Molecular Sequence Effect on the ^{13}C Carbonyl Chemical Shift Shielding Tensor

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Abstract: The ^{13}C chemical shift tensor of the carboxyl carbon has been determined from the ^{13}C dipole-coupled chemical shift powder patterns for oxalic acid dihydrate and glycine and for the carbonyl carbon of glycine in the dipeptide glycylalanine and the tripeptide valylglycylalanine. The most shielded component, σ_{33}, is found to be perpendicular to the CCO plane for all four compounds, whereas σ_{22} shows variation in magnitude and orientation.

Introduction

The ^{13}C chemical shift anisotropy of peptide carbonyls can be used to provide information on the orientation and conformation of peptide planes in polypeptides and proteins.[1,2] To obtain information about molecular structure and dynamics from the chemical shift interaction, both the magnitude and the orientation of the chemical shift tensor must be known for the site of interest. This is usually determined from single-crystal studies,[3] and the peptide carbonyl ^{13}C shift tensor of glycylglycine·HCl·H_2O, the simplest dipeptide,[4] has served as the model system for larger molecules.

The single-crystal method requires a large crystal of known crystal structure and orientation. The powder pattern obtained from a polycrystalline sample can give the magnitudes of the principal values of the chemical shift tensor, but the orientation of the tensor cannot be determined from the powder pattern with use of the chemical shift interaction alone. However, when the nucleus is dipolar coupled to a bonded atom, then it is possible to determine the relative orientation of the chemical shift and dipolar tensors from the dipole-coupled chemical shift powder pattern. Since the dipolar tensor is to a good approximation axially symmetric with a unique axis collinear with the inter-nuclear vector,[5] the orientation of the chemical shielding tensor can be directly related to the bond axis from the perturbation of the chemical shift powder pattern by the dipolar coupling.[6,7] The approximation of axial symmetry for the ^{13}C–^{13}C dipolar tensor is well-justified due to the low magnetic moment of ^{13}C and the structural isolation of the interacting carbon pairs.

Drobny and co-workers have determined the principal values and molecular orientations of the glycine carbonyl ^{13}C chemical shift tensors from ^{15}N dipole-coupled ^{13}C powder spectra, as well as that for the amide ^{15}N chemical shift tensors from ^{13}C dipole-coupled powder patterns.[8] Both the ^{15}N and ^{13}C chemical shift tensors from the dipole-coupled powder patterns of L-[^{13}C]-alanyl-L-[^{15}N]-alanine were determined.[9] Teng and Cross[10] have used the approach of Drobny and co-workers to determine the ^{15}N chemical shift tensor orientation in the polypeptide gramicidin A using a peptide bond labeled with ^{13}C and ^{15}N. The tensor orientation obtained was very similar to the result of Hartzell et al.[9] for the model compound alanylalanine with some variations in the magnitudes.

Previous studies of carbonyl ^{13}C chemical shift tensors have indicated that σ_{33} lies approximately perpendicular to the peptide plane[3] with σ_{22} within 13° of the C=O bond.[4] Oas et al.[6] have shown a 12° range in the orientation of the chemical shift tensor in the peptide plane and the only significant variation in the principal values being changes in σ_{22}. Determination of the orientation of the carbonyl ^{13}C chemical shift tensor in the peptide coordinate system then becomes simplified to determining the angle between σ_{11} and the internuclear vector between the two dipole-coupled nuclei. A determination of the dipolar coupling constant can be made on the basis of known bond lengths.

We have obtained ^{13}C powder spectra of doubly ^{13}C-labeled compounds in order to determine the magnitude and orientation of the carboxyl or carbonyl ^{13}C shielding tensor in a series of compounds: oxalic acid dihydrate, the amino acid glycine, and the dipeptide glycylalanine (Gly-Ala) and the tripeptide valylglycylalanine (Val-Gly-Ala) enriched in ^{13}C at the α and carbonyl carbons of glycine. The tripeptide Val-Gly-Ala serves as a model for an analogue of gramicidin A, doubly ^{13}C labeled at glycine. The first three amino acids of gramicidin A are the same as that of the tripeptide.[11] We chose oxalic acid and glycine for this investigation because the shielding tensor magnitude and orientation had been determined previously from single-crystal studies. The results for oxalic acid dihydrate differ from the results of Griffin et al.,[12] who came to the conclusion that σ_{33} is tilted by $\sim$20° off the perpendicular to the COO plane. For glycine, the carboxyl ^{13}C chemical shift tensor results are in agreement with those of Haberkorn et al.[13] The dipeptide and tripeptide incorporating doubly ^{13}C-labeled glycine show small differences, in agreement with the observations of Oas et al.[6] and Ando et al.[14]

Experimental Section

Oxalic *1,2*-$^{13}C_2$ acid dihydrate (99%; ICN Biomedicals Inc., Cambridge, MA) and glycine-*1,2*-$^{13}C_2$ (92%; MSD Isotopes, Montreal, Canada) powders were used. Oxalic acid was checked for purity by solution-state NMR and by mass spectroscopy. Unlabeled oxalic acid (BDH Chemicals) and singly labeled glycine-*1*-^{13}C (MSD Isotopes) were also used.

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Peptide Synthesis. Di- and tripeptides containing ^{13}C-labeled amino acids were synthesized manually on PAM resin, using *t*-BOC protected amino acids, following standard methods.[2] The tripeptides were cleaved from the resin with use of hydrogen fluoride with anisole as the scavenger. The dipeptides were cleaved by exposure to trifluoromethanesulfonic acid containing thioanisole and ethanedithiol.[15] Following cleavage the dipeptides were separated from the acid and scavengers by adsorption on a column of cation-exchange resin (AG 50W-X2, Bio-Rad, Richmond, CA). Methanolic ammonia (1600 mL of methanol + 320 mL of concentrated ammonium hydroxide) was used to elute the peptides.

Further purification was effected by reverse-phase high-performance liquid chromatography on a 0.46 cm × 10 cm, C-18 μBondapak Radial Pak column (Waters Associates, Waltham, MA). The tripeptides were eluted from this column in a linear gradient of 0–10% acetonitrile in 0.1% trifluoroacetic acid (TFA). The dipeptides were eluted isocratically with 0.1% TFA in water. The TFA was removed by repeated lyophilization of aqueous solutions of the peptides after addition of HCl to lower the pH to 1.5–2. Removal of TFA was monitored by ^{19}F NMR.

TLC, HPLC, and ^{1}H high-resolution NMR were used to confirm the structures of the peptides. The tripeptides were also subjected to amino acid analysis.

NMR Experiments. The ^{13}C powder spectra were obtained at 75.46 MHz on a Bruker CXP300 spectrometer by cross polarization (C-P). For oxalic acid a contact time of 2 ms, a recycle time of 200 s, a 63 kHz sweepwidth, and a 80 kHz ^{1}H decoupling field were used at a temperature of 276 K. Approximately 300 scans were accumulated into 1024 data points. Glycine was run with a contact time of 1 ms, a recycle delay of 30 s, and a 50 kHz decoupling field at 272 K, with 2100 scans. Similar conditions were used for unlabeled oxalic acid dihydrate and glycine. For the singly and doubly ^{13}C-labeled dipeptide and tripeptide a recycle delay of 2 s was used otherwise the conditions were similar. Sample sizes of 5–12 mg were used and 5000–42000 scans were acquired.

Nutation Spectroscopy. A modified TT-14 pulse spectrometer operating at 15 MHz for ^{13}C and extended with a 60-MHz channel for C-P and decoupling was used.[16] Spectra were run at liquid nitrogen (77 K) or ambient temperatures. A carrier nutation frequency of 12 or 19 kHz set at 122–178 ppm for different experiments was used.

Solution Spectra. High-resolution ^{13}C and ^{1}H NMR spectra were collected on a Jeol FX90Q. ^{19}F spectra were obtained on a Varian XC 400.

Data Analysis. Calculation of powder spectra was carried out on a HP 1000F with Fortran 77. A powder spectrum is computed by simulating the superposition of the spectra from a large number of randomly oriented single crystals over all directions of the magnetic field, specified by the polar angle θ and the azimuthal angle ϕ. The polar angle θ is randomly stepped from 0 to $\pi/2$ and the azimuthal angle ϕ from 0 to 2π with a sin θ weighting. The powder pattern contains contributions from both the ^{13}C chemical shift anisotropy as well as the ^{13}C–^{13}C dipolar coupling and is therefore the sum of frequency components from two sources, the chemical shift tensor and the dipolar coupling tensor:

$$\omega_{cs}(\theta,\phi) = \sigma_{11}\cos^2\phi\sin^2\theta + \sigma_{22}\sin^2\phi\sin^2\theta + \sigma_{33}\cos^2\theta$$

where θ and ϕ are the polar angles which relate the principal axis system of the chemical-shielding tensor to the laboratory or magnet frame[3] and σ_{11}, σ_{22}, and σ_{33} are the magnitudes of the shielding tensor axes.

The dipolar coupling tensor was simulated as the sum of two axially symmetric tensors, equal in magnitude but opposite in direction $\pm D$, $\pm D, \mp 2D$, where D is $^1/_4$ of the maximal dipolar splitting, equal to $\gamma_c^2 h/r^3$ or $^3/_2\gamma_c^2 h/r^3$. The orientation of the dipolar coupling tensor to the chemical shift tensor is specified by β and α, the polar and azimuthal angles, relative to the ^{13}C–^{13}C internuclear vector (r) in the principal axis system. The contributions from the positive and negative dipole terms and chemical shift are summed to produce the frequency domain powder spectrum. The spectral simulations were performed employing 64 000 different θ,ϕ pairs. A Lorentzian line-broadening function was convoluted with the calculated frequency distribution with use of a fast Fourier transformation. Line widths corresponding to T_2s of order 2–10 ms were used.

Results

Oxalic Acid Dihydrate. The ^{13}C NMR powder spectra and best-fit simulation of doubly labeled ^{13}C oxalic acid dihydrate are shown in Figure 1. The ^{13}C shift tensor components from Griffin et al.[12] were found to give a good fit to the powder pattern of unlabeled oxalic acid dihydrate and were used to fit the powder spectra of the doubly labeled material. The dipolar coupling constant $\gamma_2 h/r^3$ was estimated from the carbon–carbon bond length 0.153 nm[17] and independently measured with use of nutation spectroscopy.[16,18] The Fourier transform of a transient nutation gives a measure of the homonuclear dipolar splitting by removing chemical shift effects, provided the rf field strength is greater than the chemical shift dispersion. A value of 5960 Hz for the maximal dipolar coupling (which is twice the nutation splitting[16,18]), corresponding to a C–C bond length of 0.156 nm, was obtained, in agreement with the X-ray result of Cox et al.[19] Figure 2 shows the nutation spectrum for doubly labeled oxalic acid dihydrate, 10 mg in 90 mg unlabeled material measured at 77 K. The same result was obtained at ambient temperatures. The C-P powder spectra were run at 177 and 277 K, giving the same powder pattern at both temperatures.

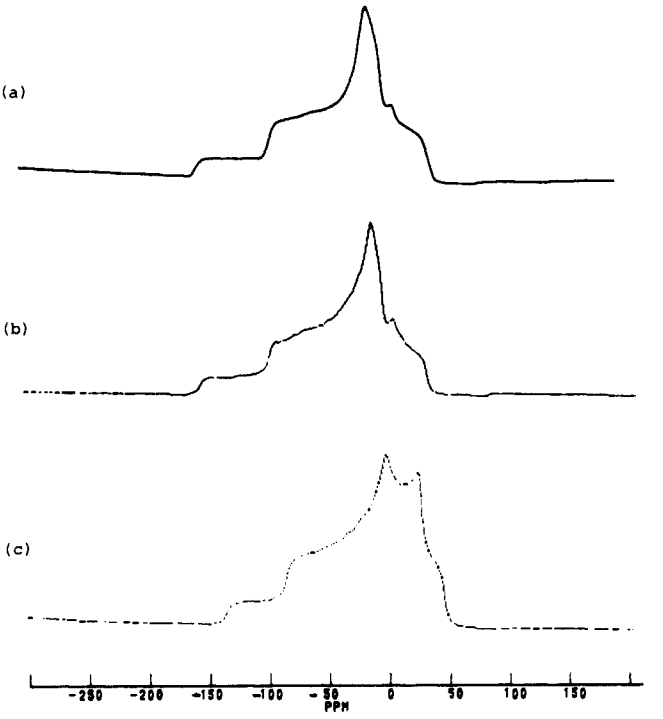

Figure 1. (a) The ^{13}C dipole-coupled powder spectrum of the ^{13}C-labeled carboxyl carbons of oxalic acid dihydrate. (b) The simulated powder spectrum obtained by using chemical shift tensor components of −85.5, 31.1, and 54.4 ppm, a dipolar coupling constant of 5980 Hz, α = 20°, and β = 90°. (c) Powder spectrum simulated with the same shielding tensor components and dipolar coupling as in spectrum b but α = 20° and β = 70°. The plot width is 510 ppm.

The simulated spectrum was calculated with use of the aestheticized (p 15,ref 3) or traceless chemical shielding tensor values of (−85.5, 31.1, 54.4) ppm, where upfield is taken as negative with use of the convention of Oas et al.[6] and a ^{13}C–^{13}C dipolar coupling constant of 5980 Hz. The bottom spectrum in Figure 1 shows the spectrum calculated by using the angles from Griffin et al.[12] (see review by Veeman[20]) with σ_{11} 20° from an axis parallel to the C–C bond and σ_{33} 20° from the perpendicular to the COO plane. A better fit to the experimental data is seen in the central spectrum of Figure 1, which differs in the orientation of σ_{33} to the perpendicular to the molecular plane. We find that σ_{33} is 90° to the COO plane, so that α = 20° and β = 90° within ±2°. This result is consistent with most reported studies of the tensor orientation for carboxyl groups, for which σ_{33} is invariably close to the perpendicular to the COO plane (see references in Veeman[20]).

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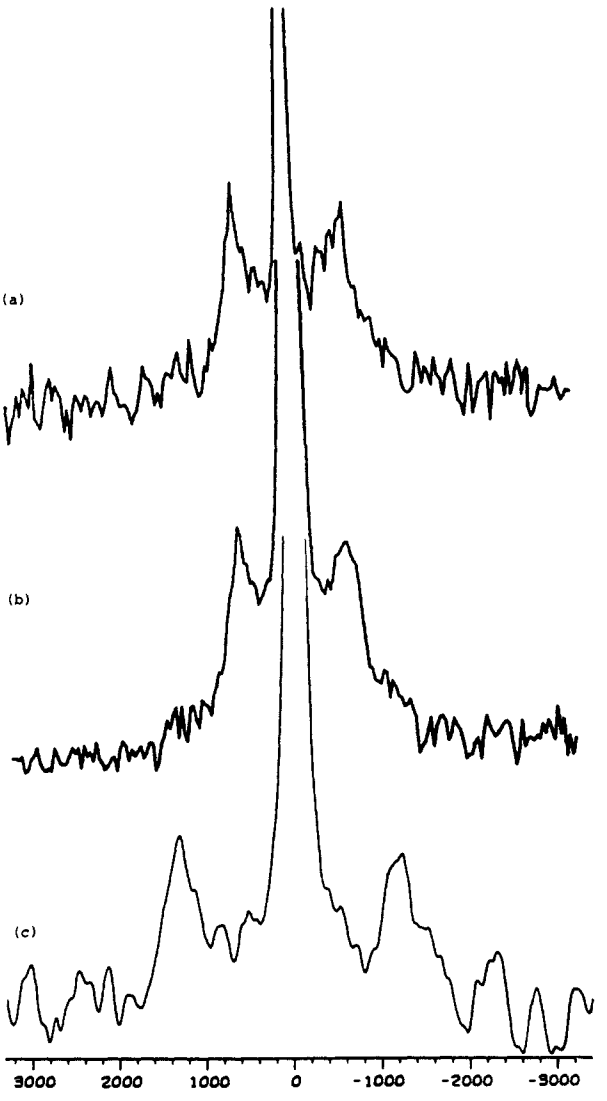

Figure 2. (a) ^{13}C nutation spectrum of doubly ^{13}C-labeled oxalic acid dihydrate at 77 K with a 12 kHz nutation frequency. The spectrum was centered at zero frequency for comparison with part c. The central line is due to isolated ^{13}C carbons. (b) The ^{13}C nutation spectrum of doubly ^{13}C-labeled glycine powder at 77 K with a 12-kHz nutation frequency. The central line due to isolated ^{13}C carbons has been centered at zero frequency for comparison with part c. (c) The ^{13}C Carr Purcell dipolar spectrum of gramicidin A powder incorporating doubly ^{13}C-labeled glycine at ambient temperature. The central line at zero frequency is due to isolated carbons and spin-locking artifacts. The plot width is 6.5 kHz.

Glycine. A similar procedure was followed for ^{13}C–^{13}C glycine. First, the magnitude of the ^{13}C carboxyl shielding tensor components was estimated by simulating the carboxyl powder pattern using ^{13}C in natural abundance. The aestheticized or traceless shielding tensor values of Haberkorn et al.,[13] (−71, −2, 73) ppm, were found to be appropriate to calculate the carboxyl powder pattern. The experimental ^{13}C powder spectrum is presented in Figure 3a together with the simulated spectrum (Figure 3b) calculated by using shielding tensor components of (−71, −2, 73) ppm and a dipolar coupling constant of 3990 Hz, a factor of two-thirds of the dipole coupling measured by nutation spectroscopy (5980 Hz) in Figure 2. The ^{13}C chemical shift difference of 131 ppm between the α carbon and the carboxyl corresponds to 2 kHz at the lower field at which the nutation experiment was carried out and 10 kHz at the higher field at which the powder pattern was acquired. The nutation experiment removes the chemical shift difference between the inequivalent carbon nuclei, resulting in the dipolar coupling constant becoming that for like spins $^3/_2\gamma^2h/r^3$. At high field the chemical shift difference reduces the ^{13}C dipolar coupling constant to γ^2h/r^3 as for unlike spins,[5] the chemical shift difference then being larger than the dipolar coupling. A similar value of 4.1 kHz was obtained by Haberkorn et al.[13] for the ^{13}C–^{13}C dipolar coupling constant for a single crystal of glycine at 74 MHz for ^{13}C. Figure 3c shows a spectral simulation with 6 kHz as the dipolar coupling constant, a value $^3/_2$ times too large. The ^{13}C shielding tensor orientation for the carboxyl carbon was determined by Haberkorn et al.,[12] with σ_{33} 3° from the perpendicular to the COO plane and σ_{11} 3° from the bisector of the OCO angle. The X-ray structure of glycine by Marsh[21] was used. Assuming the dipolar coupling tensor to be axially symmetric and oriented with the major axis parallel to the C–C bond axis, our results are in agreement with those of Haberkorn et al.[13]

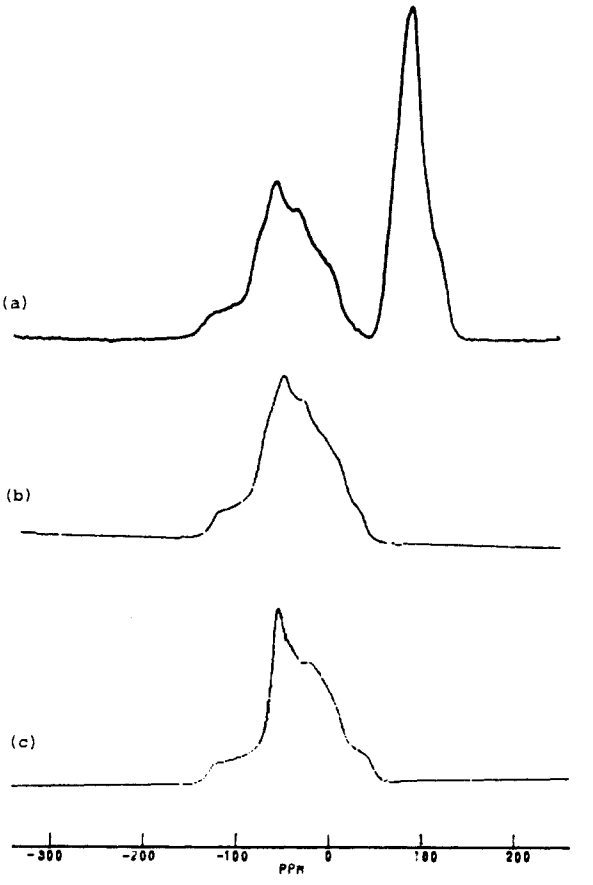

Figure 3. (a) The ^{13}C dipole-coupled powder spectrum of the ^{13}C-labeled carboxyl and α carbon of glycine. (b) The simulated powder spectrum of the glycine carboxyl obtained with use of chemical shift tensor components of −71, −2, 73 ppm, a dipolar coupling constant of 3980 Hz, α = 0°, and β = 90°. (c) The simulated carboxyl powder spectra obtained with use of the same values but with a dipolar coupling constant of 6000 Hz. The plot width is 600 ppm.

Glycine Peptides. Figure 4 illustrates the spectral differences between the dipeptide, Gly-Ala, and the tripeptide, Val-Gly-Ala, ^{13}C labeled at the carbonyl carbon. The powder pattern of the glycine carbonyl of Gly-Ala was fitted with the principal shielding tensor values of (−71, −13, 84) ±2 ppm [or (−115, −57, 40) ppm with reference to benzene], in agreement with the values of Oas et al.[6] For the carbonyl of the glycine in Val-Gly-Ala the best fit to the powder pattern spectrum was obtained with (−75, −5, 80) ± 2 ppm [or (−119, −49, 36) ppm with reference to benzene] as the magnitude of the shielding tensor axes. This represents an 8 ± 4 ppm change in the magnitude of σ_{22} and falls within the changes seen by Oas et al.[6] in glycine ^{13}C carbonyl shielding tensors in a range of dipeptides. Similarly, Ando et al.[14] obtained (−72, −6, 78) ppm for $(Ala\text{-}Gly)_n$ and (−73, −2, 76) ppm for $(Val\text{-}Gly)_n$. From single-crystal studies of [1-^{13}C]-glycyl-[^{15}N]-glycine·HCl·H_2O, Stark et al.[4] obtained values for the ^{13}C chemical shift tensor components of (−74.4, −7.4, 81.8) ppm. The

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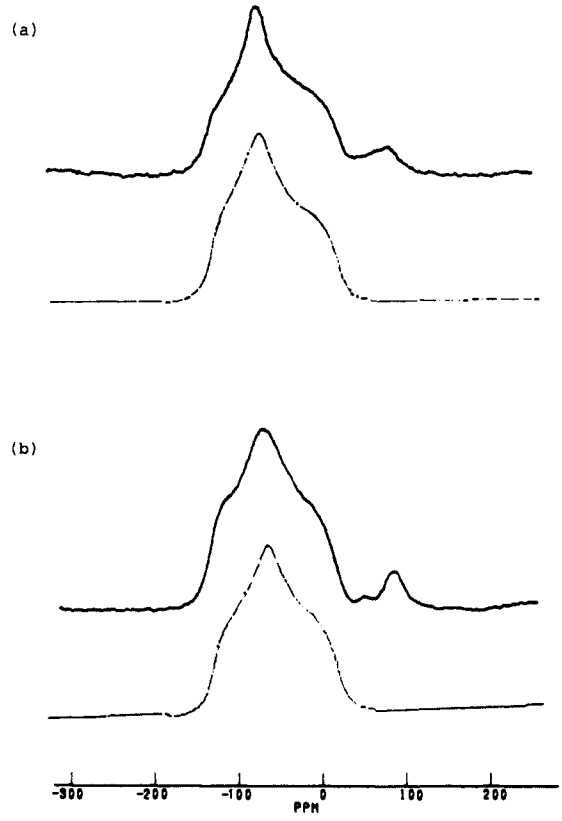

Figure 4. (a) The experimental and simulated ^{13}C powder spectrum of the dipeptide Gly-Ala ^{13}C labeled at the glycine carbonyl. (b) The experimental and simulated ^{13}C powder spectrum of the tripeptide Val-Gly-Ala ^{13}C labeled at the carbonyl of glycine. The plot width is 600 ppm. Only the carbonyl ^{13}C spectrum has been simulated.

tripeptide result is close to these values.

For determination of the orientation of the shielding tensor relative to the C–C bond, the ^{13}C carbonyl powder pattern spectrum was calculated for the doubly ^{13}C-labeled glycine incorporated in the Gly-Ala dipeptide and Val-Gly-Ala tripeptide (Figure 5). A dipolar coupling constant of 4 kHz for ^{13}C carbons with different chemical shifts and the shielding tensor values calculated for the singly labeled peptides above was used. For the dipeptide, the best fit was achieved for $\alpha = 18°$ and $\beta = 90°$, and for the tripeptide $\alpha = 20°$ and $\beta = 90°$ ($\pm 5°$), relative to the dipolar coupling tensor. Taking the OCN bond angle to be 125° from Schulz and Schirmer[22] places $\sigma_{33} \sim 10°$ off the C=O bond for the dipeptide and $\sim 8°$ for the tripeptide, similar to the dipeptide results of Oas et al. (Figure 6). There appears to be little change in the chemical shift tensor orientation between the dipeptide and tripeptide. The major change appears to be in the magnitude of σ_{22} mentioned above.

The dipeptide result was checked by using magic angle spinning techniques.[23] By fitting simultaneously the side band intensities from three different spinning rates (1.8, 2.5, 3 kHz), the following results were obtained: chemical shift anisotropy −94 ppm, asymmetry 0.66 (10% error), dipolar coupling constant 6.1 (±2) kHz, and $\alpha = 15°$ and $\beta = 90°$ (10% error).

The results for singly ^{13}C-labeled and doubly ^{13}C-labeled glycine incorporated into gramicidin A[2] are presented in Figure 7. The spectra show significant line broadening, and both spectra were able to be fitted with the same chemical shift tensor parameters. Carr Purcell dipolar spectroscopy[24] of the doubly ^{13}C-labeled gramicidin A enabled an estimate of the ^{13}C dipolar coupling. The large central peak from the single ^{13}C's in the polypeptide overlapped the 90° edges of the dipolar powder spectrum (Figure 2). However, the 0° dipolar splitting was determined to be 5.9 kHz. Due to the large natural abundance ^{13}C carbonyls in the polypeptide combined with the increase in intrinsic line broadening, no significant changes in chemical shift tensor values were able to be determined between the tripeptide and the polypeptide.

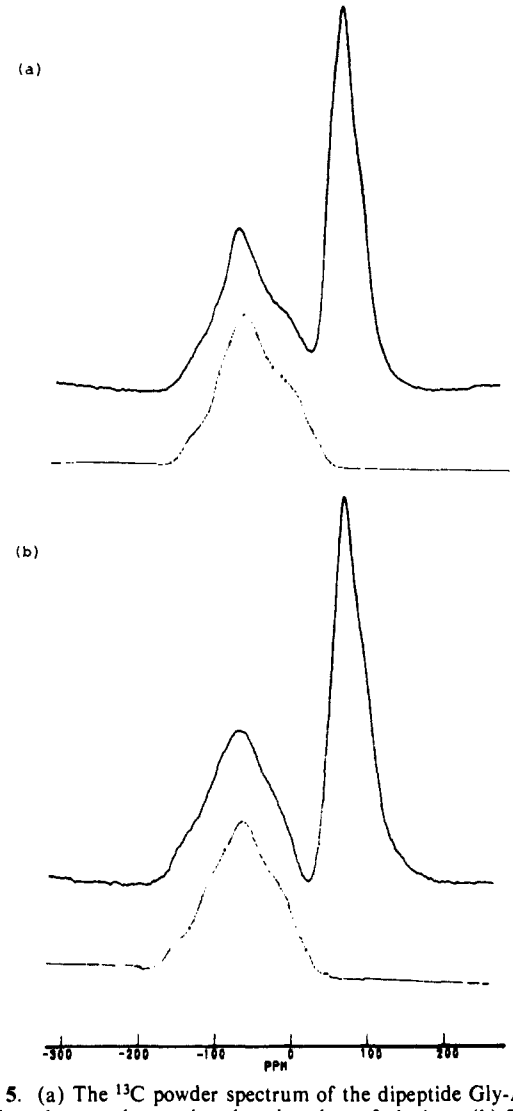

Figure 5. (a) The ^{13}C powder spectrum of the dipeptide Gly-Ala ^{13}C labeled at the α carbon and carbonyl carbon of glycine. (b) The ^{13}C powder spectrum of the tripeptide Val-Gly-Ala ^{13}C labeled at the α carbon and carbonyl carbon of glycine. Only the carbonyl ^{13}C spectrum was calculated. A plot width of 600 ppm is shown.

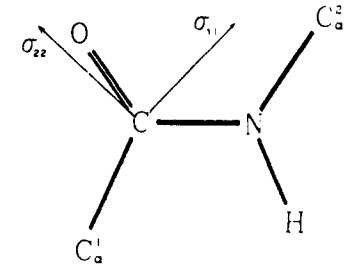

Figure 6. The orientation of the ^{13}C peptide carbonyl shielding tensor in the peptide plane. σ_{22} is approximately 10° off the C=O bond and σ_{33} is perpendicular to the plane.

Discussion

The reliability of the powder pattern fitting method was checked by comparison with the single-crystal results obtained for oxalic acid dihydrate, glycine, and glycylglycine·HCl-H_2O. The results for glycine and glycylglycine were in excellent agreement with the results of Haberkorn et al.[13] and Stark et al.[4] However, the

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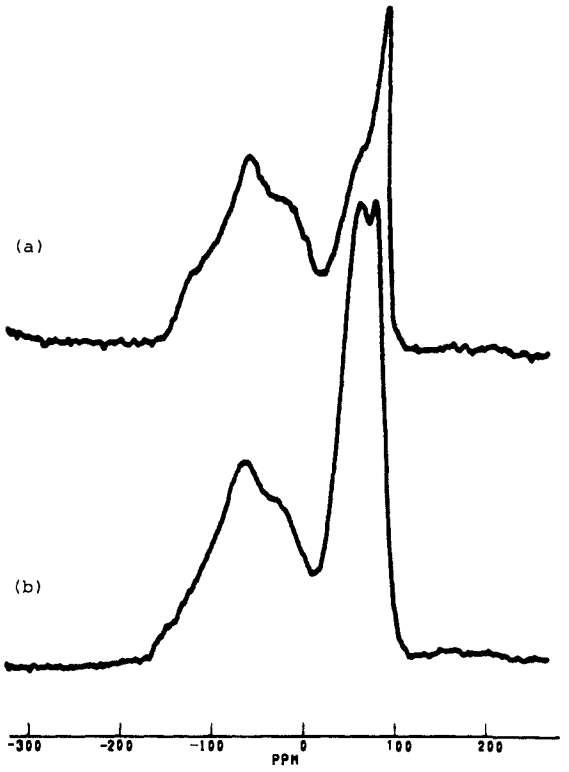

Figure 7. The ^{13}C powder spectrum of gramicidin A powder incorporating ^{13}C-labeled glycine (Gly_2 GA): (a) ^{13}C labeled at glycine carbonyl; and (b) ^{13}C labeled at both the α and carbonyl carbon of glycine. The plot width is 600 ppm.

Table I. ^{13}C Carboxyl and Amide Carbonyl Shielding Tensors[a]

	σ_{11}	σ_{22}	σ_{33}	angle σ_{22} to C=O bond
oxalic acid dihydrate	−80	31	54 (±1 ppm)	−21° (±2°) (toward N)
glycine	−71	−2	73 (±1 ppm)	−23° (±2°) (toward N)
Gly-Ala	−71	−13	84 (±2 ppm)	10° (±5°) (away from N)
Val-Gly-Ala	−75	−5	80 (±2 ppm)	8° (±5°) (away from N)

[a] Aestheticized or traceless form.

result for oxalic acid dihydrate was found to differ from that of Griffin et al.[12] in that the present work found σ_{33} to be perpendicular to the COO plane, as is the case for most carboxyl groups.[20] Oxalic acid dihydrate has been reported to exist in two crystalline forms,[23] which may have led to the difference in results. The powder pattern fitting method has the advantage over single-crystal methods and chemical-shift-dipolar magic angle spinning techniques[26-29] of instrumental simplicity and is independent of the crystal form. The use of polycrystalline material to determine the shielding tensor orientation does not depend on a knowledge of the crystal structure as does a determination from a single-crystal study. Subtle differences in the intrinsic orientation of the shielding tensor arising from different crystalline forms is the subject of further studies.

As shown in the summary of the results in Table I, no significant difference was found between the Gly-Ala dipeptide ^{13}C carbonyl chemical shift tensor and those of other dipeptides.[4,6] The major change between the dipeptide and tripeptide was in the magnitude of σ_{22}, a result consistent with the observation of other workers.[30-33] Differences in crystal packing and degrees of hydrogen bonding to carbonyl oxygen may cause variations in σ_{22}. The tripeptide ^{13}C carbonyl chemical shift tensor results were used for the interpretation of chemical shift anistropy data from ^{13}C carbonyl labels in the polypeptide gramicidin A.[2,34,35]

Conclusions

The NMR studies presented here demonstrate that σ_{22} is the most variable of the principal values of ^{13}C carbonyl chemical shift tensors in agreement with previous work and that, to within a few degrees, σ_{33} is perpendicular to the peptide plane. The σ_{22} element can be reliably oriented for peptide carbonyls, between 0° and 12° off the parallel to the C=O bond. This information is of use in analyzing ^{13}C solid-state NMR studies of larger polypeptides and proteins.

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