Determination of the ^{13}C Chemical Shift and ^{14}N Electric Field Gradient Tensor Orientations with Respect to the Molecular Frame in a Polypeptide

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Abstract: With the determination of the nuclear spin interaction tensor orientation for the site of interest in a macromolecule it is now possible to achieve very high resolution structural and dynamic information from uniformly aligned samples of a macromolecule. Here the orientation of both the carbonyl ^{13}C chemical shift and ^{14}N electric field gradient tensors are determined with respect to the molecular frame for backbone sites in polypeptides from solid state NMR spectroscopy. ^{15}N and ^{14}N dipolar coupled ^{13}C spectra were analyzed. In both cases at a 50 MHz ^{13}C frequency the presence of the dipolar coupling was plainly seen in powder pattern spectra, and for ^{14}N coupled spectra the effect of the quadrupole interaction was pronounced. Such in situ tensor characterizations are shown to substantially enhance the interpretation of structurally derived data. To make meaningful comparisons among the previously reported tensor orientations it was necessary to define the tensor elements based on their general orientation with respect to the molecular frame rather than the typical frequency based assignments. This is necessitated by the variation in tensor element magnitudes among a set of closely related compounds that makes the frequency based assignments meaningless.

Introduction

To achieve quantitative structural and dynamic data on molecules by solid state NMR it is fundamentally important to characterize nuclear spin tensor magnitudes and orientations for sites in the molecule of interest rather than in model compounds. A wide range of possible nuclear spin interactions are available for polypeptide backbone studies; some of these interactions are more readily studied than others, depending on the availability and expense of incorporating single and multiple site isotopic labels. Since solid state NMR methods are primarily limited to rare nuclei (i.e., those that do not have a significant dipolar interaction with each other), the only NMR active nucleus in the polypeptide backbone that naturally occurs at high abundance and has a nonzero nuclear spin quantum number is the ^{14}N site. This paper describes an approach for obtaining details of the ^{14}N quadrupole interaction so that high resolution structural information can be gleaned from these sites. As shown in a number of reports; dipolar interaction tensors are axially symmetric and have a unique tensor element that parallels the internuclear vector, hence this known tensor orientation with respect to the molecular frame can be used to orient chemical shift tensors.[1-3] Furthermore, Hiyami et al.[4] and recently Separovic et al.[5] have shown that the dipolar interaction between a spin = $^1/_2$ and a spin = 1 nucleus can be used similarly to orient chemical shift tensors with respect to the molecular frame. Because the data obtained for the ^{14}N sites in this report are observed via a dipolar coupling to an adjacent nucleus and because there are too many variables to achieve a unique solution directly, it has been necessary to obtain the orientation of the ^{13}C chemical shift tensor relative to the molecular frame first. Here the determination of a carbonyl ^{13}C chemical shift tensor in the polypeptide backbone of gramicidin A is achieved prior to describing information on the orientation and magnitude of the ^{14}N electric field gradient tensor.

In the backbone of protein molecules, the sites of prime structural interest are the amide amine and carbonyl groups, because of their potential for forming hydrogen bonds. Several efforts have now been made to determine the orientation of the ^{15}N chemical shift tensors from two-dimensional magic angle spinning studies,[6] single-crystal model compound studies,[7] and from powder pattern studies.[1,3,4,8,9] A more limited effort has been made to orient the carbonyl ^{13}C chemical shift tensor by single-crystal studies[10] and dipolar coupled powder pattern analysis.[2,11] A variety of two-dimensional magic angle spinning and powder pattern techniques have been developed to correlate chemical shift and dipolar tensors.[12-15] Due to a range of difficulties these approaches typically yield a low resolution determination of the relative tensor orientations. However, efforts in this area of development continue to show considerable promise.[16-18]

The first powder pattern analysis of a spin = $^1/_2$ nucleus coupled to a quadrupole nucleus was performed by VanderHart, Gutowsky, and Farrar in 1967.[19] The magnitude and orientation of the ^{14}N quadrupole coupling tensor has more recently been studied by ^{14}N NMR of single crystals. For *N*-acetylvaline Stark et al.[20] de-

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termined the magnitude of the interaction to be 3.21 MHz with an asymmetry parameter, η^q of 0.32. Furthermore it was shown that the Y component was approximately along the N–H bond. The single crystal approach for determining the orientation of the interaction tensor is based on knowing the orientation of the molecule with respect to the laboratory frame via a known crystallographic frame and then rotating the molecule so as to map out the frequencies of the nuclear spin interaction as a function of the molecular orientation with respect to the magnetic field. In simulating these plots a unique orientation of the interaction tensor can be determined with respect to the molecular frame. The application of such model compound tensor orientations for macromolecular systems is of limited value. As shown by Hiyami et al.[4] the crystal form of a molecule can dramatically affect the magnitude and potentially the orientation of the chemical shift tensor elements with respect to the molecular frame. Consequently data from single crystal studies are affected by crystal packing forces and should only be used to qualitatively orient the tensor elements for any molecular system other than the molecule in the crystal.

As noted earlier, it is also possible to determine the interaction tensor orientation without the use of single crystals. For instance, the chemical shift resonance of a spin will be split by the nuclear dipolar interactions from covalently bound nuclei with spin greater than zero. Since both the chemical shift and the dipolar interaction are dependent on their orientations with respect to the field, the splitting of the chemical shift by the dipolar interaction is also dependent on the relative orientation of these two spin interactions and, therefore, holds the necessary information to specify the relative orientation of the chemical shift tensor to the internuclear axis. Even for such a simple system involving the dipolar interaction between two spin $^1/_2$ nuclei where the dipolar interaction is axially symmetric and the chemical shift tensor is asymmetric, there are six variables that need to be solved for. This formidable number of variables can be reduced to a reasonable subset by two approaches. First, spectra of the chemical shift tensor alone can be used to provide the magnitude of each tensor element independently; secondly, powder pattern spectra represent a wealth of information that in favorable circumstances can be analyzed as a set of simultaneous equations for generating a unique solution for the remaining variables. In this way the quality of the final result is considerably enhanced. While this approach works very well for ^{15}N amide sites in a polypeptide backbone,[3] it is more difficult to implement for the amide carbonyl ^{13}C sites where it would require a triple resonance spectrometer to obtain the chemical shift powder pattern without interference from a dipolar interaction. Despite this complication, one of the primary guidelines for the studies reported here is to minimize the number of variables for which a solution is required from a single spectrum.

Another approach has been used for constraining the electric field gradient (EFG) tensor within the molecular frame of a peptide plane.[21] In magic angle spinning spectra of ^{13}C sites covalently linked to ^{14}N the dipolar coupling between these two nuclei is not completely suppressed by spinning, and the residual coupling can provide constraints for the magnitude, sign, asymmetry, and orientation of the EFG tensor. Factors such as the internuclear distance and molecular geometry complicate the analysis, which is made very difficult because of the very small residual effect in these spectra. In fact, because of the compressed frequency scale this approach is only applicable for achieving one or two of these many parameters in situations where reasonable assumptions about the other parameters can be made. The limitations of this technique does not, however, belittle the need for such quantitative information on the EFG tensor. Recently, the development of ^{14}N overtone spectroscopy[22,23] has opened the door for using natural abundant ^{14}N sites for structural constraints in biopolymer structure determination by solid state NMR techniques.[24] However, the need for a well-defined tensor orientation limits the quantitative interpretation that can presently be justified for such data sets.

The primary source for experimental data on the magnitude and asymmetry of the ^{14}N quadrupole coupling constant has been nuclear quadrupole resonance (NQR) spectroscopy. For instance, data on a variety of N-acetylated amino acids has been reported by Sadiq et al.[25] and Rabbani et al.[26] has reported on a series of polyglycine peptides. Furthermore, it has been shown that NMR powder patterns of the quadrupolar sites can be achieved via overtone spectroscopy and the discontinuities can be fit by a description of the magnitude and asymmetry of the quadrupole interaction.[22,23]

The primary polypeptide studied in this effort is gramicidin A (1880 daltons), a linear polypeptide of 15 amino acids with alternating D and L stereochemistry and blocked terminal groups.

formyl-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-
Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-ethanolamine

This polypeptide, as a dimer, forms a monovalent cation selective channel across lipid bilayers. Neither, a high resolution crystallographic nor a solution NMR structure of gramicidin in a lipid environment has been achieved. The original model of the channel describing the folding of the polypeptide backbone was proposed by Urry in 1971[27] based on conformational analysis and organic solvent studies of the polypeptide. The novel concept of folding a β-sheet structure into a helix has recently been verified by ^{15}N solid state NMR studies of oriented samples.[28] Details of this structure are vital for a high resolution understanding of how this cation channel functions. For instance, a description of the structural variability along the channel axis is sought to explain the two cation binding sites in the channel. Furthermore, the degree to which the backbone carbonyl oxygens are rotated into the channel lumen will dictate the path followed by the cations in transit across the membrane. The results described in this effort will permit the continued development of solid state NMR as an approach for protein structure elucidation as demonstrated recently by the a priori determination of backbone torsion angles in gramicidin.[29]

Experimental Section

^{15}N DL-valine and $^{13}C_1$ L-alanine were purchased from Cambridge Isotope Labs (Cambridge, MA). The valine was resolved via acetylation and enzymatic deacetylation as described previously.[3] Gramicidin A was synthesized via solid phase peptide synthesis using Fmoc chemistry and HPLC purified as described previously.[30]

The NMR spectroscopy was performed on a heavily modified IBM/Bruker WP200 NMR spectrometer equipped with a solid state NMR package and a narrow-bore 4.7 T magnet. Home-built static (i.e., nonmagic angle spinning) probes for $^1H/^{13}C$ and $^1H/^{15}N$ were used. Chemical shift spectra were obtained via cross polarization using a Hartman–Hahn match. Typical decoupling conditions were between 18 and 25 G. ^{15}N spectra are externally referenced relative to a saturated solution of $^{15}NH_4NO_3$. ^{13}C spectra are referenced to TMS via an external reference to adamantane.

For the determination of the ^{15}N and ^{13}C chemical shift tensor elements an error of ± 2 ppm is estimated for the study reported here. The determination of α_D^l has an error of $\pm 5°$ and β_D^l, an error of $\pm 2°$. The error in the determination of α_Q, β_Q, and γ_Q is greater than that for α_D^l and β_D^l and is discussed in detail in the Results section.

The simulations were carried out on a Sun Microsystems work station. The calculation of ^{13}C–^{15}N dipolar coupled powder patterns were per-

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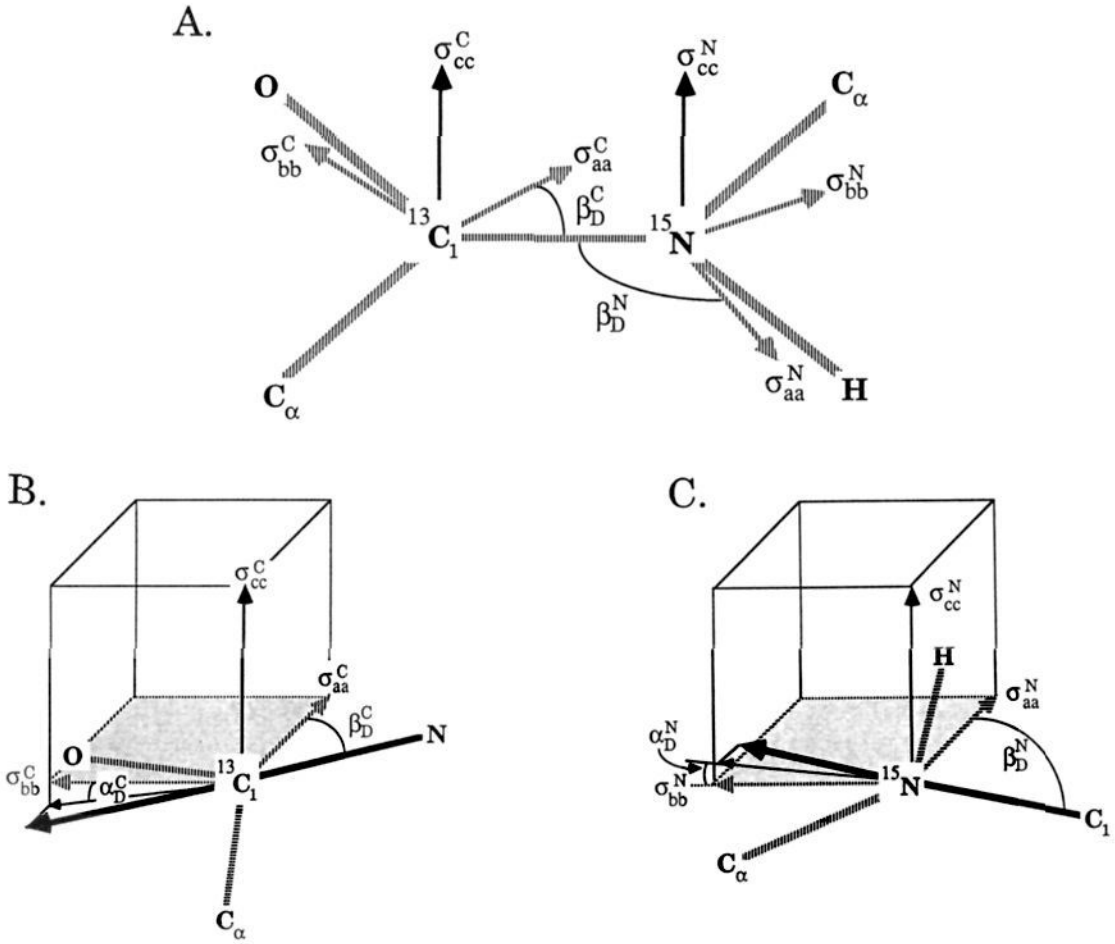

Figure 1. Orientation of ^{13}C and ^{15}N chemical shift tensors relative to the molecular frame of a peptide linkage. All broken lines indicate lines or vectors that are in the plane of the peptide linkage and in (B) and (C) the base plane of the cube is the peptide linkage plane. Also in (B) and (C) the C_1–N bond is tipped out of the base plane for the sole reason of showing the definition of α_D which has been determined to be 0 in almost all ^{15}N and $^{13}C_1$ peptide linkage tensor determinations. (A) Tensor element definitions have been chosen so that the ^{13}C and ^{15}N definitions are consistent. σ^i_{cc} is defined as perpendicular to the plane; σ^i_{aa} is the vector to which the azimuthal angle, β^i_D, is defined, and σ^i_{bb} completes the orthogonal coordinate system. α^i_D is the angle between σ^i_{bb} and the projection of the C_1–N bond onto the $\sigma^i_{cc}/\sigma^i_{bb}$ plane. (B) is the $^{13}C_1$ tensor, and (C) is the ^{15}N tensor.

formed with the program TENSORI requiring a typical calculation time of less than 1 s. The program CSQUADX was used for the calculations of both ^{14}N coupled ^{13}C powder patterns and the oriented spectra. For this latter program powder patterns required less than 3 min and oriented spectra less than 1 s of work station time. The diagonalization of the ^{14}N Hamiltonian matrix was achieved by the subroutine CH from EISPACK. The calculated data were plotted using the graphic package TGRAPH.

Theory

To achieve our primary goal of determining the orientation of nuclear spin interaction tensors with respect to the molecular frame, it is necessary to have an appropriate set of definitions for the principal elements of the tensor. For axially asymmetric tensors the standard definitions have been based on their magnitude. For instance, $\sigma_{33} \geq \sigma_{22} \geq \sigma_{11}$[31] or $|\sigma_{zz} - \sigma_{iso}| \geq |\sigma_{xx} - \sigma_{iso}| \geq |\sigma_{yy} - \sigma_{iso}|$[32] (where σ_{iso} represents the trace of the tensor) are convenient for characterizing spectra, but they are inconvenient for characterizing electronic shielding in a set of molecules or molecular sites. The inconvenience arises in ^{15}N chemical shift tensors where σ_{11} and σ_{22} have similar magnitudes. Typically σ_{11} is in the plane of the peptide linkage and σ_{22} is perpendicular to the plane, but on occasion the magnitude of the perpendicular component relative to σ_{iso} becomes greater than the in-plane element.[9] It is not appropriate to think that the tensor element, which was in the plane of the peptide linkage, is now perpendicular to the plane. There has not been any gross change in tensor orientation but rather a subtle change in the magnitude of the tensor elements. Furthermore, the tensor is oriented with respect to the molecular frame by the angles, β_D and α_D (Figure 1). If these angles are defined relative to a tensor element, for instance σ_{11}, then the definitions of these angles must also change when the tensor element labeled σ_{11} shifts from in the plane to perpendicular to the plane. Therefore, we describe here (Figure 1C) a set of definitions for the ^{15}N chemical shift elements that is based on their approximate orientation with respect to the molecular frame: σ^N_{cc} is approximately perpendicular to the peptide plane, σ^N_{aa} lies in the C_1NH bond angle, and σ^N_{bb} completes the orthogonal coordinate system. As previously defined, β^N_D is the angle between the C_1–N bond and the $\sigma_{33} = \sigma^N_{aa}$ axis. α^N_D is the angle between the projection of the C_1–N bond onto the σ^N_{bb}–σ^N_{cc} plane and the σ^N_{bb} element.

In this paper the superscripts on the tensor element magnitudes and orientational angles distinguishes these new definitions from previous definitions. When "N" for ^{15}N and "C" for ^{13}C is not specified the letter "i" is used to indicate either ^{15}N or ^{13}C for the new definitions. Furthermore, in defining the magnitude of the dipolar interactions, $\nu_\parallel^{14N}$ is used for the dipolar interaction between ^{14}N and ^{13}C, while $\nu_\parallel^{15N}$ is used for the dipolar interaction between ^{15}N and ^{13}C.

There is also an ambiguity in the $^{13}C_1$ chemical shift tensors where the powder pattern is essentially $\eta^{cs} = 1$. Here, a slight change in the magnitude of σ_{yy} can reverse the definition of σ_{xx} and σ_{zz} elements.[11] Once again, this could be misinterpreted as a dramatic change in tensor orientation rather than a subtle change

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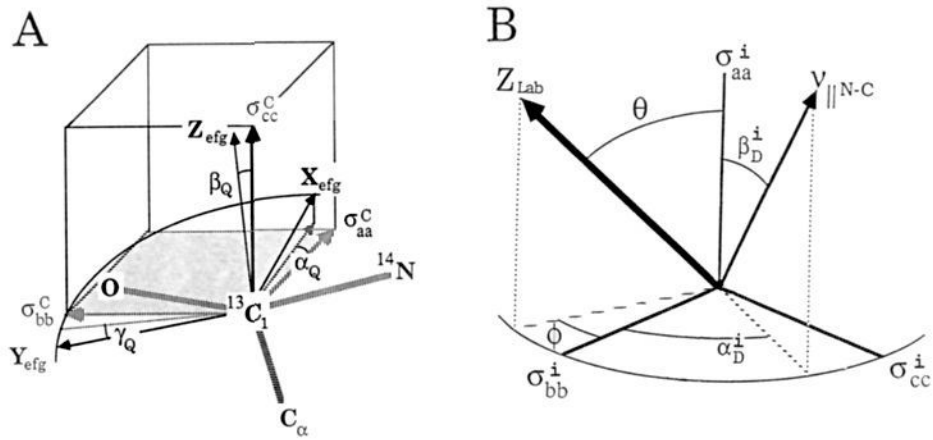

Figure 2. (A) Orientation of the electric field gradient tensor with respect to the ^{13}C chemical shift principal axis system. The base plane of the cube is the peptide linkage plane; all broken lines and vectors lie in this plane. The series of rotations, γ_Q, β_Q, and α_Q, achieves coincidence of the chemical shift and the EFG tensors. (B) The orientation of the chemical shift tensor with respect to the laboratory Z axis, Z_{lab}, which is the static magnetic field direction, given by θ and ϕ. Another view of the relative orientation of the dipolar and chemical shift tensors is also presented.

in the magnitude of a tensor element. Therefore, we establish σ^c_{cc} as approximately perpendicular to the plane as we did for the ^{15}N tensor and σ^c_{bb} as oriented in the vicinity of the carbonyl bond, while σ^c_{aa} completes the orthogonal coordinate system in the NC_1O bond angle (Figure 1B). The definitions of α_D and β_D for the ^{13}C chemical shift tensor are also changed. While β_D has typically been used to orient the element perpendicular to the peptide plane, we have chosen definitions similar to the ^{15}N chemical shift tensor. Consequently, β^c_D is defined here as 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} element and the projection of the C_1–N vector onto the σ^c_{bb}–σ^c_{cc} plane.

We will continue to use the frequency based definitions when discussing the spectra and only use the new definitions when describing the orientation of the tensor within the molecular frame. For specific molecular sites it will then be appropriate to assign the spectrally defined magnitudes of the tensor elements to the molecularly oriented frame.

The Spin $^1/_2$, Spin $^1/_2$ System. The powder pattern spectrum of a dipolar coupled nuclear site can be expressed in the following form:[12]

$$S(\nu) = \sum_i S(\nu_i) = \sum_i \int_{\theta=0}^{180} \int_{\phi=0}^{360} g_i[\nu, \nu_i(\theta, \phi, \alpha_D, \beta_D)] \sin\theta \, \partial\theta \, \partial\phi \quad (1)$$

where θ and ϕ are polar angles that describe the orientation of the chemical shift PAS with respect to the magnetic field fixed in the laboratory frame (Figure 2B) and α_D and β_D are rotations that transform a dipolar interaction into the PAS of the chemical shift tensor. ν is the observed frequency and $\nu_i(\theta, \phi, \alpha_D, \beta_D)$ is the transition frequency for a particular orientation of the chemical shift and dipolar tensors. g_i is a line shape function for $\nu_i(\theta, \phi, \alpha_D, \beta_D)$. For the calculation of theoretical line shapes a line broadening function such as a Lorentzian or Gaussian function is used to simulate various broadening mechanisms.

The dipolar interaction affects the chemical shift spectral line shape by an orientational dependent splitting of the chemical shift resonances:

$$\nu_i(\theta, \phi, \alpha_D, \beta_D) = \nu_{CS} \pm \nu_D \quad (2)$$

$$\nu_{CS} = \sigma^i_{bb} \sin^2\theta \cos^2\phi + \sigma^i_{cc} \sin^2\theta \sin^2\phi + \sigma^i_{aa} \cos^2\theta \quad (3)$$

$$\nu_D(\theta, \phi, \beta_D, \alpha_D) = \nu^{^{15}N}_{\|} \frac{1 - 3\,[\sin\beta_D \sin\theta \cos(\phi - \alpha_D) + \cos\beta_D \cos\theta]^2}{2} \quad (4)$$

$$\nu^{^{15}N}_{\|} = \frac{h\gamma_{^{15}N}\gamma_{^{13}C}}{4\pi^2 r^3} \quad (5)$$

ν_{CS} is the chemical shift frequency and ν_D is the shift in the frequency caused by the coupling with the dipolar interaction. $\nu_{\|}$ is the magnitude of the unique element of the dipolar tensor. The powder pattern simulations are accomplished by transforming the dipolar interaction tensor into the chemical shift tensor. Then both interaction tensors are transformed into the laboratory frame via the polar angles θ and ϕ. For powder spectra, integrations over θ and ϕ are performed for values of α_D and β_D. For the study reported here a typical value of the peptide bond length (1.34 Å) determined from diffraction studies is used.[33] The value of $\nu^{^{15}N}_{\|}$ obtained with this bond length is 1.26 kHz. This value has been verified in the process of determining the ^{15}N chemical shift tensor orientation.

It was shown previously[3] for the orientations of the ^{15}N chemical shift tensors from ^{15}N–^{13}C labeled gramicidin that the orientational variables β^N_D and α^N_D are a pair of independent variables. The dipolar splitting about σ_{33} (equivalent to σ_{aa} here) while dependent on the angle β^N_D is independent of α^N_D. The dipolar splitting about σ_{22} (σ^N_{cc}) and σ_{11} (σ^N_{bb}) are dependent on a combination of α^N_D and β^N_D. This is a direct consequence of the definitions of the orientational angles and the relative magnitude of the tensor elements.

The Spin $^1/_2$, Spin 1 System. In the presence of the ^{13}C–^{14}N quadrupole interaction the spectral line shape is not only dependent on the orientation of the ^{13}C chemical shift with respect to the molecular frame but also dependent on the ^{13}C tensor orientation with respect to the ^{14}N electric field gradient (EFG) principal axis system (PAS). The frequency, ν_i, in eq 1 becomes dependent upon three additional variables, γ_Q, β_Q, and α_Q rotations that transform the ^{14}N EFG into the ^{13}C chemical shift PAS. The relationship of these rotations with the principle axes of the ^{13}C chemical shift tensor is shown in Figure 2.

To study the characteristics of the tensors in a specific molecular system, an intrinsic reference frame needs to be chosen for the tensor coordinate frames. In the magic angle spinning studies, the quadrupole EFG PAS has to be used as a reference frame into which the dipolar interaction is transformed. This is because the ^{13}C chemical shift is averaged to its isotropic value so that the PAS of the ^{13}C chemical shift tensor becomes totally symmetric. In the circumstance of magic angle spinning, only the relative orientation of the dipolar interaction to the ^{14}N EFG PAS is considered. However, for a static sample the chemical shift tensor is associated with the other spin interactions. Among these spin interactions the chemical shift is the unique asymmetric spin-field interaction. The spin interactions such as spin–spin interactions (dipolar interactions and J couplings) and the electric interaction (quadrupole interaction) will affect the chemical shift resonances so that the line shape of the chemical shift spectrum is altered by splitting the energy levels of the nuclear spin states. This suggests that the addition of one of these interactions to the

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chemical shift interaction will expand the frequency range for the resonances while causing a different spectral line shape. Therefore, it is reasonable to define the chemical shift tensor PAS as the reference frame for the tensor orientation study of static samples.

The Hamiltonian for the ^{13}C spin includes the Zeeman, ^{13}C chemical shift, and ^{13}C–^{14}N dipolar interactions

$$\mathcal{H}_C = \mathcal{H}^z + \mathcal{H}^{cs} + \mathcal{H}^d \tag{6}$$

$$\mathcal{H}^d = C^d \sum_{m=-2}^{+2} (-1)^m T^d_{2,m} R^d_{2,-m} \tag{7}$$

where m is the magnetic quantum number, R^d is the orientation dependent operator, and T^d is the spin dependence of the dipolar interaction Hamiltonian, $\mathcal{H}^d$. R^d can be represented by the Wigner rotational matrix. [The Wigner rotational matrix is the same as that of Spiess[34] except γ and α are exchanged. The order of the transformation is γ, β, and then α in our definition.]

$$R^d_{2,-m} = \sum_{m'=-2}^{+2} D^{(2)}_{m',-m}(0, \theta, \phi) D^{(2)}_{0,m'}(0, \beta_D, \alpha_D) \rho^d_{2,0} \tag{8}$$

It is known from quantum theory that a Hermitian operator containing a single step operator, $I_\pm$, (either step-up or step-down operator) will yield vanishing diagonal elements because of the orthogonality of the spin state functions. Therefore, the spin-dependent part of the ^{13}C–^{14}N dipolar interaction Hamiltonian can be simplified as follows:

$$T^d_{2,0} = \frac{2}{\sqrt{6}} I^C_z I^N_z + \frac{1}{\sqrt{6}} (I^C_{+1} I^N_{-1} + I^C_{-1} I^N_{+1}) \rightarrow \frac{2}{\sqrt{6}} I^C_z I^N_z \tag{9a}$$

$$T^d_{2,\pm1} = \frac{1}{\sqrt{2}} (I^C_z I^N_{\pm1} + I^C_z I^N_{\pm1}) \rightarrow \frac{1}{\sqrt{2}} I^C_z I^N_{\pm1} \tag{9b}$$

$$T^d_{2,\pm2} = I^C_{\pm1} I^N_{\pm1} \rightarrow 0 \tag{9c}$$

Substituting these equations into eq 7 yields

$$\mathcal{H}^d = \sqrt{6} \nu^{^{14}N}_{\parallel} \sum_{m=-1}^{+1} (-1)^m T^d_{2,m} \sum_{m'=-2}^{+2} D^{(2)}_{m',-m}(0, \theta, \phi) D^{(2)}_{0,m'}(0, \beta_D, \alpha_D) \tag{10}$$

In the ^{13}C–^{14}N interaction basis, the eigenfunctions of the ^{13}C spins can be represented by the product function of ^{13}C spin states ($|\alpha^C\rangle$, $|\beta^C\rangle$) and ^{14}N spin states ($|\phi_n{}^N\rangle$) where n represents the three spin states of ^{14}N.[21] These nitrogen spin state functions can be expanded in terms of the Zeeman spin states ($|m\rangle$) and the coefficients, $C^N{}_{mn}$, which form a 3×3 matrix

$$|\phi^N_n\rangle = \sum_{m=-1}^{+1} |m\rangle\langle m|\phi^N_n\rangle$$

$$= \sum_{m=-1}^{+1} C^N_{mn} |m\rangle \qquad n = -1, 0, +1 \tag{11}$$

The coefficients $C^N{}_{mn}$ can be determined by diagonalizing the Hamiltonian matrix of the ^{14}N nuclear spin that includes the ^{14}N Zeeman interaction, $\mathcal{H}^z$, and the ^{14}N electric quadrupolar interaction, $\mathcal{H}^q$.[21]

$$\mathcal{H}_N = \mathcal{H}^z + \mathcal{H}^q$$

$$= -h\gamma_N B_0 I_z + C^q \sum_{m=-2}^{+2} (-1)^m T^q_{2,m} R^q_{2,-m} \tag{12}$$

where

$$R^q_{2,-m} = \sum_{m'=-2}^{+2} D^{(2)}_{m',-m}(0, \Theta, \Phi) \rho^q_{2,m'} \tag{13}$$

where Φ and Θ are the Euler angles that transform the EFG PAS of the ^{14}N spins into the laboratory frame. Before $\mathcal{H}_N$ is calculated, the electric field gradient PAS of the ^{14}N quadrupolar interaction needs to be transformed into the ^{13}C chemical shift tensor PAS by rotations γ_Q, β_Q, and α_Q so that the effect of the quadrupolar interaction on the ^{13}C–^{14}N dipolar interaction can be evaluated in the reference frame. Then

$$R^q_{2,-m} = \sum_{m'=-2}^{+2} D^{(2)}_{m',-m}(0, \theta, \phi) \sum_{m''=-2}^{+2} D^{(2)}_{m'',m'}(\gamma_Q, \beta_Q, \alpha_Q) \rho^q_{2,m''} \tag{14}$$

By combining C^q with $\rho^q{}_{2,m}$, the ^{14}N quadrupole Hamiltonian can be expressed as

$$\mathcal{H}^q = \sum_{m=-2}^{+2} (-1)^m T^q_{2,m} \sum_{m'=-2}^{+2} D^{(2)}_{m',-m}(0, \theta, \phi) \sum_{m''=-2}^{+2} D^{(2)}_{m'',m'}(\gamma_Q, \beta_Q, \alpha_Q) \rho^q_{2,m''} \tag{15}$$

where $\rho^q{}_{2,m}$ is defined as

$$\rho^q_{2,0} = \sqrt{\frac{3}{2}} C^q$$

$$\rho^q_{2,\pm1} = 0$$

$$\rho^q_{2,\pm2} = -\frac{1}{2}\eta C^q \tag{16}$$

where C^q is the quadrupole coupling constant

$$C^q = \frac{e^2 q Q}{h}$$

The allowed dipolar resonances are given by the following:

$$\nu^d_n = \nu^{^{14}N}_{\parallel} \{2R^d_{2,0}(C^*_{n,+1}C_{n,+1} - C^*_{n,-1}C_{n,-1}) + \sqrt{3}R^d_{2,-1}(C^*_{n,0}C_{n,-1} + C^*_{n,+1}C_{n,0}) - \sqrt{3}R^d_{2,+1}(C^*_{n,-1}C_{n,0} + C^*_{n,0}C_{n,+1})\} \tag{17}$$

The spectral frequency of the ^{13}C chemical shift coupled with ^{14}N is calculated by substituting eqs 3 and 17 into the following:

$$\nu_i(\theta, \phi, \alpha_D, \beta_D, \alpha_Q, \beta_Q, \gamma_Q) = \sum_{n=-1}^{+1} (\nu^{cs} + \nu^d_n) \tag{18}$$

thereby yielding a 1:1:1 "triplet" for each chemical shift frequency.

Results

To illustrate the determination of chemical shift tensor orientations in a polypeptide of interest, data are presented for the Ala_5-Val_6 peptide linkage in gramicidin A. Shown in Figure 3 (parts A and C) are the ^{15}N chemical shift powder pattern spectra of ^{15}N-Val_6- and $^{13}C_1$-Ala_5-^{15}N-Val_6 gramicidin A, respectively. The analysis of these spectra is performed similarly to that for the Ala_3 and Leu_4 sites in gramicidin A.[3] The simulation of the single site labeled spectrum (Figure 3B) provides a solution for the magnitudes of the chemical shift tensor elements alone (σ_{33} = 202, σ_{22} = 62, σ_{11} = 37 ppm). While σ_{11} and σ_{22} are most frequently in the peptide plane and perpendicular to the plane, respectively, σ_{33} is always in the plane and in the vicinity of the N–H bond, consequently $\sigma_{33} = \sigma^N_{aa}$. The spectrum in Figure 3C clearly shows the effect of a dipolar interaction between two spin = 1/2 nuclei. Without depending on the determination of the tensor element magnitudes, the value of β^N_D can be estimated from $\Delta\nu^N_a$ = 1.0 kHz in the spectrum of the double label (Figure 3C, β^N_D = 105°). Full spectral simulation as shown in Figure 3D using the tensor elements derived above and allowing for a limited range of angles about the β^N_D estimate, results in a unique determination of β^N_D = 105° and α^N_D = 0° and assigns $\sigma_{22} = \sigma^N_{cc}$ and $\sigma_{11} = \sigma^N_{bb}$. Furthermore, the results are entirely consistent with an N–C_1 bond length of 1.34 Å.

The analysis of the ^{13}C spectra shown in Figure 4 (parts A and C) for $^{13}C_1$-Ala_5-^{15}N-Val_6 and $^{13}C_1$-Ala_5 gramicidin A, respectively, is considerably more difficult. The spectrum of the single site labeled molecule is clearly not just dependent on the chemical shift tensor elements. In addition there are the effects of a dipolar interaction with ^{14}N, a quadrupole nucleus with a nuclear spin quantum number of 1. Such a dipolar interaction will split the intensity of a unique molecular orientation into a triplet as shown

(34) Spiess, H. W. *NMR Principles and Progress*; Springer Verlag: New York, 1978; Vol. 15.

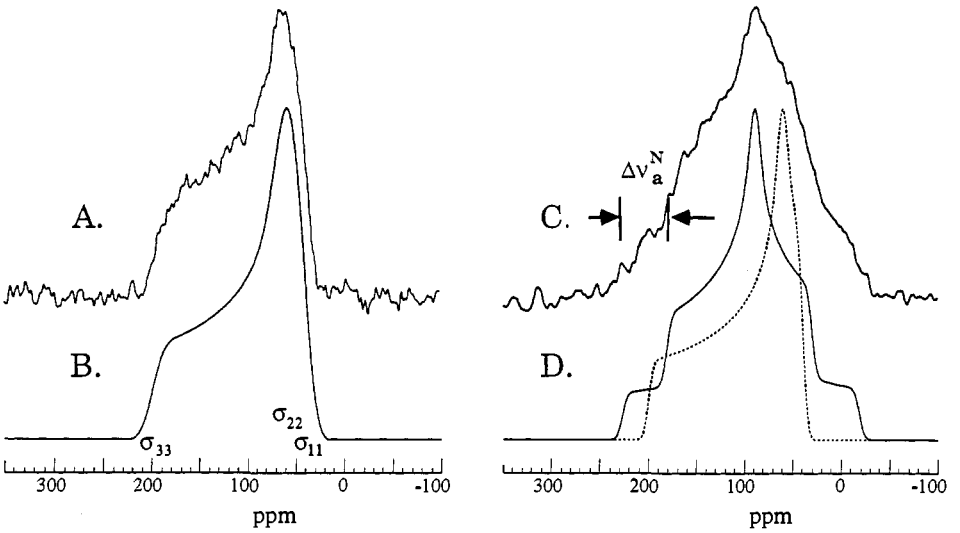

Figure 3. ^{15}N chemical shift powder pattern spectra and spectral simulations. (A) Spectrum of ^{15}N-Val$_6$ gramicidin A obtained with 26 000 data acquisitions and a recycle delay of 6 s. (B) Spectral simulation of (A) using spectral parameters of $\sigma_{11} = 37$, $\sigma_{22} = 62$, and $\sigma_{33} = 202$ ppm. (C) Spectrum of $^{13}C_1$-Ala$_5$-^{15}N-Val$_6$ gramicidin A obtained with 13 500 data acquisitions and a recycle delay of 6 s. The dipolar splitting about $\sigma_{aa}^N = \sigma_{33}$ is shown, $\Delta\nu_a^N$. (D) Spectral simulation of (C) using spectral parameters from (B) as well as $\nu_\parallel^{15N} = 1.26$ kHz, $\alpha_D^N = 0°$, and $\beta_D^N = 105°$. For comparison the chemical shift anisotropy alone is shown with dotted lines.

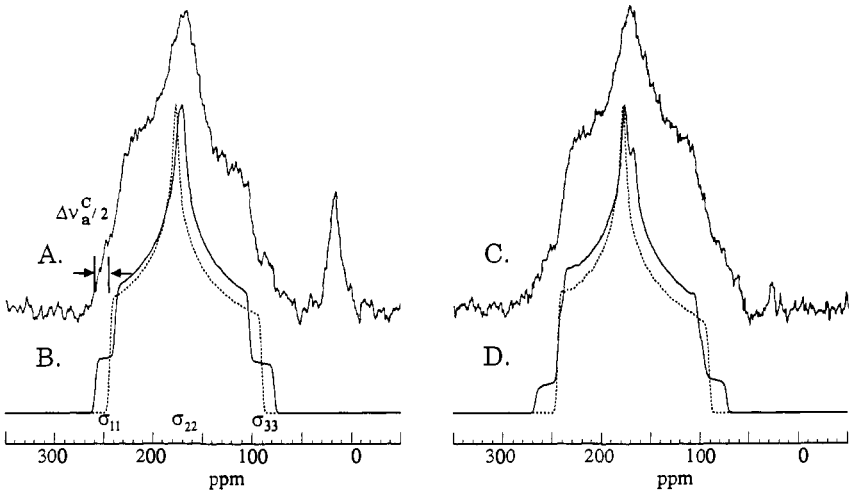

Figure 4. ^{13}C chemical shift powder pattern spectra and spectral simulations. For comparison the chemical shift anisotropy alone is shown in dotted lines in (B) and (D). Most of the natural abundance intensity has been removed from the experimental spectrum by subtracting a natural abundance spectrum of gramicidin A. (A) Spectrum of $^{13}C_1$-Ala$_5$-^{15}N-Val$_6$ gramicidin A obtained with 18 800 data acquisitions and a recycle delay of 5 s. (B) Spectral simulation of (A) using spectral parameters of $\sigma_{11} = 244$, $\sigma_{22} = 178$, $\sigma_{33} = 90$ ppm, $\nu_\parallel^{15N} = 1.26$ kHz, $\alpha_D^c = 0°$, and $\beta_D^c = 38°$. (C) Spectrum of $^{13}C_1$-Ala$_5$ gramicidin A obtained with 10 178 data acquisitions and a recycle delay of 6 s. (D) Spectral simulation of (C) using spectral parameters from (B) (except that $\nu_\parallel^{14N} = 898$ Hz) as well as $\alpha_Q = 0°$, $\beta_Q = 0°$, $\gamma_Q = 0°$, $C^q = -3.2$ MHz, and $\eta^q = 0.31$.

in Figure 5D. Consequently, the chemical shift spectrum of a single site $^{13}C_1$ labeled polypeptide is also dependent on the orientation of the dipolar interaction (α_D^c and β_D^c) and its magnitude ($\nu_\parallel^{14N}$). Furthermore, the sign (+/−), magnitude (C^q), and orientation (α_Q, β_Q, and γ_Q) of the quadrupole interaction effects the triplet splitting for a given molecular orientation (Figure 5 (parts F and H)) resulting in too many variables to achieve a unique solution set without more data. These problems are further compounded by the background intensity from natural abundance signals that can be high enough to conceal informative signals. Natural abundance for ^{13}C is about 3-fold larger than for ^{15}N, and both protein and lipid molecules are rich in carbon but have few nitrogens, consequently, natural abundance signals are a much more significant problem for ^{13}C than for ^{15}N. The ^{13}C spectra shown here have had the majority of the ^{13}C natural abundance intensity removed by subtracting a natural abundance spectrum of gramicidin A.

For the $^{13}C_1$ sites, the spectra of the double label molecules (^{13}C and ^{15}N) are considerably simpler than spectra of the single site labeled gramicidins. In figure 4 (parts A and B) the experimental and theoretical spectra are shown respectively for $^{13}C_1$-Ala$_5$-^{15}N-Val$_6$ gramicidin. Here the dipolar interaction is between two spin $= {}^1/_2$ nuclei, and a quadrupole nucleus is not involved. As with the ^{15}N spectra of the same sample, these ^{13}C spectra are dependent on just six variables, the three chemical shift tensor elements, and the orientation and magnitude of the dipolar interaction. This final variable has already been determined from ^{15}N spectra and is considered fixed ($\nu_\parallel^{15N} = 1.26$ kHz) in this analysis. As with the ^{15}N tensor it is possible to achieve an initial estimate of β_D^c, but unlike the ^{15}N analysis the determination of the orientational variables are not independent of the tensor element magnitudes for carbonyl sites. This results in an unequal splitting of the dipolar shoulders about σ_{aa}^c. This element is known from previous studies to be the downfield element labeled in this spectrum as σ_{11}, consequently $\sigma_{11} = \sigma_{aa}^c$. The ^{15}N tensor is nearly axially symmetric with the unique element being σ_{aa}^N, but for the carbonyl ^{13}C sites the powder pattern is nearly an $\eta^{cs} = 1$ pattern. The asymmetry of the splitting, $\Delta\nu_a^c$ (about σ_{11}), results from the difference in the anisotropies for these two spectra and the specific orientation dependence of the ^{13}C tensor. However, by taking the difference between the outer (i.e., downfield) dipolar shoulder and the σ_{aa}^c chemical shift tensor element as $\Delta\nu_a^c/2$ it is possible to

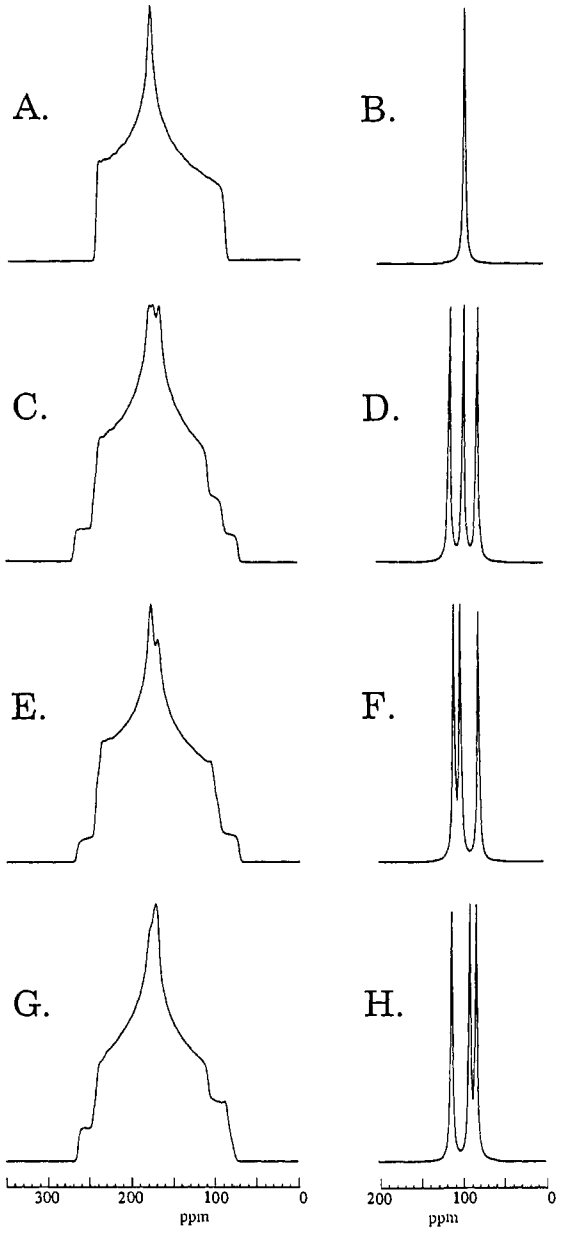

Figure 5. The effect of the ^{13}C–^{14}N dipolar and ^{14}N quadrupolar interaction on the ^{13}C chemical shift spectra of the Ala_5 gramicidin A carbonyl carbon. (A) ^{13}C chemical shift powder pattern spectrum in the absence of ^{14}N influences. (B) ^{13}C chemical shift spectrum for a single and arbitrary orientation of the carbonyl site with respect to the magnetic field ($\theta = 10°$, $\phi = 0°$). (C) To the simulation in (A), the ^{13}C–^{14}N dipolar interaction ($\nu_{\parallel}^{N} = 898$ Hz) has been added without the addition of the effects due to the quadrupole interaction. (D) For the arbitrary orientation chosen in (B) the ^{13}C–^{14}N dipolar interaction results in splitting the resonance into a 1:1:1 triplet. (E) To the simulation in (C) the effects of the quadrupole interaction have been added ($C^q = -3.2$ MHz and $\eta^q = 0.31$). (F) To the simulation in (D) the effects of the quadrupole interaction have been added resulting in a distortion in the splitting that accounts for the collapse of two shoulders in (E). (G) Same as (E) except that the sign of C^q is positive. (H) Same as (F) except that the sign of C^q is positive resulting in a reversal in the distortion of the dipolar splitting, consequently a different pair of shoulders in (G) coalesce.

determine a reasonable value for the β_D^c angle. The magnitude of $\Delta\nu_a^c/2$ is approximately 0.60 kHz, consequently the estimate for the β_D^c is 36°. In Figure 4D the spectral simulation is shown with $\sigma_{aa}^c = 244$, $\sigma_{22} = \sigma_{bb}^c = 178$ and $\sigma_{33} = \sigma_{cc}^c = 90$ ppm and the orientational parameters of $\alpha_D^c = 0°$ and $\beta_D^c = 38°$.

Having achieved a solution for the chemical shift tensor elements it is now possible to revisit the single site labeled samples and to search for a reasonable solution to the remaining variables. Several facts are obtainable by direct observation of the powder pattern spectra in Figure 4C. First, the dipolar interaction is not self-decoupled from the chemical shift interaction as would be the case if the T_1 relaxation of the ^{14}N site were very efficient.[35-37] Secondly, the sign of the quadrupole interaction is clearly negative as can be seen by comparison of the data to Figure 5 (parts E and G) where it is seen that the upfield shoulder has a much lower intensity than would be expected for a positive quadrupole coupling constant. Six of the potential variables involved in the simulation of this single site $^{13}C_1$ labeled molecule have been fixed by the analysis of the ^{13}C and ^{15}N spectra of the doubly labeled molecule. This includes the magnitude of the dipolar interaction which is fixed by a knowledge of the C–N bond length ($\nu_{\parallel}^{N} = 898$ Hz). Furthermore, a reasonable assumption can be made about the magnitude and asymmetry of the quadrupole interaction. On the basis of the single-crystal ^{14}N NMR study of *N*-acetylvaline,[20] an NQR study,[25] and the ^{13}C magic angle spinning study of the same molecule[21] we have used values of $C^q = -3.2$ MHz and η^q of 0.31. Unlike the ^{13}C–^{15}N dipolar coupled spectra it is not possible to estimate β_D^c from the dipole splitting at σ_{aa}^c because the quadrupole interaction has distorted the coupling. However, as noted above the orientation of the chemical shift tensor with respect to the unique dipolar axis is fixed from a knowledge of the ^{15}N–^{13}C analysis for this site. The full spectral simulation results in a good fit to the experimental spectra with $\alpha_Q = 0°$, $\beta_Q = 0°$, and $\gamma_Q = 0°$.

Figure 5 shows the effects of the dipolar interaction between a spin = $^1/_2$ and a spin = 1 nucleus. Spectral simulation of both powder pattern and oriented samples are shown. The powder pattern shown in Figure 5A has spectral parameters typical of a carbonyl carbon site. The molecular orientation chosen for the spectrum in Figure 5B is arbitrary ($\theta = 10°$ and $\phi = 0°$). Because its frequency is close to σ_{33}, the molecular orientation is such that σ_{33} is close to the magnetic field direction. The dipolar coupling splits each chemical shift frequency into a 1:1:1 triplet (Figure 5D). The quadrupole effect results in a distortion of the splitting separation (Figure 5F) and for the powder pattern the largest effect is in the vicinity of σ_{33} although a number of other subtle differences occur throughout the spectrum (Figure 5E). The change in sign of the quadrupole interaction, C^q, has a major effect on the distortions. The pattern of distortion in the triplet is reversed (Figure 5G) which again changes the pattern of shoulders in the vicinity of σ_{33} (Figure 5H).

Figure 6 shows another powder pattern spectrum of a polypeptide (cyclo[Val_1-Pro_2-Gly_3-Val_4-Gly_5]) that is single site labeled with a carbonyl ^{13}C at Val_1. In this spectrum the natural abundance ^{13}C signals, which gives rise to a small amount of aliphatic intensity, have not been subtracted out of the spectra. The T_2 relaxation properties are particularly inefficient in this sample leading to excellent definition of the low intensity shoulders of the pattern as well as the splitting observed at σ_{22}. This simulation of the powder pattern was not obtained with the rigorous manner used for the gramicidin powder pattern analysis, because the double labeled polypeptide was not available. However, from the success of using $C^q = -3.2$ MHz and $\eta^q = 0.31$ these values were assumed along with the magnitude of the dipolar interaction $\nu_{\parallel}^{N} = 898$ Hz to be appropriate for this sample as well. For the orientation of the ^{13}C chemical shift tensor with respect to the molecular frame reasonable values for α_D^c and β_D^c of 0° and 34°, respectively, have been used for this simulation. Interestingly, the best fit simulation was achieved with $\alpha_Q = 90°$, $\beta_Q = 0°$, and $\gamma_Q = 0°$ which describes a 90° rotation of the EFG tensor compared to that determined here in gramicidin and orientations previously determined by NMR.[21,37] As shown in Figure 6C the relatively small but significant differences caused by a 90° α_Q rotation include elimination of a second shoulder on both the downfield and upfield sides of the powder pattern, a decrease in

(35) Spiess, H. W.; Haeberlen, U.; Zimmerman, H. *J. Magn. Reson.* **1977**, *25*, 55–66.
(36) Jonsen, P. *J. Magn. Reson.* **1988**, *77*, 348–355.
(37) Wang, C.; Teng, Q.; Cross, T. A. *Biophys. J.*, in press.

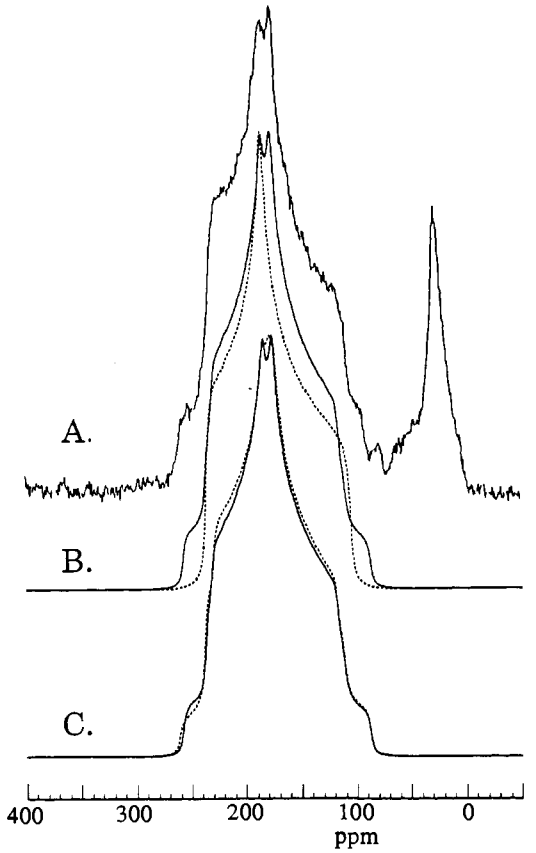

Figure 6. (A) ^{13}C powder pattern spectrum of cyclo{$^{13}C_1$-Val-Pro-Gly-Val-Gly} obtained with 2000 data acquisitions and a recycle delay of 8 s. The sample was crystallized from DMSO-d_6/H_2O and observed in the crystallization solvent. (B) Spectral simulation of (A) using the following spectral parameters: σ_{11} = 236, σ_{22} = 185, σ_{33} = 105 ppm, $\nu_{\|}^{^{14}N}$ = 898 Hz, α_D^c = 0°, β_D = 34°, α_Q = 90°, β_Q = 0°, γ_Q = 0°, C^q = −3.2 MHz, and η^q = 0.31. For comparison the chemical shift powder pattern alone is shown in a dashed line. (C) As in (B) except that a second simulation is shown with a dashed line where the only parameter difference is α_Q = 0°.

the downfield dipolar splitting, and the formation of a dipolar splitting in the vicinity of σ_{22}. The magnitude of the chemical shift tensor elements for both of the simulations are $\sigma_{11} = \sigma_{aa}^c$ = 234, $\sigma_{22} = \sigma_{bb}^c$ = 184, and $\sigma_{33} = \sigma_{cc}^c$ = 105 ppm.

In the simulation of the ^{15}N coupled ^{13}C chemical shift powder patterns, the line shape is much more sensitive to the value of β_D^i than to α_D^i, because α_D^i has a value of 0° for the sites studied. The error in β_D^i is estimated to be ±2°, while the α_D^i determinations have an error of ±5°. The accuracy in the simulation of the ^{14}N coupled ^{13}C chemical shift powder pattern spectra is largely dependent on the quality of the determination of the ^{13}C chemical shift tensor orientation and magnitude as well as the quality of these spectra. Achieving good line shape requires that attention be given to both the sample preparation and to the spectroscopy being conducted. While the line shape for the cyclic peptide is considerably better than for gramicidin, the lack of a ^{15}N coupled ^{13}C spectrum adds to the number of variables to be solved from this one spectrum and consequently the error in the determination of α_Q, β_Q, and γ_Q is approximately ±10°. The data for both the cyclic peptide and for gramicidin is summarized in Table I.

Discussion

It has been very important for this work and it will be even more important for the future development of this effort to define the variables for the chemical shift tensor elements within the molecular framework. It has also been important to redefine how the chemical shift tensors are oriented with respect to the peptide bond. While the quality of a ^{13}C spectral simulation is independent of how the orientational variables are defined, it is important for initial estimates. The standard definitions of α_D and β_D for carbonyl carbons would require an analysis of the splitting about the upfield chemical shift element which is severely compromised by natural abundance carbon signals. The definitions presented here utilize the splitting about the downfield element for the unique dependence on β_D^c. The ability to achieve such an estimate enhances the reliability of the final spectral analysis. Furthermore, these new definitions are designed so that the elements for the ^{15}N and ^{13}C tensors have similar meanings.

Table I. ^{13}C Powder Pattern Spectral Parameters[a]

parameter	$^{13}C_1$-Ala$_5$ gramicidin A	cyclo{$^{13}C_1$-Val-Pro-Gly-Val-Gly}
σ_{11} (ppm)	244 ± 2	236 ± 3[c]
σ_{22} (ppm)	178 ± 2	185 ± 3[c]
σ_{33} (ppm)	90 ± 2	105 ± 3[c]
α_D	0° ± 5°	0° ± 10°
β_D	38° ± 2°	34° ± 3°
α_Q[b]	0° ± 10°	90° ± 10°
β_Q[b]	0° ± 10°	0° ± 10°
γ_Q[b]	0° ± 10°	0° ± 10°

[a] The following values were invariant: $\nu_{11}^{^{14}N}$ = 898 Hz; $\nu_{11}^{^{15}N}$ = 1.26 kHz; C^q = −3.2 MHz; η^q = 0.31. [b] A thorough error analysis for the determination of the error in these determinations is beyond the scope of this paper but is currently under study. [c] Because of the unavailability of a $^{13}C/^{15}N$ doubly labeled cyclo peptide these error values are somewhat greater.

It has been possible to minimize the number of variables for the determination of the ^{15}N chemical shift tensor orientation for polypeptide backbone sites and in so doing increase the reliability of the determination. As more data become available it will be possible to show how variable the tensors are in protein environments. Here, one more ^{15}N site, D-Val$_6$ in gramicidin A, is described in detail, and the results are identical with the previous determination of the tensor orientation for D-Leu$_4$ gramicidin A and virtually identical with the L-Ala$_3$ gramicidin A site (β_D^N = 104° vs 105°[3]). However, in crystalline and polycrystalline model compounds a much greater variability has been observed. It has been very clearly demonstrated that the magnitude of the ^{15}N tensor elements vary not only among different dipeptides[9] but also for the same dipeptide in two different crystal forms.[4] This later study showed tensors having asymmetries of 0.06 and 0.44 for two crystal forms. It has yet to be demonstrated directly whether such variability exists in proteins. The variability in tensor element orientations is limited to a range of β_D^N angles of about 7° in the limited number of studies that have been performed so far. But even such limited variability can have a dramatic consequence on the interpretation of spectral data for structure and dynamics elucidation. Therefore, it is important to determine the tensor orientation for each site of interest in the polypeptide.

For the amide ^{13}C sites in a polypeptide backbone it is more difficult to minimize the number of variables that need to be determined. While it is possible to isolate the ^{15}N chemical shift tensor it is not so easy to isolate the ^{13}C chemical shift tensor in the polypeptide backbone because there are no readily available isotopes of nitrogen which do not have a spin. To isolate this interaction a triple resonance experiment would have to be conducted in which the nitrogen, either as ^{15}N or as ^{14}N (via overtone spectroscopy[38]) would have to be decoupled. Consequently, the tensor element magnitudes as well as their orientation are maintained as variables in the analysis of the ^{15}N coupled ^{13}C carbonyl powder patterns. It has, however, been possible to fix the N–C_1 bond length from our earlier work with the ^{13}C coupled ^{15}N powder patterns, thereby eliminating one of the variables. Furthermore, as was shown for the orientation of the ^{15}N chemical shift tensors in gramicidin,[3] analytical estimates of the azimuthal angle can be achieved for the ^{13}C chemical shift tensor directly from the dipolar coupled chemical shift spectra. These estimates substantially restrict the range of solutions for this set of variables and enhances the probability for achieving an accurate determination for the orientation and magnitude of the chemical shift

(38) Ramanathan, K. V.; Opella, S. J. *J. Magn. Reson.* **1988**, *78*, 367–370.

tensor elements. The L-Ala$_5$ gramicidin carbonyl ^{13}C site represents only the second such site to have its chemical shift characterized in detail. Previous work from this lab has shown that the tensor orientation for $^{13}C_1$-Gly$_2$ gramicidin A had the same zero value for α_D^c but a value for β_D^c that differed by 4° (38° for Ala$_5$ versus 34° for Gly$_2$[37]). Like ^{15}N the determined ^{13}C tensor orientations have placed σ_{cc}^c exactly (within the experimental error) perpendicular to the peptide plane. But in the plane with only two sites to compare, it is already possible to state that significant tensor orientation differences have been obtained in a macromolecule. These two values of β_D^c result in a 250-Hz difference in the dipolar splitting at σ_{aa}^c, a difference that is readily observed in the experimental data.

While there is good qualitative agreement between the α_D and β_D values determined in this study with respect to previous determinations, the difference with respect to the single-crystal study is significant. The only single-crystal study for a carbonyl carbon in a peptide linkage has a determined $\alpha_D^c = 0°$ and $\beta_D^c = 48°$.[10] Oas et al.[11] determined the ^{13}C carbonyl tensors for four dipeptides and observed a range of β_D^c from 34.5 to 46.6. While there is unanimous agreement among all studies to date that $\alpha_D^c = 0$, β_D^c appears to be variable.

The presence of the ^{13}C–^{14}N dipolar coupling has been well recognized in magic angle spinning spectra of carbon resonances covalently bonded to ^{14}N for many years.[39-41] However, throughout the literature of amide ^{13}C powder patterns the coupling to ^{14}N has been ignored, despite the unmistakable presence of telltale shoulders.[42-44] The shoulders mean that for these samples the ^{14}N dipolar interaction is not "self-decoupled" from the ^{13}C sites. Self-decoupling would result if rapid time-averaging of the ^{14}N spin states, due to a fast T_1 process, occurred.[36] This process was first experimentally observed in the ^{127}I coupling with ^1H in *trans*-diiodoethylene.[35] Sarkar et al.[42] claimed that self-decoupling occurred in an ^{14}N–^{13}C spin system in collagen, but the experimental spectra presented showed very clearly the expected shoulders for the dipolar interaction on the downfield side of the powder patterns. However, self-decoupling has been demonstrated in $^{13}C_1$-Gly$_2$ labeled gramicidin A, where the sample was prepared in a fully hydrated lipid bilayer.[37] The triplet splitting of more than 20 ppm was not observed, but rather a singlet with a full width at half height of 3.6 ppm was obtained.

Not only is the dipolar interaction present in the powder patterns shown here, but also the quadrupole interaction substantially perturbs the dipolar interaction at this field strength (4.7 T). The data obtained from the cyclopentapeptide which has particularly well-defined spectral discontinuities displays irrefutable evidence for the quadrupole effect on the ^{14}N–^{13}C dipolar interaction. Analysis of distortions in magic angle spinning spectra of ^{14}N coupled ^{13}C sites has predicted substantial quadrupole effects at field strengths up to 10 T.[45] Others have claimed that when the ^{14}N Larmor frequency is four times that of the quadrupole interaction (as it is in the present study) that the quadrupole effects on the ^{13}C spectra could be ignored.[5] Actually, it is the quadrupole effect that is to blame for the lack of recognition of the dipolar interaction by so many previous efforts. By collapsing two of the triplet components the dipolar shoulders on the powder patterns occur well down on the sides of the patterns and are frequently lost in the line broadening applied during data handling. At high fields where the quadrupole effect is less, the dipolar perturbation of the chemical shift powder pattern is relatively less, and again the interaction may go unnoticed. However, when spectra having the signal to noise and resolution such as that in Figure 6A is obtained, neither the dipolar interaction nor the quadrupole effect can be ignored.

The successful characterization of the EFG tensor here is consistent with some of the previous efforts. Stark et al.[20] showed that the Y_{efg} axis is parallel with the amide N–H bond in a single-crystal study of *N*-acetylvaline. Further studies of the ^{14}N EFG tensor in this molecule were obtained from residual dipolar interactions in ^{13}C magic angle spinning spectra.[21] This latter study concluded that the Z_{efg} was perpendicular to the peptide plane. While NQR studies confirm the Z_{efg} orientation for the amide sites in di- and triglycine, they have the Y_{efg} axis parallel to the C–N bond or approximately 30° from the orientation determined in previous and present NMR studies.[26] However, based on the cyclopentapeptide it is shown that the determination of α_Q cannot be made with great certainty, at least not at this field strength by this approach.

There is another difference between the chemical shift tensor results reported here and those in the previous literature. The magnitude of the tensor elements have primarily been obtained from ^{14}N coupled ^{13}C powder patterns in which the ^{14}N coupling has been ignored. Not only does the ^{14}N coupling effectively broaden the powder patterns and hence introduce an additional error in determining the magnitude of the tensor elements but also, because of the asymmetry in the dipolar coupling (i.e., in the triplet), a substantial distortion has been introduced into the pattern. It is clear from Figures 4D and 6B that if the chemical shift tensor was obtained from the experimental spectra without regard for the ^{14}N coupling that an error of 5–10 ppm could be made. The problem is especially acute for the determination of σ_{22} where the apparent value is shifted upfield with respect to the real value. But the problem is also substantial for σ_{11}, where the low intensity shoulder will significantly influence the determination of the tensor element magnitude. Another consequence is that the asymmetry of the previously reported carbonyl chemical shift tensors may have been substantially overestimated. These errors lead to a significantly greater error than has typically been reported (±3 ppm[42,44,46]) for tensor element determinations. An error of as little as ±1 ppm has been claimed in one study in which ^{14}N coupling was clearly present in the spectra but ignored.[43] However, not all carbonyl tensor element magnitudes have been determined from ^{14}N coupled systems. The efforts by Stark et al.,[10] Oas et al.,[11] and Hartzell et al.[2] utilized $^{13}C/^{15}N$ doubly labeled model compounds, and the analysis accounted for the spin $^1/_2$, spin $^1/_2$ dipolar interaction. The tensor magnitude (δ = 81 ppm) and asymmetry (η^{cs} = 0.82) determined here fall well within the range of these other determinations from doubly labeled compounds (magnitudes from 72 to 82 ppm and asymmetries from 0.70 to 0.97).

These errors have a very substantial impact on the calculation of chemical shifts from molecular models. Cornell and coworkers[44,46] observed the chemical shifts for numerous $^{13}C_1$ labeled sites in oriented preparations of gramicidin in hydrated lipid bilayers where there is rapid axial rotation about the channel axis. This group has predicted the chemical shift values for various molecular models based on an assumed tensor orientation and averaged tensor element magnitudes from ^{14}N coupled powder patterns. Both of these assumptions have a substantial impact on the predictions, in fact, for the $^{13}C_1$-Gly$_2$ site in gramicidin these assumptions have been shown to lead to a 14 ppm error in the prediction of the observed chemical shift frequency or a 21 ppm error in predicted motionally averaged anisotropy.[37]

Several conclusions need to be emphasized: first, accurate tensor orientations are required before high resolution structural and dynamical studies can be undertaken. Secondly, the magnitude of the tensor elements are equally important and are easily misread from the ^{14}N coupled data. Thirdly, if detailed tensor characterization is available, then very high resolution structural and

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dynamical data is available from chemical shift spectra of oriented samples. And fourthly, the determination in situ of the ^{14}N electric field gradient tensor opens up the possibility of using this nucleus for high resolution studies as pioneered by Opella and co-workers.[24]

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Ultraviolet Resonance Raman Study on the Binding Mode of Enkephalin to Phospholipid Membranes

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Abstract: Ultraviolet Resonance Raman spectra have been measured of Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) incorporated into phospholipid liposomes. The 213-nm Raman spectra in the amide I and III regions have shown that these opioid peptides take a folded conformation in the lipid-bound state. The environments of aromatic side chains of Tyr^1 and Phe^4 are monitored by the 240-nm Raman scattering intensities of the side chain vibrations. Tyr Raman bands generally gain intensity on going from aqueous solution to the liposome-bound state, indicating that the phenolic ring of Tyr is buried in the hydrophobic region of the lipid bilayer. On the other hand, Phe does not show intensity change, suggesting that the Phe side chain is exposed to the aqueous phase or located in the hydrophilic region of the bilayer. The insertion of the Tyr side chain into the membrane is deeper for Met-enkephalin than for Leu-enkephalin. Paralleling experiments on [Trp^4]Met-enkephalin (Tyr-Gly-Gly-Trp-Met) have shown that this peptide also takes a folded conformation in liposomes with the Tyr side chain inserted into the membrane hydrophobic interior. The Trp side chain is located in the hydrophilic region as in the case of Phe side chain of natural enkephalins. The degree of insertion of the Tyr side chain into membrane bilayers correlates with the receptor affinity and opiate activity of the peptide.

Introduction

The primary structure of enkephalin is Tyr-Gly-Gly-Phe-X, where X is Met (Met-enkephalin) or Leu (Leu-enkephalin). These endogenous peptides bind preferentially to δ-opioid receptors embedded in cell membranes and elicit morphine-like analgesic response.[1] The conformation of enkephalin has been studied extensively by using various experimental and theoretical methods, particularly in connection with the structure–activity relationships.[2] However, the active conformation of enkephalin in the receptor site has not been established yet. The difficulties in determining the active conformation mainly arise from the following reasons. First, the opioid receptors, which are membrane proteins, have not been isolated to such a high purity that spectroscopic or crystallographic studies can be made on their complexes with agonists. Second, enkephalin contains two Gly residues that make the pentapeptide highly flexible. The flexibility is exemplified by the conformational variety observed in solution and in the crystalline state.[2] Hence, the various conformers found in solutions and crystals or predicted by conformational energy calculations cannot be related immediately to the active conformer.

A catalytic role of cell membranes in the opiate-receptor binding process has been postulated to account for large apparent association constants between the opioid peptides and receptors.[3-5] According to the hypothesis, the polar lipid surface of the cell membrane attracts the amphiphilic opioid peptide, hydrophobic interaction facilitates the entry of the peptide into the membrane with concomitant conformational change to a specific structure suitable for binding to the receptor, and then the peptide binds to the receptor as a result of migration within the membrane. This proposed mechanism was employed to explain the correlation between the receptor subtype specificities of opioid peptides and their affinities to phospholipids.[6,7] A similar mechanism has been proposed for the binding of hormone peptides to their membrane-bound receptors.[8,9] In this connection, the interaction of enkephalin with lipid micelles or liposomes has been studied by NMR[3,10-14] and FT-IR[15] spectroscopy. The previous studies concluded that enkephalin takes a folded structure in membrane environments in contrast to an equilibrium of several unfolded conformers in aqueous solution. Currently, the mode of binding to membranes is argued in relation to the opiate activity.[14]

Ultraviolet resonance Raman (UVRR) spectroscopy is a useful tool to investigate the structures of peptides bound to lipid membranes because the Raman scattering from lipids is negligibly weak with UV excitation compared to the resonance-enhanced

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