Solid-state ^{13}C NMR spectroscopy of a ^{13}C carbonyl-labeled polypeptide

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ABSTRACT High resolution structural elucidation of macromolecular structure by solid-state nuclear magnetic resonance requires the preparation of uniformly aligned samples that are isotopically labeled. In addition, to use the chemical shift interaction as a high resolution constraint requires an in situ tensor characterization for each site of interest. For ^{13}C in the peptide backbone, this characterization is complicated by the presence of dipolar coupled ^{14}N from the peptide bond. Here the $^{13}C_1$–Gly_2 site in gramicidin A is studied both as a dry powder and in a fully hydrated lipid bilayer environment. Linewidths reported for the oriented samples are a factor of five narrower than those reported elsewhere, and previous misinterpretations of the linewidths are corrected. The observed frequency from oriented samples is shown to be consistent with the recently determined structure for this site in the gramicidin backbone. It is also shown that, whereas a dipolar coupling between ^{13}C and ^{14}N is apparent in dry preparations of the polypeptide, in a hydrated bilayer the dipolar coupling is absent, presumably due to a 'self-decoupling' mechanism.

INTRODUCTION

Before biological solid-state nuclear magnetic resonance (NMR) can achieve its promise as a significant tool for determining protein and polypeptide structure in anisotropic environments, a fundamental characterization of various nuclear spin interactions used for structural constraints must be achieved. ^{13}C spectroscopy of the polypeptide backbone of a macromolecule suffers from several complications. Because carbon is an abundant atom in proteins and because the natural abundance of ^{13}C is somewhat $>1\%$, the ^{13}C signal from a single site-labeled polypeptide (95% enriched), having a molecular mass of 2,000 D, is $<50\%$ from the labeled site. Although this is not a major problem for a sample that is magic angle spun or that is oriented, it is a major problem for powder pattern studies where resonance lineshapes overlap severely. The other critical problem with ^{13}C NMR in the polypeptide backbone is the presence of the quadrupole nucleus, ^{14}N. This spin = 1 nucleus is dipolar coupled to both the C_α and C_1 carbon sites in the backbone. Its coupling is most frequently manifested in magic angle spinning ^{13}C NMR spectra as a broadening or splitting of the spectral lines (Cross, 1981; Hexem et al., 1981). Recently, these couplings have been clearly demonstrated in powder pattern spectra where the coupling was used to determine the orientation of the electric field gradient tensor with respect to the molecular frame (Teng et al., 1992). In this paper we show that ^{13}C chemical shifts from single site-labeled gramicidin A can be used as a high resolution structural constraint for the determination of three-dimensional conformation.

Gramicidin is a 15–amino acid polypeptide that, as a dimer, forms a monovalent cation selective channel across lipid bilayers. Although no crystallographic structure of the channel has been achieved, cocrystals of lipid and polypeptide have been formed (Wallace, 1983). However, the first backbone torsion angles have recently been determined for this conformation from a variety of ^{15}N solid-state NMR experiments (Teng et al., 1991). Despite the paucity of high resolution structural data, a model for the channel conformation was first described in 1971 (Urry, 1971). This model proposed a unique folding pattern, a β-sheet structure wrapped into a helix. This was possible only because of the alternating stereochemistry of the amino acid residues, which generated a conformation having all of the sidechains directed radially to the outside leaving a 4-Å pore lined by the peptide backbone. This folding motif has recently been supported by the ^{15}N chemical shift data of the backbone obtained from oriented samples. However, the proposed left-handed helical sense (Urry et al., 1982) has now been refuted by experimental solid-state NMR data (Nicholson and Cross, 1989).

It has been, in part, the success of the ^{15}N chemical shift analyses of oriented preparations that prompted this study into the feasibility of ^{13}C carbonyl chemical shift spectroscopy. Actually, there have been a variety of studies on the carbonyl carbons of gramicidin, but they have been plagued by natural abundance ^{13}C intensity and by very broad lines (Cornell et al., 1988, 1989; Smith et al., 1989, 1990). Despite these problems, Smith et al. (1989) have attempted to rule out certain structural

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models and to qualitatively describe some of the backbone dynamics. These interpretations have been hampered by a third problem, that of a poorly characterized chemical shift tensor. Recently, a variety of carbonyl ^{13}C chemical shift tensors have been determined in dipeptides, showing that the magnitude of the tensor elements and the asymmetry of the tensor varies significantly (Hartzell et al., 1987; Oas et al., 1987). Furthermore, it has been recently demonstrated that the ^{15}N and ^{13}C chemical shift tensor element magnitudes and orientations can be determined for the site of interest in a macromolecule (Teng and Cross, 1989; Teng et al., 1992). Here, the couplings to a backbone $^{13}C_1$-labeled site in gramicidin are studied, the $^{13}C_1$ chemical shift is characterized, and the chemical shift of an oriented $^{13}C_1$-labeled gramicidin preparation is interpreted and compared to previous interpretations.

METHODS AND MATERIALS

Isotopically labeled amino acids were purchased from Cambridge Isotope Laboratories (Cambridge, MA) and incorporated into gramicidin A via solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl chemistry (Fields et al., 1988; Fields et al., 1989). Powder pattern samples were prepared directly from the solid-phase peptide synthesis product (>98% pure by analytical HPLC) without the addition of lipid. This was done to avoid compounding the natural abundance problem. Bilayer preparations for orientation were prepared with a 1:8 molar ratio of gramicidin/dimyristoylphosphatidylcholine (Sigma Chemical Co., St. Louis, MO). Oriented samples were prepared between glass plates as described previously (Moll and Cross, 1990), with some modification as suggested by Hing (1990), using a spacer to separate the glass plates.

^{13}C solid-state NMR spectroscopy was performed on a heavily modified spectrometer (model 200SY, Bruker/IBM, Bruker Instruments, Inc., Billerica, MA) with a homebuilt static probe operating at 50 MHz for ^{13}C. All spectra were performed under cross-polarization conditions with ~1.8-mT decoupling fields. A short (48 μs) Hahn echo was used where necessary to avoid probe ringing effects. ^{13}C chemical shifts are given relative to TMS and were determined from an external reference to adamantane. Powder pattern simulations of both the ^{15}N and ^{14}N dipole-coupled ^{13}C chemical shift spectra are described in detail elsewhere (Teng et al., 1992). Spectral simulations of uniformly aligned samples were generated from the same programs using a single orientation with respect to the field.

RESULTS

Fig. 1 shows the definitions of the nuclear spin interactions relative to the molecular frame. Recently (Teng et al., 1992), it was shown that for the purposes of comparing one chemically similar site to another it was inappropriate to define the tensor elements based on their frequency, even though definitions such as $|\sigma_{zz} - \sigma_{iso}| \geq |\sigma_{xx} - \sigma_{iso}| \geq |\sigma_{yy} - \sigma_{iso}|$ (Haeberlen, 1976) and $\sigma_{33} \geq \sigma_{22} \geq \sigma_{11}$ (Mehring, 1983) have been used for a long

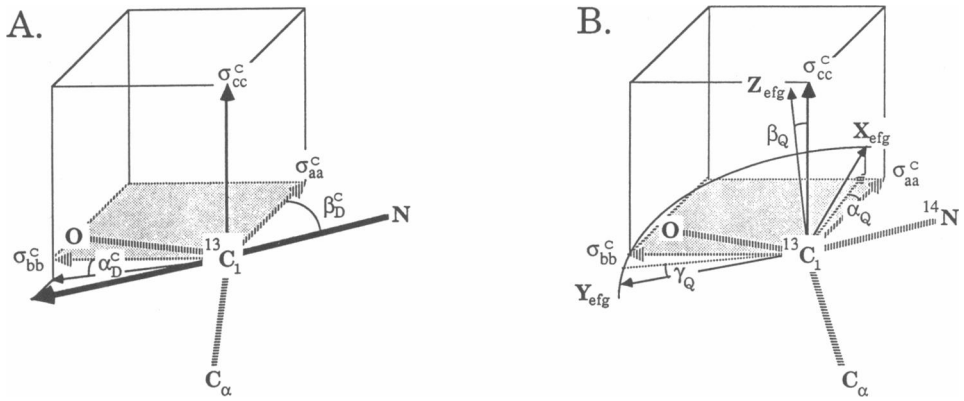

FIGURE 1 Orientation of nuclear spin interactions. (*A*) σ^c_{cc} is defined as the element approximately perpendicular to the peptide plane, shown here as the base of the cube. σ^c_{bb} is the element in the vicinity of the carbonyl bond, and σ^c_{aa} completes the orthogonal frame of reference. β^c_D is the angle between the C_1—N bond and the σ^c_{aa} element, and α^c_D is the angle between σ^c_{bb} and the projection of the C_1—N vector onto the σ^c_{bb}–σ^c_{cc} plane. (*B*) The ^{14}N electric field gradient tensor sensed at the $^{13}C_1$ site is described by the elements Z_{efg}, Y_{efg}, and X_{efg}; and they are oriented approximately parallel to σ^c_{cc}, σ^c_{bb}, and σ^c_{aa}, respectively. β_Q is the angle between σ^c_{cc} and Z_{efg}. α_Q is the angle between the σ^c_{aa} element and the projection of X_{efg} onto the σ^c_{aa}–σ^c_{bb} plane. γ_Q is the angle between Y_{efg} and the intersection of the X_{efg}–Y_{efg} and σ^c_{aa}–σ^c_{bb} planes.

time. Based on previous studies of the peptide carbonyl carbon chemical shift tensor (Stark et al., 1983; Hartzell et al., 1987, Oas et al., 1987), it is known that one of the tensor elements is approximately perpendicular to the peptide plane. Here we define this element based on its orientation to the molecular frame as σ^c_{cc} (Fig. 1 *A*). These previous studies have also shown that one of the elements, here defined as σ^c_{bb}, is in the vicinity of the carbonyl bond. σ^c_{aa} completes the orthogonal frame of reference. For the ^{14}N electric field gradient tensor sensed at the $^{13}C_1$ site, Z_{efg}, Y_{efg}, and X_{efg} are approximately parallel to σ^c_{cc}, σ^c_{bb}, and σ^c_{aa}, respectively (Fig. 1 *B*).

The dipolar interaction between two spin = ½ nuclei, ^{13}C and ^{15}N, is clearly seen in Fig. 2 *A* from a powder pattern sample of $^{13}C_1$–Gly_2 ^{15}N-Ala_3 gramicidin A. The magnitude of this dipolar interaction, $\nu_\parallel^{15N}$, can be calculated from the bond length (1.34 Å) as 1,260 Hz and has been confirmed by analysis of the ^{15}N spectra of this same sample (Teng and Cross, 1989). In ^{15}N powder pattern spectra, it is possible to estimate the value of β^N_D from an observation of the dipolar splitting about σ^N_{aa}, defined as $\Delta\nu^N_a$. This observation was independent of a quantitative determination of σ^N_{aa}, because the dipolar shoulders were symmetric about the chemical shift tensor element. For the ^{13}C studies, the dipolar splitting is not symmetric about this tensor element; however, an estimate for the value of β^c_D is possible by observing the outer $\Delta\nu^c_a/2$ between the outer dipolar shoulder at 257 ppm in Fig. 2 *A* and the σ^c_{aa} tensor element at 243 ppm. From

$$\Delta\nu^c_a = \nu_\parallel^{15N}[3\cos^2\beta^c_D - 1],$$

where $\Delta^c_a/2$ is observed to be 14 ppm (700 Hz), an estimate for β^c_D of ±33° is obtained. This estimate has been refined with the spectral simulation shown in Fig 2 *B* using $\beta^c_D = 34°$ and $\alpha_D = 0°$. Also σ^c_{11} is evaluated in this simulation with $\sigma^c_{aa} = 243$, $\sigma^c_{bb} = 173$, and $\sigma^c_{cc} = 91$ ppm.

Similarly, Fig. 2 *C* shows the ^{13}C NMR powder pattern spectrum of $^{13}C_1$–Gly_2 gramicidin A and very clear evidence for a dipolar interaction, this time between the spin = 1 quadrupole nucleus, ^{14}N, and ^{13}C. Such powder patterns are dependent on as many as 10 variables: the magnitude and asymmetry of the chemical shift and electric field gradient tensor elements; the magnitude, $\nu_\parallel^{14N}$ and orientation, α^c_D, and β^c_D of the dipolar interaction with respect to the chemical shift tensor; and the orientation of the electric field gradient tensor with respect to the chemical shift tensor, given by α_Q, β_Q, and γ_Q. However, five of these variables have been determined from the ^{15}N and ^{13}C spectra of $^{13}C_1$–Gly_2 ^{15}N-Ala_3 gramicidin A. Because the gyromagnetic ratios for ^{14}N and ^{15}N differ, here $\nu_\parallel^{14N}$ is 898 Hz. Reasonable initial estimates of the magnitude ($C_Q = -3.2$ MHz) and asymmetry ($\eta = 0.31$) of the electric field gradient

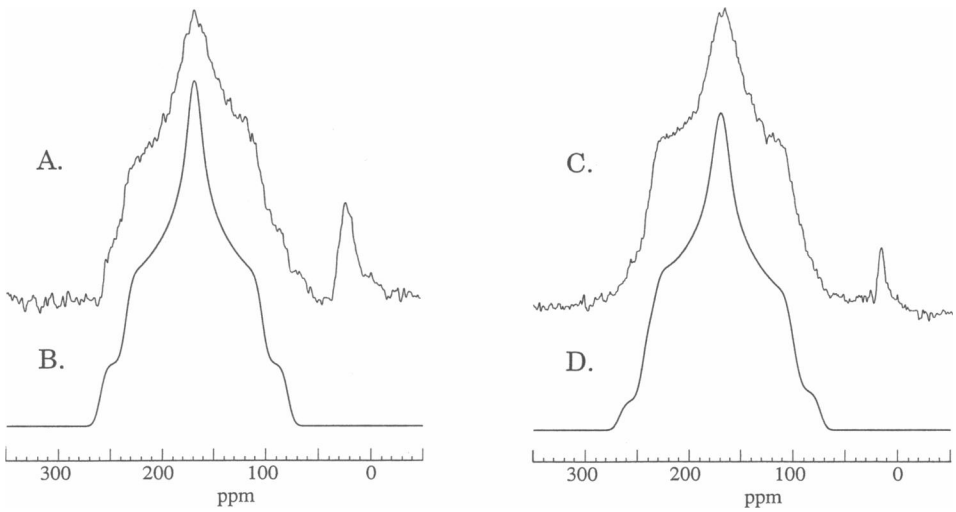

FIGURE 2 Powder pattern spectra of $^{15}N/^{13}C_1$ doubly labeled gramicidin A(*A* and *B*) and $^{13}C_1$ single site-labeled gramicidin (*C* and *D*). Natural abundance ^{13}C signals have been subtracted from both of the experimental spectra (*A* and *C*). Residual natural abundance intensity is seen near 20 ppm relative to TMS. (*A*) Spectrum of $^{13}C_1$–$Gly_2$$^{15}N$–$Ala_3$ gramicidin A obtained with 9,984 signal acquisitions and a 5-s recycle delay. (*B*) Best-fit simulation using a predetermined value for $\nu_\parallel^{15N} = 1.26$ kHz and optimized values of $\beta^c_D = 34°$, $\alpha^c_D = 0°$, $\sigma^c_{aa} = 243$, $\sigma^c_{bb} = 173$, and $\sigma^c_{cc} = 91$ ppm. (*C*) Spectrum of $^{13}C_1$–Gly_2 gramicidin A obtained with 9,872 signal acquisitions and a 3-s recycle delay. (*D*) Best-fit simulation was determined with values of the variables determined for the double label and a quadrupole coupling constant magnitude of $C_Q = -3.2$ MHz and asymmetry of $\eta = 0.31$. The orientational angles were determined to be $\alpha_Q = 0°$; $\beta_Q = 0°$, and $\gamma_Q = 0°$.

tensor can be made from previous studies, and these values were successfully maintained for the best fit solutions. The spectral fit, which is primarily determined by the magnitude and asymmetry of the dipolar coupling at each chemical shift tensor element, was very sensitive to the magnitude and sign of the quadrupole coupling constant but not to the asymmetry parameter. With the number of unknowns reduced to just a few, the spectrum can be simulated (Fig. 2 D) and a solution set achieved with a high degree of confidence for $\alpha_Q = 0°$, $\beta_Q = 0°$, and $\gamma_Q = 0°$. A quantitative estimate of errors for each angle will be determined in future studies. A qualitative analysis of errors is presented elsewhere (Teng et al., 1992).

Fig. 3 *B* shows a ^{13}C spectrum of $^{13}C_1$–Gly_2 gramicidin A in a fully hydrated lipid bilayer environment oriented such that the bilayer normal is parallel with the magnetic field direction. The natural abundance spectrum of gramicidin in lipid bilayers at the same molar ratio (1:8) is shown in Fig. 3 *C*. In this latter spectrum, signals from two carbonyl sites in the lipid are clearly seen and can be subtracted from the spectrum of the isotopically labeled sample, yielding the spectrum in Fig. 3 *A*. The resulting spectrum shows a single sharp resonance for the Gly_2 carbonyl site in the polypeptide backbone, indicating a uniformly oriented sample in which all of the ^{13}C sites throughout the sample have the same orientation with respect to the magnetic field. Furthermore, this resonance represents $\sigma_{\parallel}$, and therefore the chemical shift tensor has been dramatically averaged by the global motion of the channel about the bilayer normal (Fields et al., 1988; Smith and Cornell, 1986).

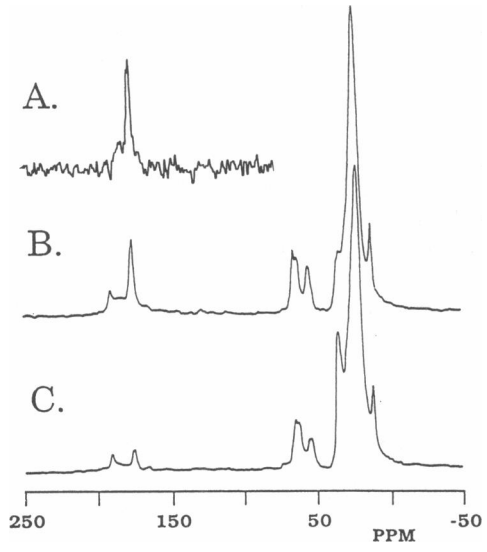

FIGURE 3 ^{13}C spectra of $^{13}C_1$–Gly_2 gramicidin A oriented in hydrated lipid bilayers with the bilayer normal and the channel axis parallel with respect to the magnetic field. (*A*) *B* minus *C*. (*B*) Spectrum of $^{13}C_1$–Gly_2 gramicidin A obtained with 7,248 acquisitions and a 7-s recycle delay. (*C*) Spectrum of natural abundance gramicidin A obtained with 8,000 acquisitions and a 7-s recycle delay.

Fig. 4 shows predicted spectra for the labeled carbonyl site of the oriented sample chacterized in Fig. 3. Using the chemical shift tensor element magnitudes and orientation from the powder pattern analysis in Fig. 2 and the experimentally derived conformation of gramicidin at the Gly_2 site (Teng et al., 1991), the chemical shift of the labeled site can be predicted. Here the predictions are based on the known static values of the chemical shift tensor; however, the local motional amplitude is known to be very small (10° rms; Nicholson et al., 1991). This predicted frequency of 173.5 ppm (Fig. 4 *A*) is very close to the observed value of 175 ppm. However, this prediction does not take into account the ^{14}N–^{13}C dipolar interaction. From the known bond length and the known orientation of this site with respect to the magnetic field, the triplet splitting due to this dipolar interaction can be calculated and is shown in Fig. 4 *B*. Even these latter spectra do not take into account the effect of the quadrupole interaction on the dipolar splitting. From the determined orientation of the electric field gradient tensor, it is possible to calculate this effect and the spectra are shown in Fig. 4 *C*. It is clear that the $^{13}C_1$–Gly_2 site in hydrated lipids is not dipolar coupled to ^{14}N, whereas the same site in a polycrystalline preparation (Fig. 2) is dipolar coupled to this quadrupole nucleus.

DISCUSSION

As has been shown via a variety of ^{15}N studies, the preparation used here has a single dominant conformation giving rise to a sharp motionally averaged resonance from oriented samples. The problem of natural abundance is not very severe for these preparations; however, it does add significant error to the powder pattern analyses, especially for the interpretation of the upfield splittings, which are in the vicinity of the aliphatic intensity. However, with the tensor orientation definitions used here, β_D^c is the angle between N—C_1 bond and the downfield tensor element, not the upfield tensor element as in previous definitions. Consequently, reasonable estimates of β_D^c are observable directly from the spectra.

The data in Fig. 3 and calculations in Fig. 4 clearly show the phenomena of self-decoupling in which the ^{14}N T_1 relaxation time is presumably rapid enough so that the ^{13}C spin states sense a time-averaged ^{14}N spin state. This phenomenon was first described by Spiess et al. (1977) when they described self-decoupling in single

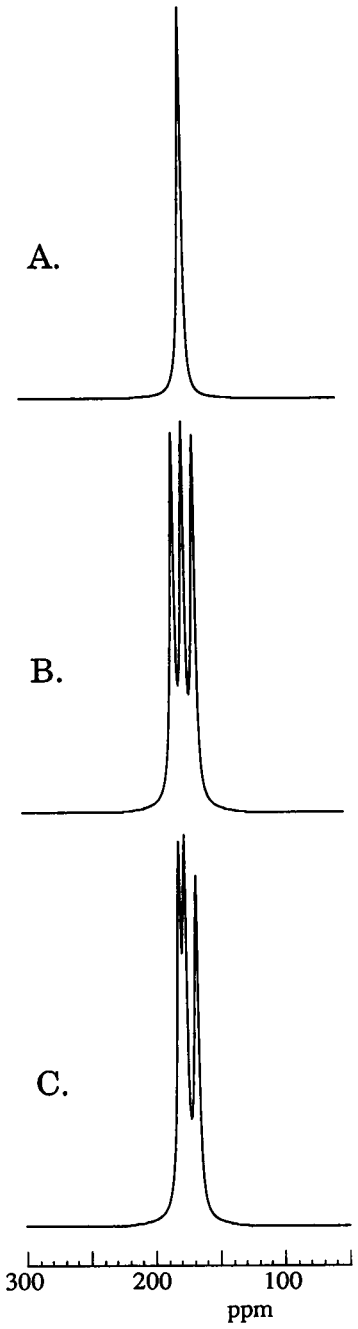

FIGURE 4 Spectral simulations of the labeled site in oriented $^{13}C_1$–Gly_2 gramicidin A broadened to a width at half-height of 3.6 ppm. (*A*) Chemical shift calculated from the recently determined conformation of this site (Teng et al., 1991). (*B*) Chemical shift spectrum dipolar coupled to a spin = 1 nucleus using the experimentally determined dipolar parameters, but arbitrarily setting $C_Q = 0$. (*C*) Spectrum as in *B* but using the experimentally determined quadrupole coupling parameters.

crystal spheres of *trans*-diiodoethylene. In this molecular system, the ^{127}I nuclei were self-decoupled from the protons. For the special case of magic angle sample spinning, Jonsen (1988) has described in a theoretical study the effects of self-decoupling on the residual dipolar interaction between ^{14}N and ^{13}C. The dramatic reduction in the dipolar coupling observed in the spectra here is the first definitive observation of self-decoupling in an ^{14}N–^{13}C spin system. Cornell et al., (1988) had suggested that self-decoupling might be occurring in hydrated bilayers of gramicidin, but the resolution in their experiments prevented them from making a definitive conclusion.

The $^{13}C_1$–Gly_2 site has been previously observed both as a dry powder and as a fully hydrated bilayer preparation oriented between glass plates (Cornell et al., 1988; Smith et al., 1989). The discontinuities in the powder pattern spectrum showed evidence of dipolar coupling, but these effects were ignored, as they have been throughout the literature of peptide carbonyl patterns. Consequently, the magnitude and asymmetry of the chemical shift tensor are significantly in error, and this coupling can be used to refine the orientation of the electric field gradient tensor with respect to the ^{13}C chemical shift tensor. Instead of the chemical shift interaction magnitude being 82 ppm, $\delta = 78$ ppm; and instead of the interaction asymmetry being 0.82, $\eta = 0.90$. These errors in determination result from an effective broadening of the discontinuities and from the asymmetry in the dipolar triplet resulting from the quadrupolar interaction. The characterization of the electric field gradient tensor is consistent with previous determinations. Stark et al. (1978), in their single crystal study of *N*-acetyl valine, showed that Y_{efg} was parallel with the N—H bond, a result that is identical within a couple of degrees to that reported here. Furthermore, studies of the residual dipolar interaction in magic angle spinning spectra (Hexem et al., 1982) of the ^{13}C carbonyl site of the same molecule confirm the single crystal study as well as placing the Z_{efg} component perpendicular to the peptide plane, as is confirmed in this study. However, these NMR results are inconsistent with the NQR studies of di- and triglycine (Rabbani et al., 1987). Whereas these latter studies show that Z_{efg} is perpendicular to the peptide plane, they conclude that Y_{efg} is parallel to the C—N bond and consequently $\sim 30°$ from the orientation determined here.

Previous spectra of oriented $^{13}C_1$-labeled gramicidin have shown very broad linewidths, especially for sites near the NH_2 terminus. Data on the Ala_3 site were first simulated as a 25-ppm (1,900 Hz) linewidth (Cornell et al., 1988); later these data were reinterpreted as a 13-ppm (975 Hz) linewidth (Smith et al., 1989). The Gly_2

site was also analyzed in this later study as having an 11-ppm (825 Hz) resonance linewidth. In the present work the observed linewidth (Fig. 3 *A*) is 3.6 ppm (183 Hz). Smith et al. (1989) have postulated that the variation in linewidths that they observe between NH_2- and COOH-terminal sites is correlated with molecular motions. Near the channel mouth the linewidths appeared to be sharper, implying greater mobility near the bilayer surface. The observation of a 3.6-ppm linewidth for a site near the NH_2 terminus suggests that the much greater linewidths observed by Smith et al. were not dominated by a relaxation phenomena as they proposed. Recent conclusions from ^2H NMR studies of the channel (Prosser et al., 1991) suggest that there is no substantial variation in the amplitude of molecular motions along the channel axis. Our reevaluation of the ^{13}C linewidths eliminates the discrepancy between the conclusions in these recent studies.

The observation of a narrow resonance from oriented samples results in a very significant structural constraint for the observed site; however, it does not define a unique conformation. The experimental error associated with this constraint is largely dictated by how well defined the tensor element magnitudes are and how well the orientation is defined with respect to the molecular frame. Typically, tensor characterizations have been achieved from single crystal studies for model compounds such as glycylglycine for which $\alpha_D^c = 0$ and $\beta_D^c = 48°$ (Stark et al., 1983). Here the tensor has been characterized for the site of interest, and the β_D^c angle differs by 14° from that observed for the model compound. This range of β_D^c has been observed in a set of dipeptides studied by Oas et al. (1987) showing the importance for a determination in situ, such as that described here. Previously the $^{13}C_1$ chemical shift frequency for the glycine site was interpreted in light of the single crystal study described above with $\beta_D^c = 48°$ (Cornell et al., 1988). This 14° error dramatically reduces the quality of the structural constraint and severely compromises efforts to distinguish between any but the most widely divergent of the structural models. With the tensor characterization presented here and the recently published structure for this site in the gramicidin backbone (Teng et al., 1991), an excellent agreement is achieved between the predicted chemical shift for this structure and the observed chemical shift. The predicted chemical shift would be in error by > 13 ppm if the single crystal characterization of Stark et al. (1983) was used. Furthermore, even if the tensor characterization for acetylglycylalanine (Oas et al., 1987) was chosen to model the Gly_2Ala_3 peptide in gramicidin, the predicted chemical shift would be in error by 10 ppm. By using the in situ characterization, the predicted and observed frequencies differ by <2 PPM; in other words, the predicted frequency is within the experimental half-width at half-height (1.8 ppm). For ^{15}N NMR we have previously (Chiu et al., 1991) shown the importance for determining the tensor orientation for each site of interest. Here that importance is extended to ^{13}C NMR; and it is becoming apparent that tensor element magnitudes and orientations are modulated not only by the covalent environment of the site but by other interactions, such as crystal packing forces, solvation effects, etc. Although this has been suspected for many years (Cross and Opella, 1985), the argument lacked the detailed evidence that this paper presents.

The observed chemical shifts from uniformly aligned samples have been shown in this study to be very sensitive to the tensor element characterization. On the other hand, the narrow linewidths of the observed data and the resultant enhancement in sensitivity combine to constitute an extraordinarily high resolution approach to conformational analysis, provided that the tensor element characterization can be performed for the site of interest. Here we have demonstrated this capability with a specific $^{13}C_1$-labeled site in the gramicidin A channel.

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