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Carbon-13 Chemical Shielding Tensors in L-Threonine*

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The carbon-13 chemical shielding tensors of the amino acid L-threonine have been determined by solid state proton-enhanced NMR of a single crystal at 3.52 T. For the first time a carbinol shielding tensor is determined quantitatively and unambiguously: the most shielded axis lies 4° from the C–O bond. A coordinate system which approximates the alpha carbon tensor orientation is proposed, and it is suggested that this "local symmetry" may be general to many amino acid alpha carbons. The carboxyl and methyl tensor orientations closely approximate previously established "local symmetry," although the methyl (C^{γ}) C_{3v} symmetry is slightly disrupted, apparently by the neighboring (O^{γ}) oxygen. The influence of the carbon–nitrogen dipolar interaction on the observed spectra is discussed, and used to make unique assignments for the carboxyl, alpha, and beta carbon tensors.

INTRODUCTION

For some time now, there has been considerable interest in determining the static and dynamic structures of amino acids, polypeptides, and proteins by means of nuclear magnetic resonance spectroscopy—both in solution and in the crystalline solid state (*1–3*). In favorable cases, it is possible to determine inter alia the rates and types of motion of various chemical groups, bond lengths, bond angles, and the orientations of various residues (*1–4*). We have recently become interested in determining the details of the dynamic structure of a variety of amino acid side chains in membrane proteins—especially those of bacteriorhodopsin, the sole protein of the photosynthetic "purple" membrane of the obligate halophile, *Halobacterium halobium* R_1. We have used exclusively deuterium (^2H) NMR techniques to probe sidechain motions of a number of amino acids (*5–7*). Unfortunately, however, it appears in a number of instances that rather complex sidechain motions must occur, for example, in methionine (*6*) and in leucine (*8*). Thus, it is clearly desirable to develop methods which permit study of the motions of *each* atom in the molecule. We believe the most promising candidate technique is carbon-13 NMR spectroscopy, in which the natural abundance (1.1%) of ^{13}C may be used, at least in studies of model systems.

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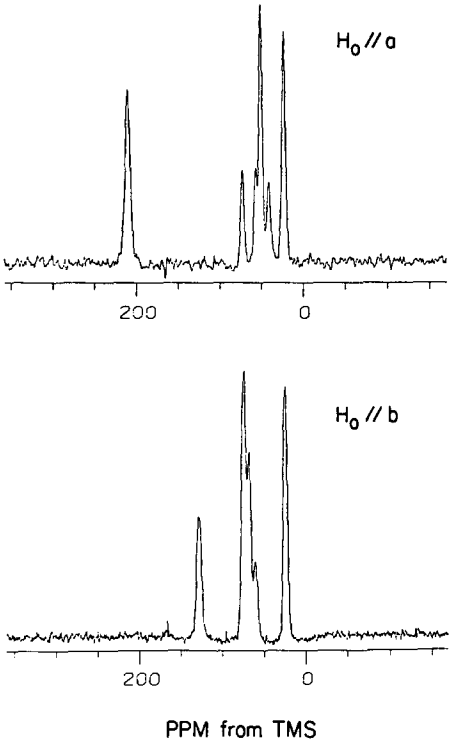


FIG. 1. Spectra showing magnetic equivalence when H_0 is parallel to the crystallographic axes, *a* and *b*, respectively.

For ^{2}H NMR, isotopic enrichment is almost always necessary for solid state studies, and production of multiply labeled amino acids is, in general, a complex procedure.

As is now well known, combination of so-called cross-polarization (*9*) and "magic-angle" sample spinning (*10*) techniques can yield high-resolution ^{13}C NMR spectra of solids. Unfortunately, of course, only the trace of the shielding tensor is obtained in such experiments. Lineshape analysis of static powder spectra (*9*), or liquid crystal experiments (*11*) can yield chemical shift tensor principal values and anisotropies, respectively, as can some two-dimensional techniques (*12*), but orientational information is lost. Only single crystal studies, therefore, yield the maximum possible information. Consequently, we have begun a program of study of the ^{13}C chemical shift tensors of a variety of single crystal amino acids. We are particularly interested in studying their temperature dependencies to permit analysis of the types of molecular motion occurring, and in addition we hope to be able to use two-dimensional NMR techniques (*13*) to determine ^{13}C–^{1}H dipolar interactions, and to investigate T_1^C and $T_{1\rho}^C$ relaxation processes (*14*), all to better understand details of amino acid dynamics in more complex systems, such as proteins. Understanding such processes in enzymes may enable us to better understand their modes of action.

In this first paper, we present results for the ^{13}C shielding tensors of the amino acid L-threonine. This work represents the first unambiguous and quantitative determination of a hydroxyl shielding tensor, and all tensor assignments (except for the

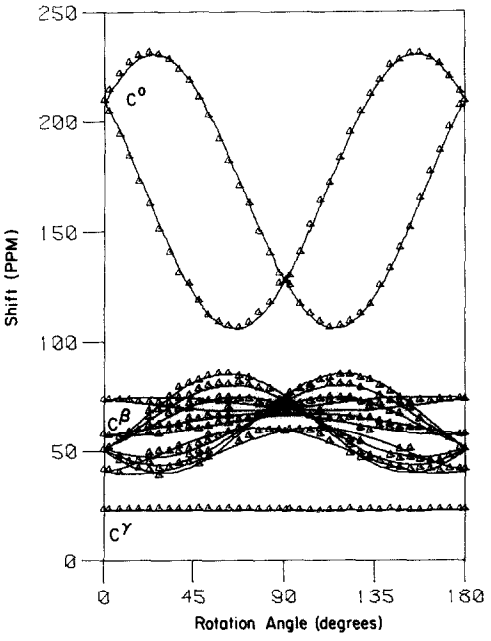
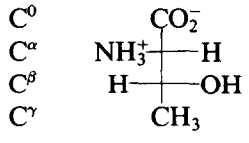


FIG. 2. Rotation pattern in the *ab* plane together with the best theoretical fit. The methyl off-diagonal element in the crystal axis system is nearly zero and the line is unsplit. Note the complicated patterns exhibited by the alpha and beta carbons due to the nitrogen dipolar interaction. Weak dipolar splitting of the carboxyl carbon was eliminated by exponential multiplication, and the average shift is shown.

methyl) are determined uniquely, without resorting to local symmetry arguments, which may of course be suspect for carbons with no obvious local symmetry—in this case the alpha and beta carbons. L-threonine has the following structure:



and was chosen for our initial study because it has a known crystal structure (*15, 16*) and for the prosaic reason that in preliminary screenings of all of the amino acids, we obtained the largest and most well formed crystals of L-threonine first. More detailed studies of other, more complex amino acids, are in progress, and will be reported separately.

EXPERIMENTAL

Spectroscopic aspects. All spectra were obtained at room temperature using the technique of proton enhanced nuclear induction spectroscopy (*9*). The "home-built" double resonance spectrometer (37.8 MHz for carbon-13) described previously (*17*) was used, but it is now equipped with a Nicolet 1280 computer system. We also used a "home-built" double resonance probe equipped with a goniometer, similar to those described previously by others (*18, 19*). Cross polarization was established through

TABLE 1

L-THREONINE CARBOXYL ^{13}C CHEMICAL SHIELDING TENSOR PRINCIPAL VALUES AND DIRECTION COSINES WITH RESPECT TO LOCAL SYMMETRY FOR SITE 1 IN THE UNIT CELL[a]

	Principal values[b,c]	Direction cosines[d]	Reference local symmetry[d]	
σ_{33}	240.2 (0.6)	−0.8442	−0.8630	Along C^{α}–C^{0} bond
		−0.4047	−0.3952	
		+0.3513	+0.3147	
σ_{22}	164.7 (0.9)	−0.3627	−0.3345	In sp^2 plane
		−0.0511	−0.0199	perpendicular to
		−0.9305	−0.9422	C^{α}–C^{0} bond
σ_{11}	105.0 (0.2)	+0.3946	+0.3838	Perpendicular to
		−0.9129	−0.9159	sp^2 plane
		−0.1037	−0.1169	

Deviation from local symmetry[c]

σ_{33}	2.5° (0.9)	C^{α}–C^{0} bond direction
σ_{22}	2.5° (1.1)	In sp^2 plane, perpendicular to C^{α}–C^{0} bond
σ_{11}	1.4° (0.5)	Perpendicular to sp^2 plane

[a] Results shown reflect an average over four sites but are presented in terms of site 1; other sites are related by the symmetry properties referred to in the text.

[b] In ppm from TMS.

[c] Values in parentheses are standard deviations of the four sites with respect to the average.

[d] With respect to the *a*, *b*, *c* crystallographic axes.

a matched Hartmann–Hahn condition (*20*) at radiofrequency field strengths of 40 G for ^{13}C and 10 G for ^{1}H. A single contact of 750 μsec was employed, using a 2 sec recycle time. Phase alternation was used throughout to eliminate baseline and intensity artifacts. The crystal was aligned optically, according to crystal morphology and considerations of magnetic equivalence along the crystallographic axes.

Crystal structure and symmetry considerations. Single crystals of L-threonine (free base) were grown from an aqueous solution at room temperature by slow solvent evaporation. The crystals belonged to the orthorhombic space group, $P_{2_12_12_1}$. The unit cell dimensions are (*15, 16*) a = 13.630, b = 7.753, and c = 5.162 Å. There are four magnetically inequivalent molecules per unit cell, related pairwise by three mutually perpendicular screw axes. In any crystallographic plane the molecules become pairwise equivalent, since the screw axes become effective mirror planes, thereby reducing the number of magnetically inequivalent sites from four to two. When the static field is parallel to a crystallographic axis—at the juncture of two effective mirror planes simultaneously—all sites are magnetically equivalent. The crystal was thus rotated through 180° in the three perpendicular crystallographic planes, and spectra recorded at 5° intervals, to facilitate the resolution of the alpha and beta carbons. The isotropic shifts of these carbons are only 5 ppm apart, and spectra are further complicated by the nitrogen dipolar interaction, which splits the resonance of C^{α} into a triplet. This yields a possible 12 lines per plane for the alpha and beta carbons alone.

TABLE 2

L-THREONINE HYDROXYL (BETA) CARBON ^{13}C CHEMICAL SHIELDING TENSOR PRINCIPAL VALUES AND DIRECTION COSINES WITH RESPECT TO LOCAL SYMMETRY FOR SITE 1 IN THE UNIT CELL[a]

	Principal values[b,c]	Direction cosines[d]	Reference local symmetry[d]	
σ_{11}	83.4 (0.2)	+0.1860		
		−0.7859		
		−0.5897		
σ_{22}	74.1 (0.4)	−0.5428		
		+0.4181		
		−0.7284		
σ_{33}	38.8 (0.2)	+0.8190	+0.7802	Along C^{β}–O bond
		+0.4556	+0.5166	
		−0.3488	−0.3527	

ANGULAR RELATIONSHIP BETWEEN PRINCIPAL AXIS SYSTEM AND BOND DIRECTIONS

Tensor component	Angle[c]	Bond direction
σ_{11}	30.9° (1.0)	C^{β}–H
σ_{22}	18.9° (0.7)	C^{β}–C^{γ}
σ_{33}	4.2° (0.2)	C^{β}–O

[a] Results shown reflect an average over four sites, but are presented in terms of site 1; other sites are related by the symmetry properties referred to in the text.

[b] In ppm from TMS.

[c] Values in parentheses are standard deviations of the four sites with respect to the average.

[d] With respect to the *a*, *b*, *c* crystallographic axes.

Spectral analysis. Using first order perturbation theory the observed shielding can be related to the symmetrical second rank tensor (*21*) in the laboratory or crystallographic axis system by the unit vector **k** as follows,

$$\sigma_{obs} = \mathbf{k} \cdot \sigma_{lab} \cdot \mathbf{k} \qquad [1]$$

where **k** represents the crystal orientation with respect to the static field, and is expressed in spherical coordinates (*22*). Diagonalization of the tensor in the laboratory axis system yields the three principal values of the chemical shielding tensor and the corresponding direction cosines, which contain orientational information. In practice, a variety of factors complicate our analysis. Primarily, the presence of the spin $I = 1$ nucleus nitrogen-14 causes additional line splittings (*23*). Since the nitrogen-14 nucleus has a quadrupole moment, there is an orientational dependent mixing of the nitrogen Zeeman states. If the electric quadrupole interaction is of the order of the nitrogen Zeeman interaction, there is a significant admixture of the nitrogen Zeeman states. In the high field limit, carbon resonances near to nitrogen-14 nuclei are split into a symmetric triplet. At 3.5 T, however, the electric quadrupole interaction is roughly one tenth of the nitrogen Zeeman interaction, and in general a slightly asymmetric triplet results ($e^2Qq = \pm 1.158$ MHz (*24*), $\gamma_N \hbar H_0 = 10.86$ MHz).

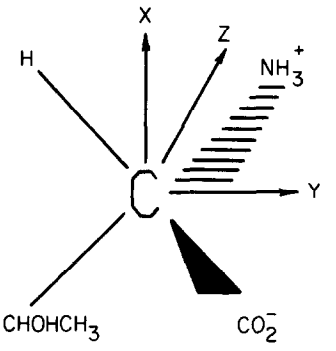


FIG. 3. Coordinate system used to reference the alpha carbon tensor orientation. *Y* bisects the NC$^{\alpha}$C° angle. *X* is perpendicular to this plane. *Z* is perpendicular to *X* and *Y*.

Assuming axial symmetry of the nitrogen electric field gradient, the analysis as carried out in Ref. (*25*) shows a small shift of up to 2 ppm due to the admixture of nitrogen Zeeman states. This assumption should introduce only several tenths of a ppm uncertainty for threonine (^{14}N η = 0.36, Ref. (*24*)) in light of data for glycine, where despite a large asymmetry, η = 0.54, V_{zz} is only 5° from the C–N bond (*4*). Other effects which contribute to the observed shieldings and are more difficult to assess quantitatively include intermolecular shielding and susceptibility effects, due to crystal size and shape, as well as goniometer geometry. An uncertainty of about 0.5 ppm may be due to the former, based on estimates of 0.8 ppm in the aromatic species ferrocene (*26*), while an absolute uncertainty of about 1 ppm is likely due to the latter as estimated from Osborn's calculations (*27, 28*) concerning the demagnetizing field of an ellipsoid. In view of these considerations, an absolute uncertainty of $\pm(1.5 + \delta)$ is assigned to the tensor values, where δ is the standard deviation of the four tensors (corresponding to the four sites in the unit cell) from the average value. The results in this publication are presented in terms of molecule I in the unit cell, with direction cosines (l, m, n). The other three sites can be obtained by symmetry transformation of the direction cosines as follows: ($-l$, $-m$, n), (l, $-m$, $-n$), ($-l$, m, $-n$).

Uniqueness. The dipolar interaction briefly discussed above is crucial to unique tensor assignments in L-threonine. In most previous studies, "commonsense" local symmetry arguments have been used to make assignments. However, in L-threonine neither the alpha nor the beta carbons exhibit any obvious local symmetry. In the absence of the dipolar interaction, the observed chemical shift in the ij plane in the crystal axis system is (*29*)

$$\sigma_{obs}(\theta) = \sigma_{ii} \cos^2 \theta + 2\sigma_{ij} \sin \theta \cos \theta + \sigma_{jj} \sin^2 \theta. \quad [2]$$

Along a crystallographic plane in a $P_{2_12_12_1}$ crystal, two magnetically inequivalent sites lead to two resonance lines in the rotation pattern. The diagonal elements of the two tensors are identical, while the off-diagonal elements differ in sign. More concretely, in the ij plane there are two resonances per functionality designated *A* and *B*. We need to know if the source of line *A* in the ij plane is the source of line *A* or *B* in the jk or ik plane. For three planes with two magnetically inequivalent

TABLE 3

L-THREONINE ALPHA CARBON ^{13}C CHEMICAL SHIELDING TENSOR PRINCIPAL VALUES AND DIRECTION COSINES WITH RESPECT TO THE COORDINATE SYSTEM SHOWN IN FIG. 1[a]

	Principal values[b,c]	Direction cosines[d]	Reference local symmetry[d]	
σ_{33}	69.0 (0.4)	−0.0194	−0.1999	
		+0.8503	+0.8392	*X*
		+0.5260	+0.5057	
σ_{22}	58.9 (0.4)	−0.8559	−0.8754	
		+0.2743	+0.0790	*Y*
		−0.4384	−0.4771	
σ_{11}	52.6 (0.3)	−0.5172	−0.4403	
		−0.4484	−0.5380	*Z*
		+0.7290	+0.7188	

ANGULAR RELATIONSHIP BETWEEN PRINCIPAL AXES AND LOCAL SYMMETRY FOR L-THREONINE, L-ALANINE (*18*), AND GLYCINE (*4*)

Degree of shielding	Local symmetry	L-Thr[c]	L-Ala	Gly
Least	*X*	10.4° (0.7)	7.7°	15°
Intermediate	*Y*	11.4° (2.2)	10.7°	10°
Most	*Z*	6.8° (1.5)	13.0°	11°

[a] Results shown reflect an average over four sites, but are presented in terms of site 1; other sites are related by the symmetry properties referred to in the text.

[b] In ppm from TMS.

[c] Values in parentheses are standard deviations of the four sites with respect to the average.

[d] With respect to the *a*, *b*, *c* crystallographic axes.

sites per plane there are eight (2^3) possible combinations. Four combinations are correct and identical within experimental error (after accounting for the symmetry relations between the sites). Four combinations are artifacts and identical in the same manner. All eight traces are equal. The principal values do differ so that, in principle, the correct set of tensors could be obtained from a lineshape analysis of a powder pattern. Once the correct set of tensors is chosen, however, ambiguity remains. The four correct tensors must be assigned to specific sites within the unit cell. These assignments can fortunately be made unambiguously for nuclei close to nitrogen. The orientational dependence of the dipolar interaction causes the two otherwise magnetically equivalent sites to become, in general, magnetically nonequivalent, and thus indistinguishable.

In the case of the methyl carbon, the dipolar splitting was unfortunately too small for such an analysis. By chance, however, the off-diagonal element in the *AB* plane was nearly zero and only one line was observed in the rotation pattern. Four combinations ($1 \times 2 \times 2$) were possible—all were correct and local symmetry arguments were used to assign each tensor to a particular site.

TABLE 4

L-THREONINE METHYL ^{13}C CHEMICAL SHIELDING TENSOR PRINCIPAL VALUES AND DIRECTION COSINES WITH RESPECT TO LOCAL SYMMETRY FOR SITE 1 IN THE UNIT CELL[a]

	Principal values[b,c]	Direction cosines[d]	Reference local symmetry[d]	
σ_{11}	32.1 (0.5)	−0.2551		
		+0.7990		
		+0.5446		
σ_{22}	23.1 (0.2)	−0.9533		
		−0.3016		
		+0.0165		
σ_{33}	1.4 (0.3)	−0.1608	−0.2412	Along C^{β}–C^{γ} bond
		+0.5200	+0.4797	
		−0.8389	−0.8436	

Deviation from local symmetry: σ_{33} 5.2° (1.1)

Departure from local symmetry	L-Threonine (*16*)	Acetic acid (*33*)
	Bond lengths (Å)	
CH^1	1.075	1.050
CH^2	1.097	1.052
CH^3	1.113	1.078
	Proton–oxygen close approach (Å)	
H^1	3.35	—
H^2	2.61	—
H^3	2.74	2.41

[a] Results shown reflect an average over four sites, but are presented in terms of site 1; other sites are related by the symmetry properties referred to in the text.

[b] In ppm from TMS.

[c] Values in parentheses are standard deviations of the four sites with respect to the average.

[d] With respect to *a*, *b*, *c* crystallographic axes.

RESULTS AND DISCUSSION

Typical CP single crystal spectra and rotation plots for L-threonine are shown in Figs. 1 and 2. The principal values of the carboxyl tensor are similar to those of glycine (*4*) and L-alanine (*18*); the major difference is the magnitude of σ_{22}, which is 15–20 ppm more shielded, further from axial symmetry. As a result, the solid state isotropic shift is slightly more shielded as well. The tensor orientation reflects quite closely the carboxyl local symmetry, the principal axes differing from the local symmetry by less than 3°. A summary of the results is given in Table 1.

The carbinol (beta carbon) shielding tensor has a small asymmetry, $\eta = 0.35$. The principal values are deshielded with respect to the principal values of the ethanol hydroxyl carbon (*11*), but more shielded than the principal values of the ammonium

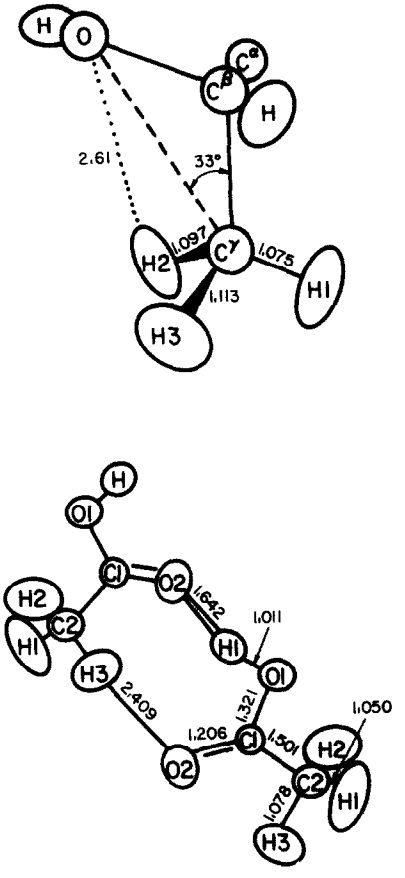


FIG. 4. Comparison of the geometries of L-threonine and acetic acid, showing the oxygen–methyl carbon close approach. The alpha carbon substituents of L-threonine are omitted for simplicity.

D-tartarate hydroxyl carbon (*30*). The difference is greatest for the most shielded value, whereas the less shielded values are all similar. The strong variation of the most shielded value among various systems is reflected in the tensor anisotropies, $\Delta\sigma = \sigma_{33} - 1/2(\sigma_{11} + \sigma_{22})$. The anisotropy for the L-threonine hydroxyl carbon tensor (−40 ppm) is smaller than that for ethanol (−57 ppm), but larger than that of ammonium D-tartarate (−29 ppm). The only previous study of a hydroxyl carbon shielding tensor orientation (*30*) involved assumptions concerning local pseudosymmetry and concluded that the most shielded axis was roughly coincident with the C–O bond direction. Our unambiguous results reinforce that conclusion. The most shielded axis is tilted away from the C–O bond direction by only 4° in L-threonine. The results are summarized in Table 2. The tensor exhibits no sign of motional averaging due to rotation about the C^{α}–C^{β} bond, suggesting immobility on the millisecond timescale.

The alpha carbon exhibits no obvious local symmetry; therefore, it is essential that the tensor determination be made uniquely. The tensor is much more isotropic than found in glycine (*4*) or L-alanine (*18*). The difference between the most and least

shielded values is only about 16 ppm, compared to 40 ppm for glycine and 34 ppm for L-alanine. The orientation of the principal values does not correspond to any bond direction; rather, there is a correspondence with the typical methylene symmetry exhibited by the alpha carbon of glycine (*4*) and depicted in Fig. 3. Manipulation of the data for L-alanine (*18*) using neutron diffraction data (*31*) reveals a similar correspondence, suggesting that this symmetry may be general for many amino acid alpha carbons. The results are presented in Table 3. The sign of the quadrupole coupling constant cannot be determined in nuclear quadrupole resonance or nitrogen NMR, but can be determined in ^{13}C NMR. Analysis of the carbon–nitrogen dipolar interaction shows a positive quadrupole coupling constant (*18*).

The methyl carbon shielding tensor is axially asymmetric, but the asymmetry is not large. The tensor orientation, principal values and solid state isotropic shifts are similar to those of L-alanine (*18*) and *p*-xylene (*32*). The most shielded axis is tilted away from the rotor axis by 5°, directly towards the neighboring oxygen. Disruption of the C_{3v} symmetry of the methyl rotor can apparently be traced to this oxygen as well. The two methyl protons staggered about the oxygen exhibit elongated bond lengths (*33*) and exhibit a close approach of 2.6 and 2.7 Å, as shown in Table 4.

Disruption of methyl C_{3v} symmetry by a nearby oxygen has previously been implicated as a cause of the anomalous methyl carbon shielding tensor of acetic acid (*34, 35*), where the most shielded axis is tilted 19° away from the rotor axis. Unlike the L-threonine case, this effect is intermolecular, there being a close (2.4 Å) approach between a nearby carboxyl oxygen and a methyl proton, apparently causing the carbon–proton bond to become elongated. The tensor's most shielded axis is tilted directly toward that elongated bond. The geometries of these two systems are depicted in Fig. 4. The methyl–proton bond lengths are more varied in L-threonine, as shown in Table 4, yet the methyl tensor is rather typical. The precise origins of these differences are unclear at present, and further studies will be required to more fully analyze them.

In the amino acids, such effects may to some extent be controlled by crystallizing different amino acid forms—racemic D, L mixtures, free base (zwitterionic) or salt (e.g., hydrochloride) forms, and so on. We are currently carrying out such studies on a variety of systems to try to determine more precisely the extent to which inter- and intramolecular interactions may affect observed chemical shift anisotropies.

CONCLUSION

We have determined all carbon-13 chemical shielding tensors in the L-threonine molecule, which, because of the presence of small isotropic chemical shift separations (for C^{α}, C^{β}) and the presence of ^{13}C–^{14}N dipolar interactions, represents one of the most complex species to be investigated to date using ^{13}C NMR spectroscopy. Nevertheless, because our results were obtained at relatively low field (3.52 T), there is every reason to believe that at high field ($\geq$8.45 T) all of the naturally occurring amino acids may be investigated using such methods. When combined with temperature dependence and two-dimensional NMR studies (Ref. (*13*) and work in progress) it should thus be possible to describe in considerable detail the dynamic structure of any amino acid sidechain.

In L-threonine, our results indicate that the hydroxymethyl group can only undergo very slow rotational motion about C^{α}–C^{β} (at 23°C), since the tensor has a breadth

(≈1700 Hz) and orientation consistent with it being immobile on the millisecond timescale. A similar conclusion was drawn, but on a much shorter timescale, from deuterium NMR (*36*). These results strongly suggest the presence of strong hydrogen-bond networks in the crystalline amino acid, consistent with the reported diffraction structure determinations (*15, 16*).

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