by the $RMSD_t$ chemical shift for all sites and the $RMSD_l$ for only those sites where the chemical shift tensor has been experimentally determined rather than assumed. These RMSDs suggest that the Arseniev model has the poorest fit to the observed data and the Jakobsson model, the best; however, it must be kept in mind that the predicted chemical shifts do not specify a unique structural solution but only provide a high-resolution constraint. This conformational ambigu-

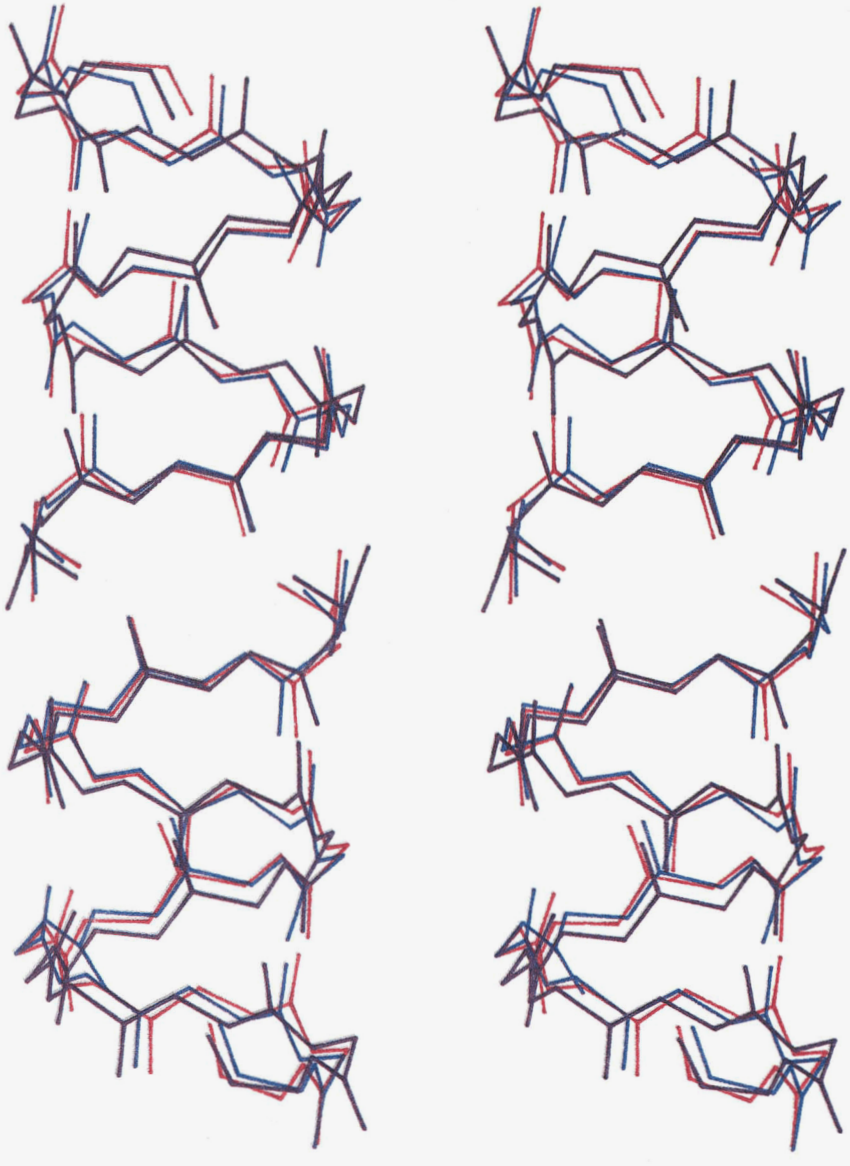

Fig. 8. Superposition of structural models for the gramicidin A cation channel. These models are color coded: blue, red, and black represent Roux, Jakobsson, and Arseniev structures, respectively. These structures, especially the Roux and Jakobsson structures are very similar as judged by the backbone RMSD for distance.

ity arises from axially asymmetric tensors where it is possible to have a considerable range of orientations result in the same chemical shift. For instance, Ala_3, Trp_{11}, and Trp_{15} have very narrow ranges for the predicted chemical shifts, and the observed chemical shift is in very close agreement. It has been previously shown that a 7° change in the orientation can result in a 21-ppm shift in frequency; therefore the narrow range of predicted chemical shifts for these three sites (approximately 3 ppm) could result in a very narrow orientational range. These sites demonstrate the danger in using chemical shift alone to "determine" structure and show that chemical shift can be both very sensitive and insensitive to orientation with respect to the magnetic field. The N-H orientation has been calculated from each model at the three sites, and the range in N-H orientations is 19, 14, and 18° for Ala_3, Trp_{11}, and Trp_{15} sites, respectively. Clearly, more data, such as the ^{15}N–^{1}H dipolar interaction (LoGrasso et al., 1989; Teng et al., 1991) are needed to absolutely define the structure.

The fact that the RMSD for those sites where the tensor orientation has been determined is smaller than the total RMSD suggests that the determination of tensor orientations is important. For Jakobsson's model the RMSD could be greatly reduced by improvement at one site, in particular, Leu_{10}. Interestingly, the other models predict the chemical shift very well for this site. Another site that stands out is Val_8, one of the sites where the predicted values do not span the observed chemical shifts. These are clearly potential sites for a tensor orientation determination.

The narrow error bar on the determination of the tensor element magnitudes, tensor orientations, and observed chemical shifts culminate in the generation of exception-

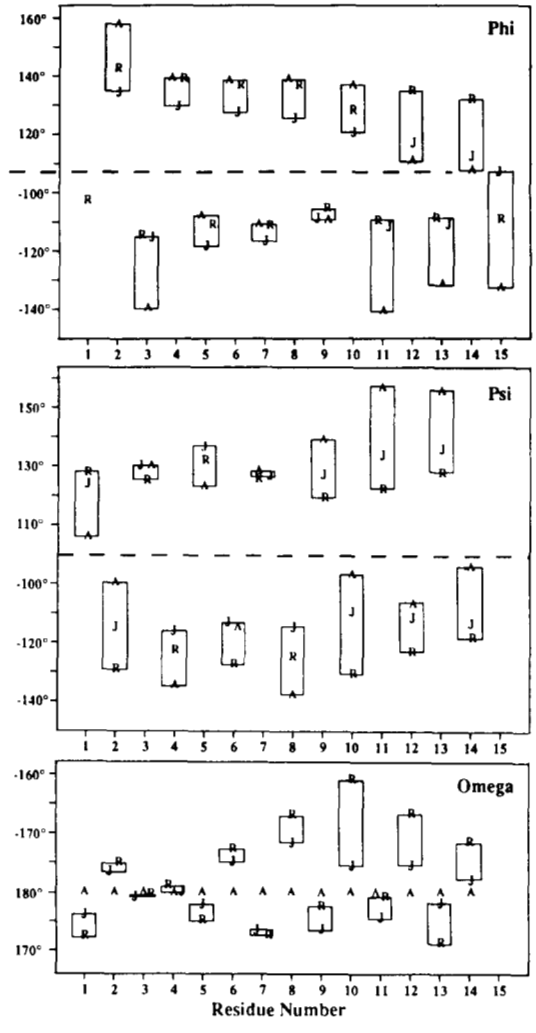

Fig. 9. Backbone torsion angles for three structural models denoted by "J" for Jakobsson, "R" for Roux, and "A" for Arseniev's model. Despite the excellent RMSD for the distances between backbone atoms there are numerous sites in the backbone where the torsion angle variability is large.

ally high-resolution structural constraints. While agreement between predicted and observed is not conclusive, it is severely constraining to the structure in an easily quantifiable way. Furthermore a disagreement between predicted and observed chemical shifts is conclusive in that the native and model structures are not the same. The importance for characterizing the tensor element magnitudes has been documented here, while variability in tensor orientation is still an open question. Qualitatively there is remarkable agreement between predicted and observed chemical shifts, but the quality of the fit is indeed less good for the Arseniev structure, suggesting that modeling the membrane environment with SDS micelles may not be beneficial. Furthermore, the best fit to the observed data comes from a computational effort starting from a uniform structural model (Jakobsson) rather than starting from the experimental data (Roux).

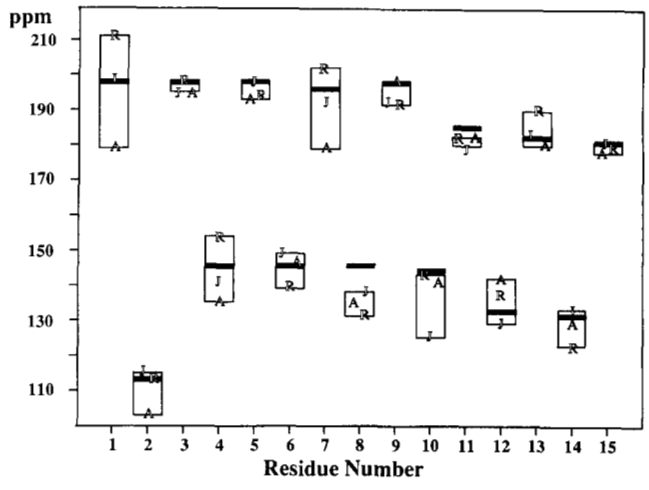

Fig. 10. Predicted (denoted by "J" for Jakobsson, "R" for Roux, and "A" for Arseniev's model) and observed (solid line) chemical shifts as a function of residue number for the ^{15}N amide backbone sites in gramicidin from oriented lipid bilayer preparations. The agreement is deceptively good – just because there is close agreement does not necessarily mean that there is a close agreement between the conformation of the model and the native channel conformation. However, significant deviations of the predicted and observed chemical shifts does mean that the model and native conformations differ. This is particularly true for those sites where we have determined the orientation of the chemical shift tensor with respect to the molecular frame (residues 1–4, plus 6 and 11).

Materials and methods

[$^{13}C_1$]formyl-[^{15}N]L-valine was synthesized by dissolving 3.5 mmol [^{15}N]L-valine (Cambridge Isotope Labs) in 3.5 mL 1 N NaOH. *p*-Nitrophenyl formate (3.5 mmol) (Muramatsu et al., 1964; synthesized in situ from *p*-nitrophenol and [^{13}C]formic acid [Cambridge Isotope Labs]) in 5 mL tetrahydrofuran was added dropwise in an ice bath while the solution was stirred. After reacting for 2 h in an ice bath the reaction was continued at room temperature for 20 h. To the solution, 30 mL of water was added, and the pH was adjusted to 4 with 1 N HCl. The solution was extracted twice with 20 mL ethyl ether, and the extracted mixture was vacuum dried. The solid was then refluxed in 30 mL of acetone, followed by centrifugation and drying of the solution to yield a white solid with a 16% yield. The solid was characterized by ^1H NMR to be [$^{13}C_1$]formyl-[^{15}N]L-valine.

Single-site and double-site isotopically labeled gramicidin A was synthesized via solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. For single-site-labeled gramicidin, 0.5 mmol of isotopically labeled Fmoc amino acid was used, followed by 1 mmol of unlabeled amino acid for recoupling. Double-site-labeled gramicidin required one mmol of each labeled Fmoc amino acid. The synthetic peptides were identified by high performance liquid chromatography (HPLC) and purified, when necessary (i.e., if purity was <98%), by

HPLC. Details on the peptide synthesis and the HPLC purification have been reported elsewhere (Fields et al., 1989).

Uniformly oriented samples were prepared by cosolubilizing 9 mg of isotopically labeled gramicidin and 26 mg of DMPC (1:8 molar ratio) in benzene/ethanol (95/5 by volume). The solution was placed in a freezer overnight. After thawing at room temperature, it was spread in equal aliquots onto 26 clean glass coverslips and dried under vacuum overnight. These glass coverslips were then stacked into a 13 × 8-mm square tube with HPLC-grade water added to achieve 45–50% by weight water. After sealing both ends, the sample was incubated at 45 °C for at least 4 days to allow the complete alignment of the gramicidin-containing bilayers. This preparation has been shown to yield the channel conformation (Moll & Cross, 1990). The degree of orientation was monitored by ^{31}P NMR. Powder pattern samples were prepared by codissolving 55 mg of specific site-labeled gramicidin and 165 mg of DMPC (1:8 molar ratio) in 8 mL of 5% ethanol in benzene. The mixture was frozen in liquid nitrogen and then immediately placed under vacuum to dry overnight.

Spectra of uniformly aligned and randomly dispersed samples were obtained on a narrow-bore IBM/Bruker WP200 NMR spectrometer modified for solids capabilities. Oriented samples were placed in a home-built static probe equipped with variable temperature control in such a way that the channel axis is parallel with the static magnetic field. Cross-polarization between the abundant ^{1}H pool and the rare ^{15}N nuclei followed by a Hahn echo was used to enhance sensitivity and to minimize probe ringing. Typical experimental conditions were: 6 μs 90° pulse length for cross polarization, 1 ms mixing time for polarization transfer, 48 μs for echo delay, 7 s for recycle delay, and a sweep width of 62.5 kHz.

The determination of the chemical shift tensor orientation for specific sites was achieved by orienting the chemical shift tensor relative to the ^{15}N–$^{13}C_1$ dipolar interaction (Oas et al., 1987; Teng & Cross 1989; Teng et al., 1992). The principal components of the chemical shift anisotropy σ_{11}, σ_{22}, and σ_{33} were obtained by simulating the experimental powder pattern spectra of single-site-labeled samples. For the purposes of comparing the chemical shift tensor elements from one site to another it is important to define the tensor elements relative to the molecular frame (σ_{aa}, σ_{bb}, and σ_{cc}; Teng et al., 1992) rather than based on their magnitudes (e.g., σ_{11}, σ_{22}, and σ_{33}). This is particularly important for the glycine site. Amino acids typically have their σ_{22} element perpendicular to the peptide plane; however, it has been shown (Oas et al., 1987) that on occasion, for glycine, the σ_{22} element can lie in the peptide plane. This is not the result of a 90° rotation of the tensor, but rather a small change in the magnitudes of the σ_{11} and σ_{22} elements. A similar problem occurs for the C_2 site in the tryptophan side chain where the asymmetry, η, is approximately equal to 1 and for different indoles σ_{zz} is forced, by definition, to switch from being upfield to being the most downfield element (Cornell et al., 1988; Separovic et al., 1991). Consequently, definitions for the tensor elements of the amide nitrogens relative to the molecular frame have been established: σ_{cc} is approximately perpendicular to the peptide plane, σ_{aa} lies in the C_1NH bond angle, and σ_{bb} completes the orthogonal coordinate system (see Fig. 2). Once determined, the magnitudes of the principal values are used as input to simulate the ^{13}C dipolar-coupled ^{15}N powder pattern spectra for obtaining α_D and β_D, the angles for rotating the dipolar interaction into the principal axis system of the chemical shift tensor. α_D is the angle formed between σ_{bb} and the projection of the N–C_1 bond onto the σ_{cc}/σ_{bb} plane. β_D is the angle between the N–C_1 bond and σ_{aa} component and it can be estimated from the observed dipolar splitting about $\sigma_{33} = \sigma_{aa}$ frequency using the equation $\Delta\nu_{33} = \nu_{\parallel}(1 - 3\cos^2\beta_D)$ (Teng & Cross, 1989).

The chemical shift in an oriented sample varies with the orientation of the tensor elements to the magnetic field ($\nu_{obs} = \sigma_{11}\cos^2\theta_{11} + \sigma_{22}\cos^2\theta_{22} + \sigma_{33}\cos^2\theta_{33}$). Calculation of the chemical shift for each site from different models is possible after the orientation of the ^{15}N chemical shift tensor relative to the molecular frame at the specific site of interest is known. The calculation is done through a series of unitary transformations from the principal axis system of the chemical shift tensor to the laboratory-fixed reference frame via the molecular symmetry axis frame (Chiu et al., 1991). The principal axis frame was first transformed to the molecular symmetry axis frame using the angles α_D and β_D. Rotation via a second set of angles brings the molecular symmetry axis frame to the laboratory frame where the Z axis parallels the static magnetic field.

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