How Tetrahedral Are Methyl Groups in Proteins? A Liquid Crystal NMR Study

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Abstract: A small degree of protein alignment with an external magnetic field can be obtained in a dilute aqueous liquid crystalline solution of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC). It is demonstrated that residual one-bond $^{13}C-^{13}C$ and $^{13}C-^{1}H$ dipolar couplings of methyl groups in weakly aligned human ubiquitin can be measured with high accuracy. Experimentally, the ratio between $^{13}C-^{1}H$ and $^{13}C-^{13}C$ dipolar couplings is found to be -3.17 ± 0.03. Assuming a static conformation of the methyl group, rapidly spinning about its 3-fold symmetry axis, this ratio corresponds to an average C−C−H bond angle of $110.9 \pm 1°$, which is larger than the ideal tetrahedral value of 109.5°. Data indicate that the geometry of the various methyl groups is quite uniform, but that small ($\leq 1°$) deviations between the C−C vector and the axis connecting the methyl carbon to the geometric center of the three methyl protons may occur. The largest outlier is found for Ala[46], which has a positive ϕ backbone angle, causing its methyl group to be within van der Waals contact of the preceding carbonyl oxygen.

Introduction

Methyl groups commonly are assumed to adopt an ideal tetrahedral geometry, with H−C−H and C−C−H bond angles of 109.5°. However, small deviations from such idealized tetrahedral geometry previously were found on the basis of NMR dipolar coupling measurements of small molecules dissolved in nematic liquid crystals,[1−4] and single-crystal neutron diffraction studies of alanine and valine.[5,6] Neutron diffraction for these two amino acids yielded C−C−H angles of 110.0° and 111.9°, respectively. Small molecule liquid crystal NMR cannot determine the angle directly, unless an assumption about bond length is made. However, the ratio of the one-bond $^{1}D_{CH}$ and two-bond $^{2}D_{HH}$ dipolar couplings is strongly correlated with this angle. This ratio ranges from 0.68 in acetaldehyde[1] to 0.88 in CH_3I.[3] This is in qualitative agreement with results from gas-phase rotational spectroscopy, which yields the ideal C−C−H angle of 109.5° for the methyl group of acetaldehyde (but a very short C−H bond length of 1.073 ± 0.002 Å)[7] and 113.0° for CH_3I ($r_{CH} = 1.085$ Å).[8] Thus, these results indicate that substantial variation in methyl group geometry can indeed occur, depending on the substituent.

The issue of methyl group geometry recently has regained new interest[9−11] because ^{13}C methyl group NMR relaxation parameters present a potentially powerful probe for the study of side-chain mobility in proteins.[9,10,12−17] Relaxation of such ^{13}C signals is dominated by the dipolar interaction with the methyl protons, but fast rotation about the methyl group's C_3 symmetry axis reduces the effective dipolar couplings by $P_2(\cos\beta)$, with $P_2(x) = (3x^2 - 1)/2$, and β being the C−C−H angle. Similarly, when studying methyl group dynamics using deuterium NMR, after averaging over the 3-fold rotation the effective quadrupole coupling scales with $P_2(\cos\beta)$. In the vicinity of $\beta = 109.5°$, $P_2(\cos\beta)$ is a particularly steep function of β, and accurate knowledge of β is therefore essential for making quantitative interpretation of such relaxation measurements. Substantial differences, on the order of 30−40% between the order parameters observed for Ala $C^{\alpha}-H^{\alpha}$ and $C^{\beta}-H^{\beta}{}_3$ in the protein staphylococcal nuclease,[9] for example, potentially

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(1) Emsley, J. W.; Lindon, J. C.; Tabony, J. *J. Chem. Soc., Faraday Trans. 2* **1975**, *71*, 586−595.
(2) Khetrapal, C. L.; Saupe, A. *Mol. Cryst. Liq. Cryst.* **1973**, *19*, 367−381
(3) Wooton, J. B.; Savitsky, G. B.; Jacobus, J.; Beyerlein, A. L. *J. Chem. Phys.* **1977**, *66*, 4226−4230. Wooton, J. B.; Savitsky, G. B.; Jacobus, J.; Beyerlein, A. L.; Emsley, J. W. *J. Chem. Phys.* **1979**, *70*, 438−442.
(4) Emsley, J. W.; Longeri, M.; Veracini, C. A.; Catalone, D.; Pedulli, G. F. *J. Chem. Soc., Perkin Trans. 2* **1982**, 1289−1296.
(5) Lehnmann, M. S.; Koetzle, T. F.; Hamilton, W. C. *J. Am. Chem. Soc.* **1972**, *94*, 2657−2660.
(6) Koetzle, T. F.; Golic, L.; Lehnmann, M. S.; Verbist, J. J.; Hamilton, W. C. *J. Chem. Phys.* **1974**, *60*, 4690−4696.
(7) Duncan, J. L. *J. Mol. Struct.* **1970**, *6*, 447−459.
(8) Iijima, T.; Kimura, M. *Bull. Chem. Soc. Jpn.* **1969**, *42*, 2159−2164. Iijima, T.; Tsuchiva, S. *J. Mol. Spectrosc.* **1972**, *44*, 88−107.
(9) Nicholson, L. K.; Kay, L. E.; Torchia, D. A. In *NMR Spectroscopy and its Application to Biomedical Research*; Sarkar, S. K., Ed.; Elsevier: New York, 1996; pp 241−279.
(10) Wand, A. J.; Urbauer, J. L.; McEvoy, R. P.; Bieber, R. J. *Biochemistry* **1996**, *35*, 6116−6125.
(11) Chatfield, D. C.; Szabo, A.; Brooks, B. R. *J. Am. Chem. Soc.* **1998**, *120*, 5301−5311.
(12) Jones, W. C.; Rothgeb, T. M.; Gurd, F. R. N. *J. Biol. Chem.* **1976**, *251*, 7452−7460. Richarz, R.; Nagayama, K.; Wüthrich, K. *Biochemistry* **1980**, *19*, 5189−5196.
(13) Nicholson, L. K.; Kay, L. E.; Baldisseri, D. M.; Arango, J.; Young, P. E.; Bax, A. ; Torchia, D. A. **1992**, *31*, 5253−5263.
(14) Muhandiram, D. R.; Yamazaki, T.; Sykes, B. D.; Kay, L. E. *J. Am. Chem. Soc.* **1995**, *117*, 11536−11544. Yang, D. W.; Mok, Y. K.; Forman-Kay, J. D.; Farrow, N. A.; Kay, L. E. *J. Mol. Biol.* **1997**, *272*, 790−804. Yang, D. W.; Mittermaier, A.; Mok, Y. K.; Kay, L. E. *J. Mol. Biol*. **1998**, *276*, 939−954.
(15) LeMaster, D. M.; Kushlan, D. M. *J. Am. Chem. Soc.* **1996**, *118*, 9255−9264.
(16) Straus, S. K.; Bremi, T.; Ernst, R. R. *J. Biomol. NMR* **1997**, *10*, 119−128.
(17) Lee, A. L.; Urbauer, J. L.; Wand, A. J. *J. Biomol. NMR* **1997**, *9*, 437−440.

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could be explained by increasing the C−C−H angle by only 3° from its ideal value. However, ab initio calculations on individual amino acids or molecular dynamics calculations on the intact protein yield rather uniform C−C−H angles of ca. 110.5°,[11] suggesting that other factors must play a role too.

Recently, it has become possible to measure dipolar couplings in macromolecules aligned with the magnetic field in a dilute lyotropic liquid crystalline phase of phospholipid particles,[18] known as bicelles.[19] Alternatively, such alignment now can also be obtained in media containing filamentous phages,[20] purple membrane fragments,[21] or a dilute lamellar phase of nonlipid molecules.[22] In such media, it is possible to accurately measure one- and two-bond dipolar interactions in isotopically enriched proteins.[18,20−22] We have shown that comparison of the one bond $^{13}C-^{1}H$, $^{13}C-^{15}N$, $^{15}N-^{1}H^{N}$, and $^{13}C-^{13}C$ dipolar interactions in the protein ubiquitin yields information on the ratios of the effective bond lengths, corrected for rapid angular fluctuations.[23] The ratio for the r_{CN} and r_{CC} bond lengths was found to be in excellent agreement with results from a high-resolution crystal structure database,[24] but the effective r_{CH} and r_{NH} bond lengths were about 3% larger than their equilibrium values, when using r_{CN} and r_{CC} as a reference. This latter result is not unexpected because it is known that r_{NH} and r_{CH} undergo larger angular librations than r_{CN} and r_{CC},[25] resulting in a decrease in dipolar coupling and thereby in the increased effective bond lengths of 1.041 (r_{NH}) and 1.117 Å (r_{CH}).[23]

Here, we demonstrate that it is possible to accurately measure both the $^{13}C-^{1}H$ and $^{13}C-^{13}C$ one-bond dipolar couplings for most methyl groups in ubiquitin. Assuming that r_{CH} is uniform, our results indicate that there is very little variation in the C−C−H angle. If a librationally corrected effective bond length of 1.117 Å is used, the average C−C−H angle is found to be 110.9 ± 0.2 °. There is some indication that either the C_3 axis can make a small angle with the C−C bond or that the three methyl protons do not span an exactly equilateral triangle. This latter finding is consistent with small deviations from axial symmetry observed for ^{2}H powder patterns of certain methyl groups in solids.[26]

Experimental Section

An aqueous liquid crystalline sample containing uniformly $^{13}C/^{15}N$-enriched ubiquitin (VLI Research, Southeastern, PA) was prepared as described previously.[27] Bicelles consisted of a mixture of dimyristoylphosphatidylcholine (DMPC), dihexanoylphosphatidylcholine (DHPC), and cetyltrimethylammonium bromide (CTAB).[28] Final sample conditions were: 0.7 mM protein, 5% (w/w) DMPC/DHPC/CTAB in a molar ratio of 30:10:1 in water, 10 mM phosphate buffer, pH 6.6, 7% D_2O.

NMR spectra were recorded on a Bruker DMX750 spectrometer operating at a ^{1}H resonance frequency of 750 MHz equipped with a triple resonance, three-axis pulsed-field-gradient probehead. Data sets in the aligned state were recorded at 35 °C; isotropic spectra were recorded at 22 °C. Spectra were processed using the NMRPipe software package.[29]

Residual $^{1}D_{CH}$ dipolar couplings were derived from the difference of the modulation frequencies in the aligned and the isotropic states of 3D *J*-modulated constant-time [^{13}C,^{1}H] HSQC spectra[30] which were recorded as data matrices of 224* × 16 × 1024* points (*n** denotes *n* complex points), with acquisition times of 28 (t_1, ^{13}C), 28 (t_2, $^{1}J_{CH}$), and 57 ms (t_3, ^{1}H). The modulation frequencies were obtained by time-domain fitting in the t_2 constant-time dimension, as described previously.[30]

$^{1}D_{CC}$ values were derived from the difference in one-bond $^{13}C-^{13}C$ *J* splittings in the aligned and the isotropic states measured for the methyl group resonances in the 2D [^{13}C,^{1}H] HSQC spectra. These spectra were recorded as data matrices of 460* × 1024* complex points, with acquisition times of 92 (t_1, ^{13}C), and 57.0 ms (t_2, ^{1}H). Acquired data were apodized with a squared sine bell in the directly and a sine bell in the indirectly detected dimension, both shifted by 72° and truncated at 176°. Data were extensively zero-filled prior to Fourier transformation to yield high digital resolution. Peak positions were determined by contour averaging using the program PIPP,[31] as described previously.[32] In both the aligned and the isotropic state two 2D [^{13}C,^{1}H] HSQC spectra were recorded, one right before the 3D *J*-modulated constant-time [^{13}C,^{1}H] HSQC and one immediately after it. For all further analysis, averaged values were used.

The effect of temperature changes on the magnitude of the molecular alignment tensor is small (about 0.3% per °C).[27] Nevertheless, special care was taken to keep the temperature and sample conditions constant during acquisition of the experiments: (a) by recording all spectra in the aligned and the isotropic state, respectively, in one series without interruption, (b) by applying equal decoupling power within and between the experiments, requiring the insertion of dummy decoupling periods prior to the relaxation delay,[33] (c) by keeping the recycling periods long and constant (about 1.6 s), (d) by inclusion of an initial 5 min of dummy scans at the start of each experiment, and (e) by enclosing the 3D *J*-modulated CT-HSQC in between two 2D HSQC spectra which serve to simultaneously assess stability of the liquid crystal and to obtain an estimate for the random error in the measurement of $^{1}J_{CC}$ values.

Results and Discussion

Theoretical Background. The dipolar coupling between two nuclei, A and B, in a solute macromolecule of fixed shape is described by

$$D_{AB}(\theta,\phi) = D^{a}_{AB}\{(3\cos^2\theta - 1) + {}^{3}/_{2}\, R\,(\sin^2\theta\cos 2\phi)\} \tag{1a}$$

with

$$D^{a}_{AB} = -(\mu_o h/16\pi^3)\,\gamma_A\gamma_B\, r_{AB}^{-3}\, A_a \tag{1b}$$

where R is the rhombicity defined by D^{r}_{AB}/D^{a}_{AB}; D^{a}_{AB} and D^{r}_{AB} (in units of hertz) are the axial and rhombic components of the

(18) Bax, A.; Tjandra, N. *J. Biomol. NMR* **1997**, *10*, 289−292. Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114. Wang, Y.-X.; Marquardt, J. L.; Wingfield, P.; Stahl, S. J.; Lee-Huang, S.; Torchia, D. A.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 7385−7386. Bewley, C. A.; Gustafson, K. R.; Boyd, M. R.; Covell, D. G.; Bax, A.; Clore, G. M.; Gronenborn, A. M. *Nat. Struct. Biol.* **1998**, *5*, 571−578. Cornilescu, G.; Marquardt, J. L.; Ottiger, M.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 6836−6837.

(19) Sanders, C. R.; Schwonek, J. P. *Biochemistry* **1992**, *31*, 8898−8905. Sanders, C. R.; Landis, G. C. *Biochemistry* **1995**, *34*, 4030−4040. Ram, P.; Prestegard, J. H. *Biochim. Biophys. Acta* **1988**, *940*, 289−294. Sanders, C. R.; Prestegard, J. H. *Biophys. J.* **1990**, *58*, 447−460.

(20) Clore, G. M.; Starich, M. R.; Gronenborn, A. M. *J. Am. Chem. Soc.* **1998**, *120*, 10571−10572. Hansen, M. R.; Rance, M.; Pardi, A. *J. Am. Chem. Soc.* **1998**, *120*, 11210−11211.

(21) Koenig, B. W.; Hu, J.-S.; Ottiger, M.; Bose, S.; Hendler, R. W.; Bax, A. *J. Am. Chem. Soc.* **1999**, *121*, 1385−1386.

(22) Prosser, R. S.; Losonczi, J. A.; Shiyanovskaya, I. V. *J. Am. Chem. Soc.* **1998**, *120*, 11010−11011.

(23) Ottiger, M.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 12334−12341.

(24) Engh, R. A.; Huber, R. *Acta Crystallogr.* **1991**, *A47*, 392−400.

(25) Henry, E. R.; Szabo, A. *J. Chem. Phys.* **1985**, *82*, 4753−4761.

(26) Schwartz, L. J.; Meirovitch, E.; Ripmeester, J. A.; Freed, J. H. *J. Phys. Chem.* **1983**, *87*, 4453-4461. Hiyama, Y.; Roy, S.; Guo, K.; Butler, L. G.; Torchia, D. A. *J. Am. Chem. Soc.* **1987**, *109*, 2525−2526.

(27) Ottiger, M.; Bax, A. *J. Biomol.* NMR **1998**, *12*, 361−372.

(28) Losonczi, J. A.; Prestegard, J. H. *J. Biomol. NMR* **1998**, *12*, 447−451.

(29) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277−293.

(30) Ottiger, M.; Delaglio, F.; Marquardt, J. L.; Tjandra, N.; Bax, A. *J. Magn. Reson.* **1998**, *134*, 365−369.

(31) Garrett, D. S.; Powers, R.; Gronenborn, A. M.; Clore, G. M. *J. Magn. Reson.* **1991**, *95*, 214−220.

(32) Wang, A. C.; Bax, A. *J. Am. Chem. Soc.* **1996**, *118*, 2483−2494.

(33) Wang, A. C.; Bax, A. *J. Biomol. NMR* **1993**, *3*, 715−720.

traceless second rank diagonal tensor D given by $^1/_3[D^{zz}_{AB} - (D^{xx}_{AB} + D^{yy}_{AB})/2]$ and $^1/_3[D^{xx}_{AB} - D^{yy}_{AB}]$, respectively, with $|D^{zz}_{AB}| > |D^{yy}_{AB}| \geq |D^{xx}_{AB}|$; θ is the angle between the A−B interatomic vector and the z axis of the tensor; and ϕ is the angle which describes the position of the projection of the A−B interatomic vector on the $x-y$ plane, relative to the x axis.[34] D^a_{AB} subsumes various constants, including the gyromagnetic ratios of the two nuclei γ_A and γ_B, the inverse cube of the distance between the two nuclei, r_{AB}^{-3}, and the unitless axial component, A_a, of the molecular alignment tensor $\boldsymbol{A}$. The tensor $\boldsymbol{A}$ corresponds to the diagonalized Saupe matrix,[35] which describes the alignment of the protein relative to the magnetic field. The symbol $\boldsymbol{A}$ is used here because the symbol $\boldsymbol{S}$, commonly used for the (undiagonalized) Saupe matrix, is already used for the generalized order parameter in protein structural studies.[36]

In contrast to small molecule studies in the liquid crystalline phase, where the alignment tensor is rapidly modulated by internal dynamics of the molecule, the overall shape of a protein, and thereby its alignment tensor, is not significantly affected by small amplitude structural fluctuations such as bond angle bending or stretching motions, methyl group rotations or angular oscillations of backbone, or side-chain bonds. Moreover, such fluctuations typically occur on time scales much faster than the rotational correlation time of the macromolecule. This greatly simplifies the analysis of the effect of such dynamic processes on the observed dipolar couplings, and allows eq 1 to be replaced by either the time or ensemble average[37]

$$D_{AB}(\theta,\phi) = D^a_{AB}\{\langle(3\cos^2\theta - 1)\rangle + {}^3/_2\, R\,\langle(\sin^2\theta\cos 2\phi)\rangle\} \quad (2)$$

where the $\langle\,\rangle$ brackets indicate motional averaging. It is convenient to separate the effects of very fast internal motions which occur for all atoms, including the well ordered polypeptide backbone, from other motions such as side-chain rearrangements, larger amplitude backbone motions, and methyl group rotation. To a good approximation, averaging over the angular terms associated with the fast motions scales all dipolar interactions of a given type by the same factor,[23] and this effect can therefore be conveniently incorporated in D^a_{AB}, by redefining it according to

$$D^a_{AB} = -(\mu_o h/16\pi^3)\,\gamma_A\gamma_B\,\langle r_{AB}^{-3}\rangle\, A_a \quad (3)$$

where $\langle r_{AB}^{-3}\rangle$ equals the inverse cube of the *effective* bond length, r_{AB}^{eff}, which includes the effects of fast librational motions.[23] The angular averaging remaining in eq 2 now only concerns motions such as side-chain rearrangements, larger amplitude backbone motions, and methyl group rotation. Note that domain motions, if present, cannot be treated in the simple manner of eq 2 as they will modulate the alignment tensor. The angular terms in eq 2 can be rewritten as $\langle(3\cos^2\theta - 1)\rangle = \boldsymbol{S}(3\cos^2\theta_{av} - 1)$ and $\langle(\sin^2\theta\cos 2\phi)\rangle = \boldsymbol{S}(\sin^2\theta_{av}\cos 2\phi_{av})$, where θ_{av} and ϕ_{av} are the spherical coordinates describing the average orientation of the A−B bond vector in the frame of the alignment tensor, and $\boldsymbol{S}$ is the generalized order parameter.[36]

For a rapidly rotating, C_{3v} symmetric methyl group, the average ^{13}C−^{1}H vector is that of the C_{3v} axis, which ideally coincides with the C−C axis. This rapid rotation scales the $^1D_{CH}$ coupling by $\boldsymbol{S}_{rot} = P_2(\cos\beta)$, where β is the C−C−H angle. Other internal motions of the C−CH$_3$ fragment affect $^1D_{CH}$ and $^1D_{CC}$ equally, and the ratio of $^1D_{CH}$ and $^1D_{CC}$ is therefore given by

$$^1D_{CH}/^1D_{CC} = P_2(\cos\beta)\,(\gamma_H/\gamma_C)\,\langle r_{CH}^{-3}\rangle/\langle r_{CC}^{-3}\rangle \quad (4a)$$

which can be rewritten as

$$\beta = \cos^{-1}[\,{}^2/_3\,(\gamma_C/\gamma_H)\,(D_{CH}/D_{CC})\,(\langle r_{CC}^{-3}\rangle/\langle r_{CH}^{-3}\rangle) + {}^1/_3]^{1/2} \quad (4b)$$

Note that, barring deformation of the C_{3v} symmetry of the methyl group, eq 4a is valid independent of the degree of mobility of the methyl group as a whole or of its orientation relative to the alignment tensor.

Errors in the measured $^1D_{CH}$ and $^1D_{CC}$ values and nonidealities in the methyl group geometry have the largest effect on β for small values of the denominator, $^1D_{CC}$ in eq 4b. Thus, the average $^1D_{CH}/^1D_{CC}$ ratio, which determines the average value of β if $\langle r_{CH}^{-3}\rangle/\langle r_{CC}^{3-}\rangle$ is known, is best obtained by linear regression of plots of $^1D_{CH}$ versus $^1D_{CC}$. Ab initio calculations of ^{13}C chemical shifts and $^1J_{CH}$ coupling constants are known to be highly sensitive to the ^{13}C−^{1}H internuclear distance. Considering the narrow range of ^{13}C shifts and $^1J_{CH}$ values observed experimentally for methyl groups, it is safe to assume that variations in $\langle r_{CH}\rangle$ and $\langle r_{CC}\rangle$ among different methyl groups are negligible. Experimentally, we previously determined $\langle r_{CH}^{-3}\rangle^{-1/3} = 1.117 \pm 0.007$ Å for C$^\alpha$−H$^\alpha$, when using $\langle r_{C'N}^{3-}\rangle^{-1/3} = 1.329$ Å and $\langle r_{C\alpha C'}^{-3}\rangle^{-1/3} = 1.525$ Å as reference distances.[23] This value of 1.117 Å includes the effect of very high-frequency librations, which are of larger amplitude for C$^\alpha$−H$^\alpha$ than for the C′−N and C$^\alpha$−C′ bonds and lead to smaller dipolar interactions and thereby larger values of the effective C$^\alpha$−H$^\alpha$ distance. This lengthening is equivalent to ascribing an order parameter $\boldsymbol{S}_{lib} = 0.94$ to the effect of fast librations on the C$^\alpha$−H$^\alpha$ dipolar coupling. For methyl groups, only librations orthogonal to the direction of rotation contribute to a decrease in the dipolar coupling;[11] the effect of librations tangential to the direction of methyl group rotation is incorporated in the $P_2(\cos\beta)$ factor in eq 4. Therefore, for methyl groups $\boldsymbol{S}_{lib} = 0.97$. Assuming that in the absence of librations (i.e., only stretching vibrations) $\langle r_{CH}^{-3}\rangle^{-1/3}$ for methyl groups and C$^\alpha$−H$^\alpha$ have the same value of 1.095 Å, the effective r_{CH} for methyl groups equals 1.106 Å. Carbon−carbon bond lengths for CH−CH$_3$ and CH$_2$−CH$_3$ moieties reported by Engh and Huber are 1.521 and 1.513 Å, respectively.[24] Here, we use an average value of 1.517 Å.

Analysis of the Data. Measurements were carried out on ubiquitin, a small protein of 76 residues which yields very well-resolved spectra with narrow line widths. Figure 1 shows a small region of the [^{13}C,^{1}H] HSQC correlation map, containing about half of the methyl correlations, both in the aligned and in the isotropic states. As can be seen, the $^1J_{CC}$ splittings (Figure 1B) and the $^1J_{CC} + {}^1D_{CC}$ splittings (Figure 1A) are well resolved and can be measured at high accuracy. Because the ^{1}H−^{1}H dipolar couplings within the methyl group do not average to zero, the spectrum measured in the liquid-crystalline phase shows considerably larger line widths in the ^{1}H dimension than does the isotropic sample. For Ala[46] C$^\beta$H$_3$, which has its ^{13}C$^\alpha$−^{13}C$^\beta$ vector nearly orthogonal to the z axis of the alignment tensor, the ^{1}H−^{1}H dipolar couplings result in a resolved triplet.

(34) Clore, G. M.; Gronenborn, A. M.; Bax, A. *J. Magn. Reson.* **1998**, *133*, 216−221.
(35) Saupe, A.; Englert, G. *Phys. Rev. Lett.* **1963**, *11*, 462−465. Emsley, J. W.; Lindon, J. C. *NMR Spectroscopy using liquid crystal solvents*; Pergamon Press: New York, 1975.
(36) Lipari, G.; Szabo, A. *J. Am. Chem. Soc.* **1982**, *104*, 4546−4559.
(37) Tjandra, N.; Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1996**, *118*, 6264−6272. Tolman, J. R.; Flanagan, J. M.; Kennedy, M. A.; Prestegard, J. H. *Nat. Struct. Biol.* **1997**, *4*, 292−297.

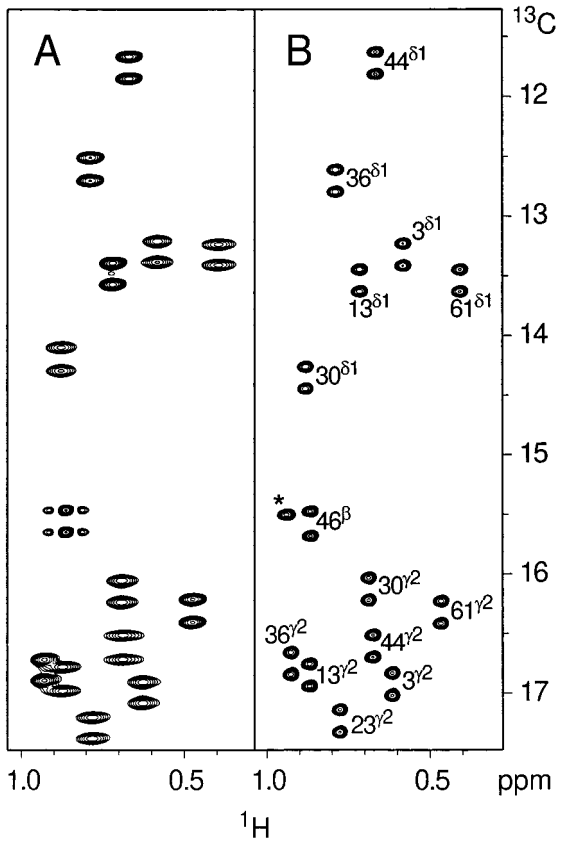

Figure 1. Small region of the 750 MHz ^{1}H$-^{13}$C HSQC spectrum of U-^{13}C/^{15}N ubiquitin, recorded in 93:7 H_2O:D_2O, pH 6.5, 50 mg/mL of a 30:10:1 molar ratio mixture of DMPC/DHPC/CTAB, (A) in the aligned state at 37 °C, and (B) in the isotropic state at 22 °C. A phospholipid natural abundance ^{13}C signal, only visible in the isotropic phase, is marked with an asterisk.

Previously, the ratio of this ^{1}H$-^1$H dipolar coupling over the $^1D_{CH}$ coupling has been used for studying methyl group geometry,[1,3,4] but for the very weak alignments used in the present study this ^{1}H$-^1$H dipolar splitting remains unresolved for most methyl groups.

Figure 2 shows the reproducibility of the ^{13}C$-^{13}$C splittings, measured in two separate experiments, which sandwiched the one-day experiment for measurement of ^{13}C$-^1$H splittings. The pairwise rmsd between the two measurements in the aligned state equals 0.075 Hz, indicating a random error of 0.04 Hz in their averaged value. With a pairwise rmsd of 0.064 Hz, the reproducibility is even slightly better in the isotropic state (Figure 2B). The carbons with the most extreme $^1J_{CC}$ couplings in the isotropic sample are labeled in Figure 2B. These data show a much larger spread than expected on the basis of literature data. $^1J_{CC}$ couplings for methyl groups measured in free amino acids cover a very small range of 34−35 Hz for all amino acids except Thr, which has $^1J_{CC} = 38.0$ Hz.[38] For extracting reliable $^1D_{CC}$ values, it therefore is essential to measure the ^{13}C$-^{13}$C splittings both in the aligned *and* isotropic states.

The largest deviation from the literature value is observed for Ala[46], which shows $^1J_{C\alpha C\beta} = 39.0$ Hz, 5 Hz larger than the value of 34.0 Hz reported for free alanine.[38] Ala[46] adopts a positive ϕ angle,[39] which positions its $C^\beta H_3$ methyl group within van der Waals distance of the Phe[45] carbonyl oxygen. This presumably is responsible for the large change in $^1J_{CC}$. The four leucine C^δ methyl groups with very small $^1J_{C\delta C\gamma}$ values confirm that $^1J_{CC}$ is sensitive to steric effects: All four Leu $C^{\delta 2}$ methyl groups that are gauche with respect to $^{13}C^\alpha$, as determined from $^3J_{C\alpha C\delta}$ couplings (Supporting Information), have $^1J_{C\delta C\gamma}$ values in the 33.0−33.6 Hz range. All $^1J_{C\delta C\gamma}$ for Leu C^δ methyl carbons that are locked in a trans conformation or which undergo rotameric averaging about the $C^\beta-C^\gamma$ bond (as determined from $^3J_{C\alpha C\delta}$), have $^1J_{C\delta C\gamma}$ values in the 34.4−35.3 Hz range, close to the values of 34.8 and 35.0 Hz observed in the free amino acid (Supporting Information). A more detailed analysis of the correlation between $^1J_{CC}$ and local geometry will be presented elsewhere.

Figure 3 shows typical interferograms through the $^1J_{CH}$-modulated CT-HSQC spectrum, together with the best-fit obtained using a constrained nonlinear least-squares fitting procedure.[30] The value of $^1J_{CH} + {}^1D_{CH}$ is actually derived from the separation of the outer components of the $^{13}CH_3$ quartet, which are separated by $3(^1J_{CH} + {}^1D_{CH})$, and which have the highest intensity in experiments which use INEPT-type polarization transfer.[30,40] On the basis of reproducibility in duplicate measurements, estimated errors in the measured $3(^1J_{CH} + {}^1D_{CH})$ splittings are less than 0.2 Hz, resulting in errors in $^1D_{CH}$ of ∼0.1 Hz.

Figure 4 shows the correlation between the $^1D_{CH}$ and $^1D_{CC}$ couplings. Linear regression yields a slope of -3.17 ± 0.03 with a correlation factor R of -0.999. Using eq 4b then yields $\beta = 110.91 \pm 0.17°$, or $\boldsymbol{S}_{rot} = 0.308$.

Systematic Errors in β. The above derived value of β represents our best estimate for the average C−C−H angle, but the standard deviation does not take into account the effect of possible systematic errors caused by the uncertainty in $\langle r_{CC}{}^{-3}\rangle / \langle r_{CH}{}^{-3}\rangle$.

Although methyl group vibrational corrections and equilibrium bond lengths are likely to be very similar from one methyl group to another, they differ to some extent from those observed for the polypeptide backbone. As mentioned above, librations tangential to the rotation direction are included in the $P_2(\cos\beta)$ factor in eq 4 and the magnitude of the effect of C−H bond vector librations on the C−H dipolar coupling in methyl groups is therefore 2-fold smaller compared to backbone C^α sites. Libration of the three methyl protons will be correlated, however, and this is likely to alter the magnitude of the observed dipolar coupling somewhat relative to the case of independent librations. Considering the relatively large value of the uncertainty reported for the effective $C^\alpha-H^\alpha$ bond length (±0.007 Å),[23] and that only librations affecting the C−C−H angle influence methyl $r_{CH}{}^{eff}$, the effect of correlated libration is expected to be much smaller than ±0.007 Å and is neglected in our analysis. The effect of stretching vibrations of the C−H bond are very similar for different types of carbons, and result in a small increase in $r_{CH}{}^{eff}$ (by ∼0.01 Å) compared to the equilibrium bond length.[25] This vibrational stretching effect is included in the previously reported $r_{C\alpha H\alpha}{}^{eff}$ value of 1.117 Å, and again ignoring the effect of correlated stretching modes, it does not significantly increase the uncertainty in the methyl $r_{CH}{}^{eff}$. However, ab initio calculations suggest the possibility of a small (up to 0.005 Å) increase for the methyl group r_{CH} compared to $C^\alpha-H^\alpha$ (D. Case, unpublished results). Combined, these factors lead to a rather wide range from 1.099 to 1.118 Å for the methyl group $r_{CH}{}^{eff}$.

(38) Eisenreich, W.; Strauss, G.; Werz, U.; Fuchs, G.; Bacher, A. *Eur. J. Biochem.* **1993**, *215*, 619−632.

(39) Vijay-Kumar, S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531−544.

(40) Morris, G. A.; Freeman, R. *J. Am. Chem. Soc.* **1979**, *101*, 760−762.

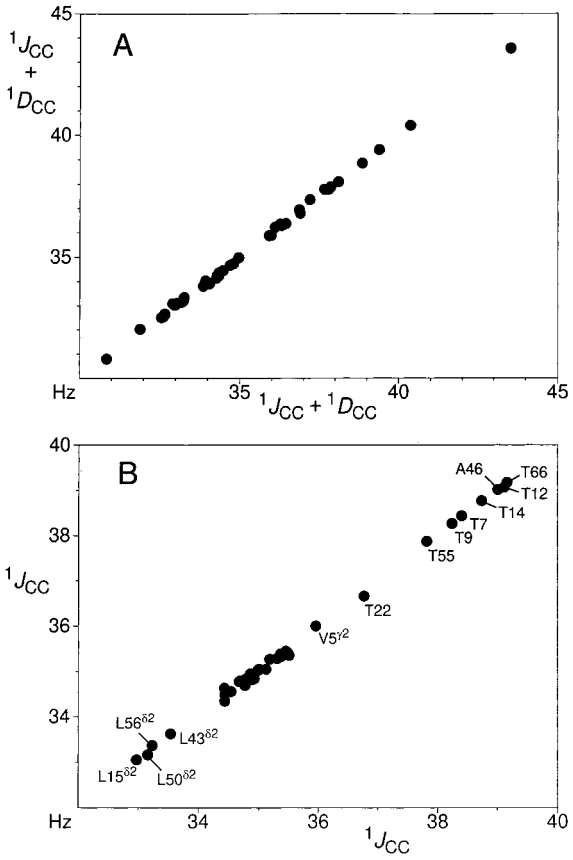

Figure 2. Reproducibility plot of pairs of $^{13}C-^{13}C$ splittings, measured for the nonoverlapping methyl groups in the 750 MHz [^{13}C,^1H] HSQC spectrum of ubiquitin, (A) in the aligned state at 37 °C, and (B) in the isotropic state at 22 °C. The Euler angles relating the alignment tensor orientation to that of the X-ray structure coordinate frame are $\alpha = 34°$; $\beta = 32°$; $\gamma = 21°$; with $D_a^{NH} = 15.8$ Hz, and $R = 0.48$, assuming $\boldsymbol{S} = 1$.

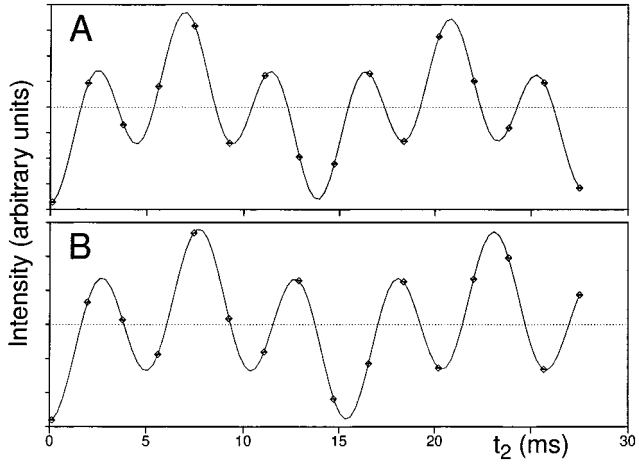

Figure 3. Interferogram showing the intensity modulation of the Ala^{46} $^1H^{\beta}-^{13}C^{\beta}$ correlation in the *J*-modulated HSQC experiment and the best fit function: $\{A\cos[\pi(^1J_{CH} + {}^1D_{CH})t_2] + B\cos[3\pi(^1J_{CH} + {}^1D_{CH})t_2]\}\exp(-Ct_2^2)$, (A) in the aligned state, yielding $^1J_{CH} + {}^1D_{CH} = 144.3$ Hz, and (B) in the isotropic state, yielding $^1J_{CH} = 130.0$ Hz.

Although vibrational corrections for the C−C bonds are negligible, there is considerable uncertainty in its equilibrium value. For example, Engh and Huber report C−C bond lengths for CH−CH$_3$ and CH$_2$−CH$_3$ which differ by 0.008 Å.[24] Here, we use their averaged value and estimate the uncertainty in r_{CC} to be 0.004 Å. The total aggregate of the uncertainties in the above-discussed equilibrium r_{CH} and r_{CC} values and their librational correction, ranges from a possible decrease by up to 4.1% in the $\langle r_{CH}^{-3}\rangle/\langle r_{CC}^{-3}\rangle$ ratio, to an increase of up to 2.7%. A 1% increase in $\langle r_{CH}^{-3}\rangle/\langle r_{CC}^{-3}\rangle$ increases β by 0.18°, and the uncertainty in β introduced by these systematic errors, in addition to the standard error in the fit, extends the possible range for β from 110.2 to 111.8°.

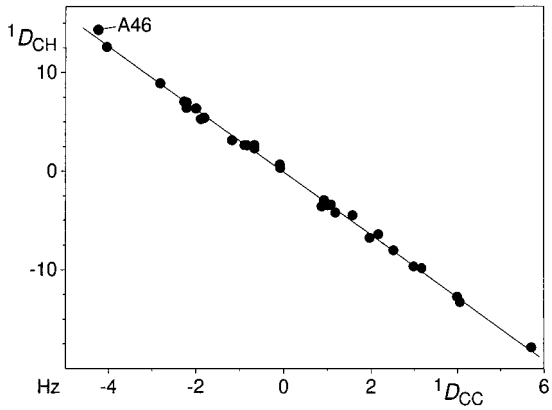

Figure 4. Plot of $^1D_{CH}$ versus $^1D_{CC}$ residual dipolar couplings of methyl groups in the protein ubiquitin, dissolved in a dilute liquid crystalline medium. The solid line represents the best fit of the data by linear regression, with a slope of -3.17 ± 0.03 and a correlation factor R of -0.999. See text for further details.

It is important to note, however, that for relaxation studies only the D_{CH} magnitude of a rapidly spinning methyl group is of interest, and it is the ratio of $D_{CH}^{methyl}/D_{CC}^{methyl}$ which is determined at very high accuracy in the present study, thereby indirectly relating D_{CH}^{methyl} and $D_{CH}^{C\alpha H\alpha}$. If effective C−H bond lengths are different for C$^{\alpha}$ and methyl sites, this affects the β value but not the $D_{CH}^{methyl}/D_{CH}^{C\alpha H\alpha}$ ratio. For a rapidly spinning methyl group, the effect of different effective C−H bond lengths is therefore indistinguishable from a change in β, and any difference in the effective bond length is included implicitly in the value we report for $\boldsymbol{S}_{rot}$.

Variations in β. As mentioned above, random errors in the measurement of the one bond ^{13}C-^{13}C and ^{13}C-^1H splittings are extremely small (0.03 and 0.07 Hz in the isotropic phase; 0.04 and 0.1 Hz in the liquid crystalline phase, respectively). As the dipolar contribution to the splitting is obtained from the difference in splitting between the liquid crystalline and isotropic phases, which are measured with exactly the same experimental protocol, it is unlikely that the difference contains a significant systematic error in the measurement, and the errors in the experimental $^1D_{CC}$ and $^1D_{CH}$ values are therefore estimated at 0.05 and 0.12 Hz, respectively.

These errors are considerably smaller than the size of the dots marking the individual pairs of $^1D_{CH}$ and $^1D_{CC}$ couplings in Figure 4. Therefore, it is interesting to consider the range of β values obtained when using eq 4 for individual $^1D_{CH}$ and $^1D_{CC}$ pairs. As discussed below, the $^1D_{CH}/^1D_{CC}$ ratios for methyl groups which yield the smallest absolute $^1D_{CH}$ and $^1D_{CC}$ couplings are very sensitive to minor distortions from ideal geometry and therefore cannot be used in such an analysis. Limiting the calculation of β to methyl groups with a $^1D_{CH}$ value larger than 7 Hz, β values obtained using eq 4b range from 112.8° for Ile^{36}−C$^{\gamma 2}$H$_3$ to 109.5° for Ala^{46}−C$^{\beta}$H$_3$. Clearly, this range presents an upper limit for the true range of variation in β values, since experimental errors in the $^1D_{CH}/^1D_{CC}$ ratios and distortions from C_{3v} symmetry of the methyl groups can cause random changes in these ratios and thereby in the derived β values.

Distorted Geometry? Methyl groups with near-zero $^1D_{CC}$ dipolar couplings have the strongest $^1D_{CC}$-dependence on the orientation of the C−C bond vector. Therefore, if the C−C vector orientation deviates from the C_{3v} symmetry axis of the CH_3 group (i.e., assuming that the three protons nevertheless form an equilateral triangle with equal C−H distances for the three protons), this can result in a considerable deviation from the $^1D_{CC}$ value expected on the basis of its $^1D_{CH}$ value. With the exception of Ala[46], Figure 4 shows that the $^1D_{CH}$ versus $^1D_{CC}$ correlation indeed is better for methyl groups with large $^1D_{CH}$ and $^1D_{CC}$ values than for those with smaller values. This suggests that deviations between the C−C vector orientation and the C_{3v} symmetry axis may indeed occur. Simulations carried out in which the C_{3v} axis of the methyl group is rotated by a random angle away from the C−*C* axis show that the spread for the correlation between the calculated $^1D_{CH}$ and $^1D_{CC}$ values for small (<7 Hz) values of $^1D_{CH}$ increases to the same value as observed in Figure 4 for a root-mean-square (rms) angle of 0.8°. If this type of methyl group distortion were the sole source of the scatter in Figure 4, this rms angle of 0.8° would represent the rms difference between the C−C bond and the C_{3v} axis. This rms value therefore must be regarded as an upper limit.

Other distortions from ideal geometry are also likely to occur. For example, if the methyl group is considered to undergo discrete hops about the C−C axis but the proton in one of the three positions is pushed away from its ideal position by a steric effect, this can also change the $^1D_{CH}/^1D_{CC}$ ratio. In this case, the effect is largest if the proton whose position is distorted has a near-zero instantaneous $^1D_{CH}$ value, and for such cases no correlation between the change in $^1D_{CH}$ from ideality with the magnitude of $^1D_{CC}$ is expected. For the experimentally observed alignment tensor of ubiquitin, a change by 1° from the idealized C−H orientation of one of the three occupied positions of a rapidly hopping methyl group proton can result in a change in $^1D_{CH}$ by up to 0.5 Hz. Significant asymmetry parameters for the ^{2}H quadrupole pattern of the methyl groups in methyl deuterated thymine and hexamethylbenzene suggest that such distortions may indeed occur in a variety of systems.[26] Ala[46] in ubiquitin is a prime candidate for such distortions because this residue has a "forbidden" positive ϕ angle which causes its $C^\beta H_3$ to clash with the carbonyl oxygen of Phe[45]. Indeed, the Ala[46] $C^\beta H_3$ is the methyl group with the largest deviation (0.8 Hz in $^1D_{CH}$) from the experimental correlation in Figure 4. Other possible support for a distortion of this methyl group may be found in the very large increase (5.0 Hz) in the $^1J_{C\alpha C\beta}$ coupling relative to that of the free amino acid. However, in this context it must also be pointed out that the $^1J_{C\beta H\beta}$ value and its $^{13}C^\beta$ and $^1H^\beta$ chemical shifts fall in the normal range.

Concluding Remarks

Because of the 3-fold higher integrated intensity and favorable relaxation properties of methyl groups relative to other sites in macromolecules, methyl group $^1D_{CH}$ and $^1D_{CC}$ dipolar couplings can be measured with exceptional accuracy. The uniformity of the observed $^1D_{CH}/^1D_{CC}$ ratios provides strong support for the absence of large deviations from ideal tetrahedral geometry of the methyl groups in ubiquitin. If we assume that the amplitude of very rapid librations of the methyl group C−H vectors are of the same magnitude as those for the backbone $C^\alpha-H^\alpha$ vectors, and exclude librations in the direction of methyl group rotation, the effective C−H bond length is 1.106 Å. The dipolar couplings measured in the present study then result in an average methyl group C−C−H angle of 110.9 ± 0.2°. If a shorter effective methyl C−H bond length of 1.095 Å is used, this angle increases to 111.5°. Conversely, for an effective methyl C−H bond length of 1.135 Å, $\beta = 109.5°$.

Our data suggest that if deviations between the average orientations of the methyl group C_{3v} axis and its C−C bond vector occur in proteins, the magnitude of such distortions is limited to less than 1°**.** The most outlying pair of $^1D_{CH}$ and $^1D_{CC}$ values is observed for Ala[46] $C^\beta H_3$ which, as a result of the unusual positive ϕ angle of this residue, clashes with the carbonyl oxygen of the preceding one. A dramatic increase of 5 Hz in the isotropic $^1J_{C\alpha C\beta}$ coupling of this residue is observed over that of the free amino acid. Again, assuming a symmetric methyl group with a 1.106 Å C−H bond length, its $^1D_{CH}/^1D_{CC}$ ratio yields an unusually small C−C−H angle of 109.5°. However, our data do not allow us to distinguish whether the exceptionally large $^1D_{CH}/^1D_{CC}$ ratio results from a geometric distortion of the methyl group, or whether it is an intrinsic property of Ala residues. High-resolution single-crystal neutron diffraction data available on free amino acids suggest a 1.5° smaller C−C−H angle for Ala relative to Val methyl groups,[7,8] and although unlikely, this could also explain the unusually large $^1D_{CH}/^1D_{CC}$ ratio. Ala[28] is the only other alanine in ubiquitin, but its resonance overlaps with the Met[1] $C^\epsilon H_3$ resonance, and no reliable C−C−H angle could be derived for its methyl group.

Overall, our data confirm that methyl group geometries in proteins are highly uniform. Rapid rotation of the methyl group, together with C−H bond vector librations orthogonal to the direction of rotation, scale the observed $^{13}C-^1H$ dipolar interaction by a factor $\boldsymbol{S}_{rot} = -0.299$ relative to the D_{CH} for a hypothetical proton on the C−C bond vector, at the commonly used $r_{CH} = 1.095$ Å. This is equivalent, however, to $\boldsymbol{S}_{rot} = -0.308$ for $r_{CH} = 1.106$ Å. These $\boldsymbol{S}_{rot}$ values contain a 0.8% uncertainty resulting from the error in the C−C bond length and 1% from the standard error in the measured D_{CH}/D_{CC} ratio. Thus, using $r_{CH} = 1.095$ Å yields $\boldsymbol{S}_{rot}^2 = 0.090 \pm 0.003$, but it is important to realize that this lower $\boldsymbol{S}_{rot}^2$ value also includes the effect of rapid C−H librations. Instead, if the effect of librations is included in the bond length (i.e., $r_{CH}^{eff} = 1.106$ Å), $\boldsymbol{S}_{rot}^2$ increases to 0.956. These values are 15−20% smaller than the $\boldsymbol{S}_{rot}^2 = 0.111$ order parameter, typically assumed for ideal methyl groups in ^{13}C relaxation studies. This smaller $\boldsymbol{S}_{rot}^2$ value considerably reduces the discrepancy between previous relaxation measurements of $^{13}C^\alpha$ and methyl $^{13}C^\beta$ in alanyl residues, although it does not resolve it entirely.

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Supporting Information Available: One table containing the one-bond $^1J_{CH}$, $^1J_{CC}$, $^1D_{CH}$, and $^1D_{CC}$ couplings for the methyl groups in ubiquitin; one table containing the $^3J_{C\alpha C\delta}$ couplings for Leu and Ile residues in ubiquitin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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