Measurement of Methyl ^{2}H Quadrupolar Couplings in Oriented Proteins. How Uniform Is the Quadrupolar Coupling Constant?

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Abstract: ^{2}H quadrupolar and one-bond ^{13}C−^{13}C dipolar couplings have been measured at methyl sites in a uniformly ^{13}C and fractionally ^{2}H labeled sample of the N-terminal *drk* SH3 domain, weakly aligned in a dilute solution of Pf1 phage. An average ratio between the ^{2}H quadrupolar and ^{13}C−^{13}C dipolar couplings of 19.2 ± 0.1 is measured. Assuming rapid rotation about the one-bond $^{13}C^{methyl}$—^{13}C axis and an angle of 109.5° between the unique principal axis of the electric field gradient tensor and the methyl averaging axis ($^{13}C^{methyl}$—^{13}C bond), an average value of 167 ± 1 kHz is obtained for the quadrupolar coupling constant, e^2qQ/h. The profile of ^{2}H quadrupolar vs ^{13}C−^{13}C dipolar couplings suggests that the use of a uniform value for the quadrupolar coupling constant is a good approximation in the analysis of ^{2}H relaxation data measured at methyl sites in proteins.

Introduction

In principle NMR spectroscopy is a powerful probe of dynamic processes in molecules.[1,2] However, accurate interpretation of NMR derived relaxation times in terms of motion requires knowledge of a number of parameters which may not be known to high accuracy. For example, bond lengths or quadrupolar coupling constants are needed in the analysis of relaxation data derived from dipolar or quadrupolar interactions, respectively. Additionally, in cases such as in the study of methyl groups, the orientation of vectors relative to averaging axes must also be known.[3] In some situations the errors introduced by uncertainties in these values may well be greater than the precision of the experimental data, severely compromising the accuracy of the extracted motional parameters. For example, in the analysis of ^{13}C relaxation data large discrepancies were noted in amplitudes of motion of the $^{13}C^{\alpha}$—$^{1}H^{\alpha}$ and $^{13}C^{\alpha}$—$^{13}C^{\beta}$ bond vectors of Ala residues in staphylococcal nuclease, despite the fact that both vectors should monitor the same dynamic processes.[4]

Recently Ottiger and Bax have described a liquid crystal NMR study reporting ^{13}C−^{13}C and ^{1}H−^{13}C dipolar couplings of methyl groups in ubiquitin.[5] On the basis of these couplings effective methyl ^{1}H−^{13}C bond lengths and H−C−C bond angles of 1.106 Å and 110.9°, respectively, were determined. These values are different from those used in published ^{13}C dynamics studies[3] (1.09 Å, 109.5°) and decrease somewhat, although not completely, the differences between motional parameters extracted from $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ relaxation times of Ala spin systems.

The difficulties in interpretation of side chain ^{13}C relaxation parameters stimulated the development of a series of experiments for measuring ^{2}H relaxation times in uniformly ^{13}C-labeled, fractionally deuterated proteins.[6,7] The attraction of this approach is based on the fact that the relaxation of a deuteron is completely dominated by the local quadrupolar interaction.[8,9] In the case of methylene and methyl groups this simplifies interpretation of relaxation data relative to studies based on ^{13}C relaxation times where interference between ^{1}H−^{13}C dipoles can complicate the analysis.[10] A number of ^{2}H-based methyl dynamics studies have now been reported,[11−15] and insight into dynamics in hydrophobic cores of proteins, as well as into changes in dynamics of side chains upon ligand binding, has emerged. Notably, much better agreement between amplitudes of motion as monitored by backbone relaxation studies and Ala ^{2}H methyl relaxation has been noted.[16] Nevertheless, extraction of accurate amplitudes and time constants describing motional processes contributing to ^{2}H relaxation is predicated on knowledge of the ^{2}H quadrupole coupling constant, e^2qQ/h. Additionally, it is important to establish whether the quadrupolar coupling is, to a reasonable approximation, constant at all methyl sites in the molecule or varies as a function of position.

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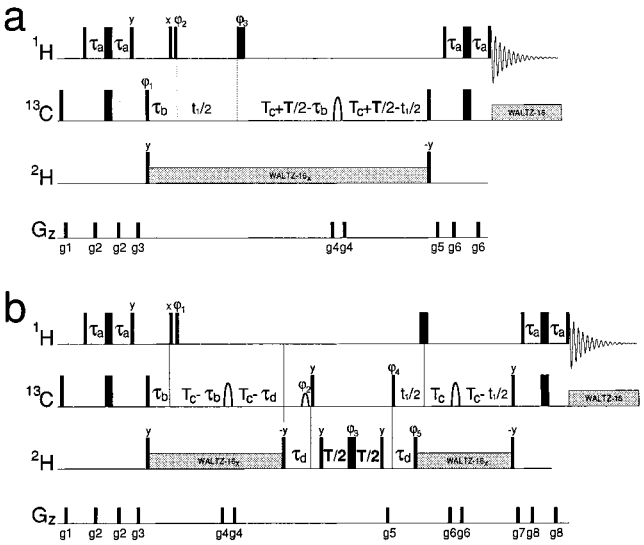

Figure 1. Pulse schemes to measure the one-bond $^{13}C^{methyl}-^{13}C$ dipolar coupling, $^1D_{CC}$, (a) and the methyl ^2H quadrupolar coupling, ν_Q (b). The pulse schemes are very similar to sequences published for measuring the relaxation rates of I_zC_z (a) and $I_zC_zD_z$ (b) in $^{13}CH_2D$ spin systems (I = ^{1}H, C = ^{13}C, D = ^{2}H); details can be found in the captions to Figures 1 and 6 in Muhandiram et al.[6] Briefly, in (a) τ_a = 1.7 ms, τ_b = 3.85 ms, T_C = 14.5 ms, and the value of **T** is varied between experiments (see Materials and Methods). The phase cycle employed is: $\phi1 = 4(x), 4(-x)$; $\phi2 = (x, -x)$; $\phi3 = 2(x), 2(-x)$; rec = $2(x, -x), 2(-x, x)$. In (b) all delays are as in (a) and τ_d is set to ~11 ms ($1/4J_{CD}$). The phase cycle is: $\phi1 = x, -x$; $\phi2 = 4(x), 4(-x)$; $\phi3 = 2(y), 2(-x), 2(-y), 2(x)$; $\phi4 = 8(x), 8(-x)$; $\phi5 = 2(y), 2(-y)$; rec = $2(x, -x, -x, x), 2(-x, x, x, -x)$.

Values of e^2qQ/h for aliphatic deuterons ranging from 168 ± 2 to 174 ± 2 kHz have been reported for a number of small compounds, including methane, ethane, butane, hexane, and cyclohexane.[17] However, to our knowledge values for methyl groups on a per-site basis in proteins have not been reported. We demonstrate here for the first time that it is possible to measure accurate ^{2}H-methyl quadrupolar couplings in small, ^{13}C-labeled and fractionally deuterated proteins oriented in solution. By comparing ^{2}H quadrupolar couplings (ν_Q) with one-bond $^{13}C^{methyl}-^{13}C$ dipolar couplings ($^1D_{CC}$) at each methyl site in a ^{13}C-labeled, fractionally deuterated sample of an SH3 domain and assuming an angle of 109.5° between the principal axis of the (assumed axially symmetric) electric field gradient tensor and the one-bond $^{13}C^{methyl}-^{13}C$ axis (also referred to in the text as the methyl averaging axis or $^{13}C^{methyl}-^{13}C$ axis) an average value for e^2qQ/h of 167 ± 1.5 kHz is obtained. Moreover, the linear relation between ν_Q and $^1D_{CC}$ suggests that assuming a constant value for e^2qQ/h in the analysis of ^{2}H methyl relaxation data is a good approximation.

Materials and Methods

^{15}N,^{13}C, fractionally ^{2}H-labeled Thr22Gly mutant N-terminal SH3 domain from the protein *drk* (T22G drkN SH3, 59 amino acids) was prepared as described previously[7] with the exception that the growth media was comprised of a mixture of 50% H_2O/50% D_2O. A 1 mM sample, 0.05 M sodium phosphate, 10% D_2O/90% H_2O, pH 6.0, 25 °C was used for all experiments. In the case of partial alignment Pf1 phage[18] was added to a final concentration of ~33 mg/mL (residual D_2O splitting of 12.8 Hz). All spectra were recorded at 600 MHz on a Varian Inova spectrometer. Data matrices were acquired with (138,578) complex (t_1,t_2) points using the experiments of Figure 1. Acquisition times of 25 and 64 ms in t_1 and t_2, respectively, were employed. F_1-quadrature was obtained with States-TPPI[19] of $\phi1$ (Figure 1a) or $\phi4$ (Figure 1b).

The values of **T** used for measuring $^1D_{CC}$ were (ms): 1.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, 60.0, 65.0, 70.0, while **T** values of 0.3, 3, 6, 9, 12, 15, 18, 21, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120 were employed for measuring ν_Q. All spectra were processed using NMRPipe/NMRDraw software[20] and analyzed with the PIPP/CAPP suite of programs.[21] Values of $^1D_{CC}$ and ν_Q were obtained by fitting the time dependence of signal intensities using MATLAB.

As described below the evolution of $^{13}C^{methyl}$ magnetization in the experiment of Figure 1a proceeds according to the relation

$$A\cos\{\pi(^1J_{CC}+{}^1D_{CC})T'\}[\Pi\cos\{\pi(^kJ_{CC}+{}^kD_{CC})T'\}]\exp(-T'/T_2)$$

where $T' = 2T_C + \mathbf{T}$, the product is over all nonzero $^{13}C^{methyl}-^{13}C^{aliphatic}$ couplings that are two-bond or greater (i.e., $k \geq 2$) that modulate the signal, A is a constant, T_2 is the effective transverse relaxation time and $^kJ_{CC}$,$^kD_{CC}$ are the k-bond $^{13}C^{methyl}-^{13}C$ scalar and dipolar couplings, respectively. The value of $^kD_{CC} \sim 0$ ($k \geq 2$), however, $^3J_{CC}$ values as large as 3−4 Hz can be observed, depending on dihedral angle χ values.[22] A number of different fitting functions derived from the above equation have been employed including $A\cos\{\pi(^1J_{CC} + {}^1D_{CC})T'\}\exp(-T'/T_2)$, $A\cos\{\pi(^1J_{CC} + {}^1D_{CC})T'\}\cos\{\pi(^kJ_{CC} + {}^kD_{CC})T'\} \times \exp(-T'/T_2)$ and $A\cos\{\pi(^1J_{CC} + {}^1D_{CC})T'\}\exp(-BT'^2)$, where the decay in signal caused by the long range $^{13}C-^{13}C$ couplings ($k \geq 2$) is approximated by the Gaussian, $\exp(-BT'^2)$.[23] Identical results were obtained in all cases.

One-bond $^{15}N-^1HN$ dipolar couplings were measured for the T22G drkN SH3 domain by the IPAP-HSQC method.[24,25] A best fit of the measured dipolar couplings to those predicted on the basis of the NMR derived structure of the wild-type protein gave values of the axial and rhombic components of the alignment tensor,[26] A_a and R, of -7.1×10^{-4} and 0.50, respectively, and Euler angles describing the transformation from the NMR coordinate frame to the alignment frame of (66°,88°,24°).

Results and Discussion

Theoretical Background. Parts a and b of Figure 1 illustrate the pulse schemes that were employed to measure the one bond $^{13}C^{methyl}-^{13}C$ dipolar (a) and the $^2H^{methyl}$ quadrupolar (b) couplings in a uniformly ^{13}C, fractionally ^{2}H-labeled sample of T22G drkN SH3. The schemes are very similar to sequences published previously by our group for measuring ^{2}H relaxation times[6] and will therefore be described only briefly. The flow of magnetization in the experiment of Figure 1a can be summarized by

$$^1H \xrightarrow{^1J_{CH}} {}^{13}C\ (\mathbf{T},\text{CT-}t_1) \xrightarrow{^1J_{CH}} {}^1H\ (t_2) \qquad (1)$$

where $^1J_{ij}$ is the active coupling responsible for the transfer and t_i (i = 1,2) is an evolution time. Note that in the case where the protein is partially aligned with the magnetic field, $^1J_{ij} = {}^1J_{ij} + {}^1D_{ij}$, where $^1J_{ij}$ and $^1D_{ij}$ are scalar and dipolar couplings,

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respectively. (In what follows couplings denoted by the italicized character "*J* " will refer to sums of dipolar and scalar terms). A series of experiments are recorded where the value, **T**, is incremented. Thus, a set of 2D planes are obtained with methyl cross-peaks at (ω^{13}C,ω^{1}H) modulated in intensity according to

$$A\cos\{\pi({}^1J_{CC}+{}^1D_{CC})T'\}\,[\,\Pi\cos\{\pi({}^kJ_{CC}+{}^kD_{CC})T'\}]\exp(-T'/T_2) \quad (2)$$

where $k \geq 2$, the product is over all $^{13}C^{methyl}-^{13}C^{aliphatic}$ couplings, $T' = 2T_C + \mathbf{T}$, T_2 is the effective transverse relaxation time of the ^{13}C signal during T' and

$${}^1D_{CC} = -\gamma_C{}^2h/(4\pi^2R_{CC}{}^3)\langle 3\cos^2\theta - 1\rangle \quad (3)$$

Note that in the case of Met residues $^1J_{CC} = {}^1D_{CC} = 0$. In eq 3 γ_C is the gyromagnetic ratio of ^{13}C, R_{CC} is the one-bond $^{13}C^{methyl}-^{13}C$ distance, and θ is the angle between the $^{13}C^{methyl}-^{13}C$ axis and the magnetic field. In the absence of media which induce alignment of the solute $\langle 3\cos^2\theta - 1\rangle = 0$ and $^1D_{CC}$ vanishes. Hence, by measuring $^1J_{CC}$ for both oriented and unoriented protein samples the value of $^1D_{CC}$ can be extracted in a straightforward manner, as described in Materials and Methods. It is noteworthy that because the interchange between inphase and antiphase $^{13}C^{methyl}$ magnetization arising from $^1J_{CC} + {}^1D_{CC}$ couplings is sufficiently fast compared to the differences in relaxation of the inphase and antiphase components a single effective relaxation time-constant can be used[27] (eq 2). This situation is to be contrasted with the evolution of ^{2}H magnetization, described below.

The transfer of magnetization in the sequence of Figure 1b proceeds as

$${}^1H \xrightarrow{{}^1J_{CH}} {}^{13}C \xrightarrow{{}^1J_{CD}} {}^2H\,(\mathbf{T}) \rightarrow {}^{13}C\,({}^1J_{CD}, \text{CT-}t_1) \xrightarrow{{}^1J_{CH}} {}^1H\,(t_2) \quad (4)$$

where the active couplings involved in magnetization transfer are indicated.

During the period of duration **T**, ^{2}H magnetization evolves according to[28]

$$D_x \rightarrow D_x\cos(\pi\nu_Q\mathbf{T}) + \{D_zD_y + D_yD_z\}\sin(\pi\nu_Q\mathbf{T}) \quad (5)$$

where the effects of relaxation have been neglected for the moment, D_i is a spin operator denoting i ($i = x,y,z$) magnetization and ν_Q is the splitting (Hz) between the two lines in deuterium spectra of an oriented deuteron. The value of ν_Q is given by

$$\nu_Q = 0.75\,(e^2qQ/h)\langle 3\cos^2\alpha - 1\rangle \quad (6)$$

where α is the angle between the principal axis of the (assumed axially symmetric) electric field gradient tensor and the magnetic field. Note that in the absence of alignment, $\langle 3\cos^2\alpha - 1\rangle = 0$ and therefore $\nu_Q = 0$ and there is no modulation of the ^{2}H signal.

In practice the effects of relaxation during the evolution of D_x cannot be neglected. It can be shown that the relaxation rates of the operators D_x and $\{D_zD_y + D_yD_z\}$ are given by

$$R_I = C[6J(0) + 10J(\omega_D) + 4J(2\omega_D)] \quad (7)$$

and

$$R_A = C[6J(0) + 2J(\omega_D) + 4J(2\omega_D)]$$

respectively, where $C = (3 \times 4\pi^2/160)\,(e^2qQ/h)^2$, $J(\omega) = S^2\tau_C/[1 + (\omega\tau_C)^2] + (1 - S^2)\,\tau_i/[\,1 + (\omega\tau_i)^2\,]$, τ_C is the overall tumbling time of the protein, S is an order parameter describing the spatial restriction of motion of the $^{13}C-{}^2H$ methyl vector on the ns−ps time scale[29,30] and $\tau_i^{-1} = \tau_C^{-1} + \tau_e^{-1}$ with τ_e the effective correlation time describing the internal motions of the $^{13}C-{}^2H$ bond vector. For the degree of alignment of solute employed in the present study it is not necessarily the case that exchange between D_x and $\{D_zD_y + D_yD_z\}$ components proceeds rapidly compared to $(R_I - R_A)$ (i.e., $4\pi^2\nu_Q^2 \gg (R_I - R_A)^2$). Note that for the T22G drkNSH3 domain $R_I - R_A$ values are predicted to vary between $\sim$2 s^{-1} and 30 s^{-1} on the basis of ^{2}H relaxation measurements of the unaligned molecule, while -67 Hz $\leq \nu_Q \leq$ 97 Hz. The evolution of D_x must therefore be described according to

$$D_x(\mathbf{T}) = A/2\exp\{-0.5(R_I + R_A)\mathbf{T}\}[\,(1-\Delta)\exp(0.5\Omega\mathbf{T}) + (1+\Delta)\exp(-0.5\Omega\mathbf{T})] \quad (8)$$

where $\Omega = \{\,(R_I - R_A)^2 - 4\pi^2\nu_Q^2\,\}^{0.5}$ and $\Delta = (R_I - R_A)/\Omega$. In the limit that relaxation can be neglected $D_x(\mathbf{T}) = A\cos(\pi\nu_Q\mathbf{T})$, consistent with eq 5. The pulse scheme of Figure 1b generates $^{13}C-{}^1H$ correlation spectra where the intensity of each cross-peak is modulated according to eq 8. The value of $|\nu_Q|$ is thus readily extracted from a fit of peak intensities as a function of **T**. Although the sign of ν_Q cannot be determined from the present set of experiments, it is possible to infer the sign for each residue by noting the sign of the corresponding $^1D_{CC}$ value. As shown below (see eq 10) ν_Q and $^1D_{CC}$ are of the same sign, assuming $e^2qQ/h > 0$ and that the angle between the methyl averaging axis and the C−D bond lies between 54.7° and 125.2°.

It is worth noting that in the experiments of Figure 1 signal derived from $^{13}CH_2D$ methyl groups exclusively is selected. Although it is not possible to produce ^{13}C labeled proteins where each methyl is labeled with only a single deuteron, it is very straightforward to generate samples which are fractionally deuterated by expressing protein in a medium containing both D_2O and H_2O. In this way, protein samples are prepared where $^{13}CD_3$, $^{13}CHD_2$, $^{13}CH_2D$, and $^{13}CH_3$ methyls are populated. Selection of only a single isotopomer is crucial to ensure spectra of high resolution, necessary for accurate quantitation of each cross-peak intensity. We choose to select for CH_2D methyls since in this case the magnetization transfer pathway can be manipulated in a straightforward manner to produce transverse ^{2}H signal with high sensitivity. Selection of single-deutero methyl groups occurs via the 90_x90_ϕ ^{1}H pulse pair in the sequence in a manner described in detail previously.[6] Figure 2 shows 2D $^{13}C-{}^1H$ correlation maps of T22G drkN SH3 recorded with the schemes of Figure 1, illustrating the level of isotopomer suppression that has been achieved.

Data Analysis. The time evolution of ^{13}C and ^{2}H magnetization from Ile 53 δ1 (a) and Leu 28 δ1 (b) methyl groups of T22G drkN SH3 dissolved in buffer with 33 mg/mL Pf1 phage is shown in Figure 3. Values of $^1D_{CC}$ and ν_Q have been measured for 20 and 19 residues, respectively. We have excluded data from 2 Leu residues (Leu 17, Leu 41) where strong $^{13}C^{\delta}-{}^{13}C^{\gamma}$ coupling complicates analysis as well as from a number of

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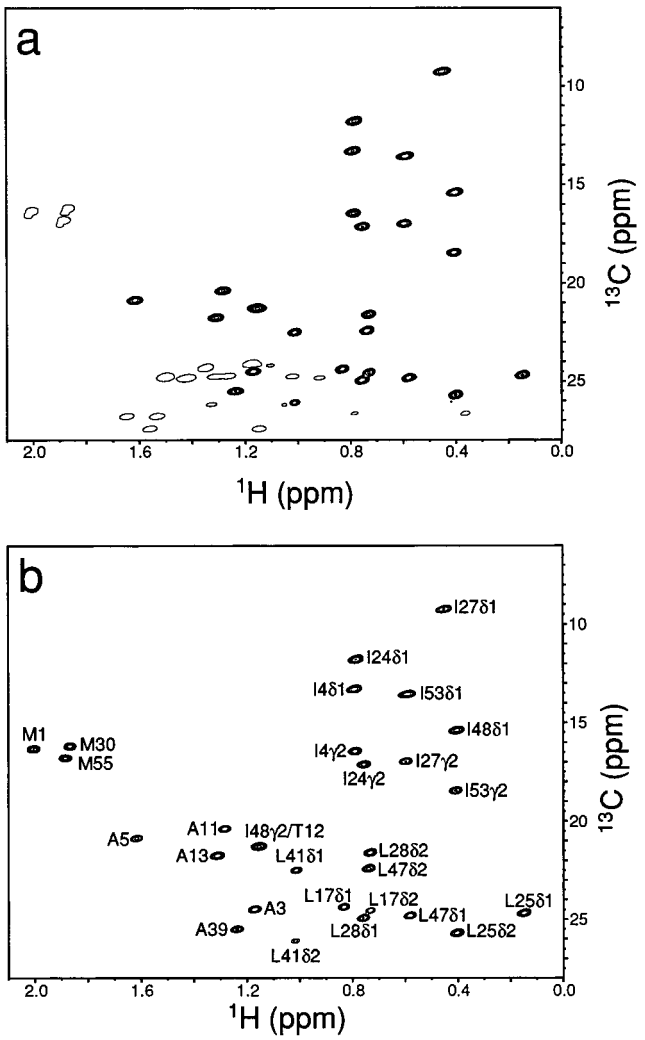

Figure 2. $^{13}C-^1H$ correlation maps of $^{15}N,^{13}C$, ~50% fractionally ^2H-labeled T22G drkN SH3 recorded at 600 MHz (^1H frequency) using the pulse schemes of Figure 1a (a) and 1b (b). Suppression of the CHD_2 and CH_3 isotopomers occurs as described in detail previously.[6] The appearance of CH_3 isotopomers in the case of Met residues (^1H chemical shift of ~2 ppm) in (a) results from the larger than average $^1J_{CH}$ coupling for Met (139 Hz vs 126 Hz) and the fact that delays for purging were optimized for a $^1J_{CH}$ of ~130 Hz. Negative peaks are drawn with a single contour. Note that in (a) CH_3 and CH groups are purged, and hence correlations involving methylene groups will be obtained. In (b) an additional filter involving selection of carbons with at least one bound ^2H spin ensures that the only correlations observed are those of CH_2D groups.

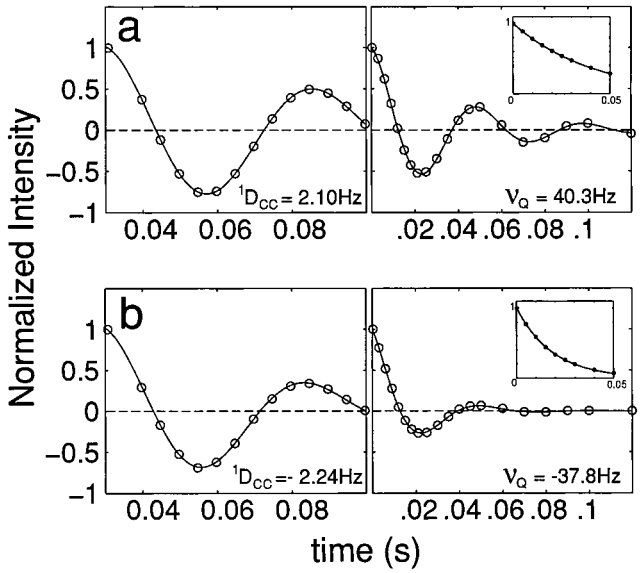

Figure 3. Evolution of ^{13}C and ^2H transverse magnetization as a function of delay **T** (see schemes of Figure 1) for Ile 53 $\delta1$ (a) and Leu 28 $\delta1$ (b) of T22G drkN SH3 partially oriented with Pf1 phage (~33 mg/mL). Insets in the upper right-hand corners show the evolution of ^2H transverse magnetization in the absence of phage.

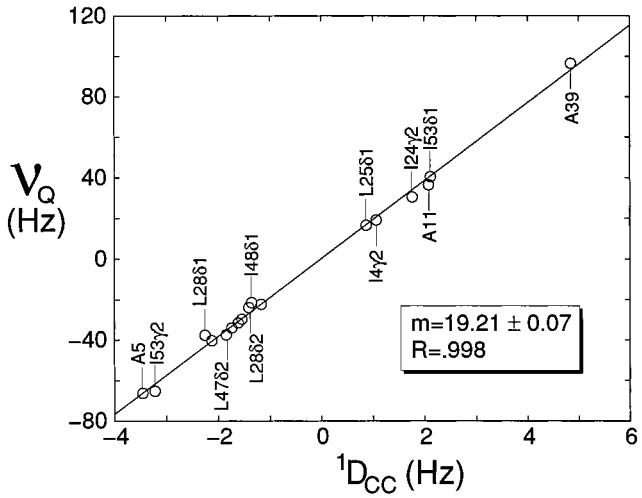

Figure 4. Correlation of the ^2H quadrupolar splitting (ν_Q) with the one-bond $^{13}C^{methyl}-^{13}C$ dipolar coupling, $^1D_{CC}$, for the T22G drkN SH3 domain.

residues (Ile 4 $\delta1$, Ile 27 $\gamma2$, Met 55 and Ala 13) where it is difficult to extract accurate ν_Q values because the signal decayed prior to the first zero crossing. The precision of $^1D_{CC}$ and ν_Q values was assessed by measuring $^1D_{CC}$ twice and ν_Q three times; reproducibility plots of dipolar or quadrupolar couplings have slopes deviating from 1 by less than 0.004 and R values greater than 0.9997.

Figure 4 shows the correlation between measured ν_Q and $^1D_{CC}$ values. A best linear fit of ν_Q vs $^1D_{CC}$ gives $\nu_Q = (19.21 \pm 0.07) \times {}^1D_{CC} + (0.20 \pm 0.12)$, while a slope of 19.20 ± 0.07 is obtained if the y-intercept is fixed at 0. From eqs 3 and 6

$$\nu_Q/{}^1D_{CC} = -0.75\,(e^2qQ/h)\,\langle 3\cos^2\alpha - 1\rangle / \{\gamma_C{}^2h/(4\pi^2R_{CC}{}^3)\,\langle 3\cos^2\theta - 1\rangle\} \quad (9)$$

Assuming that the methyl group rotates about the $^{13}C^{methyl}-^{13}C$ bond (or executes equaprobable 3-site jumps about this axis) we can write $\langle 3\cos^2\alpha - 1\rangle = 0.5\,(3\cos^2\beta - 1) \times \langle 3\cos^2\theta - 1\rangle$, where β is the angle between the principal axis of the electric field gradient and the $C^{methyl}-C$ bond. Equation 9 can therefore be simplified as

$$\nu_Q/{}^1D_{CC} = -0.75\,(e^2qQ/h)P_2(\cos\beta)/\{\gamma_C{}^2h/(4\pi^2R_{CC}{}^3)\} \quad (10)$$

where $P_2(x) = (3x^2 - 1)/2$. Note that the ratio of $\nu_Q/{}^1D_{CC}$ is independent of $\langle 3\cos^2\theta - 1\rangle$. Thus, the details of both magnetic alignment and of side chain motions, outside of rotation about the $C^{methyl}-C$ axis, are factored out by considering the ratio of couplings in eq 10.

To evaluate the product $(e^2qQ/h)P_2(\cos\beta)$ an accurate value for R_{CC} must be known. Engh and Huber report C−C bond lengths of 1.521 and 1.513 Å for $CH-CH_3$ and CH_2-CH_3

fragments,[31] respectively, and in what follows we use an average value of 1.517 Å and assume an error of ±0.004 Å. Using eq 10 and a slope of 19.21 ± 0.07 from Figure 4 it follows that $(e^2qQ/h)P_2(\cos\beta) = (-5.55 \pm 0.05) \times 10^4$ Hz. Assuming a value of 168 kHz for e^2qQ/h gives $109.5° \leq \beta \leq 109.9°$. Alternatively, if a β value of 109.5° is used, then $e^2qQ/h = 166.8 \pm 1.5$ kHz. A (β,e^2qQ/h) pair of (109.5°,167 kHz) agrees favorably with values of 109.5° and 165−168 kHz that we have used in previous analyses of ^{2}H methyl dynamics.[7,11,12] It is noteworthy that the value of β we have obtained from this analysis is approximately 1° smaller than the $H^{methyl}-C^{methyl}-C$ angle (110.9°) reported by Ottiger and Bax[5]. This difference could be due to the fact that CH_2D methyls were considered in the present study, while CH_3 groups were studied by the Bax group. In addition, what is measured here is the angle between the unique axis of the assumed axially symmetric electric field gradient tensor and the methyl rotation axis, β, and this could well be slightly different than the $H^{methyl}-C^{methyl}-C$ angle in either CH_2D or CH_3 methyl groups.

Correlation between ν_Q and $^1D_{CC}$. Figure 4 illustrates that there is a strong linear correlation between ν_Q and $^1D_{CC}$, with an rmsd between the experimental points and those predicted from the best fit line of 2.58 Hz (ν_Q) and 0.13 Hz ($^1D_{CC}$). A number of residues do, however, show deviations from the line that are in excess of the presumed errors based on the reproducibility of the measurements (on average, 0.24 and 0.01 Hz for ν_Q and $^1D_{CC}$, respectively). These deviations do not necessarily reflect nonuniform values for the electric field gradient tensor; indeed they can be explained on the basis of a number of factors including noncollinearity of the methyl rotation axis and the $C^{methyl}-C$ bond,[5] departures from axial symmetry of the electric field gradient,[32,33] variations in the angle β from one residue to the next, per-residue differences in the $^{13}C^{methyl}-^{13}C$ bond length and nonequaprobable methyl rotamer populations (resulting from the introduction of a deuteron). Differences in expected $\nu_Q/^1D_{CC}$ ratios may also arise from electrostatic contributions from the surrounding amino acids. A number of these factors are discussed below.

For a deuteron in a methyl group undergoing fast reorientation, solid state spectra show, in general, axially symmetric powder patterns.[34] Of particular importance to the study here, ^{2}H spectra of deutero-labeled methyl groups in polycrystalline solids of Ala, Val, Thr, and Leu at low temperature are axially symmetric, consistent with a dominant motion of rotation about the methyl averaging axis[34] and very small intrinsic asymmetry parameters. Thus, for the methyl containing amino acids it appears that the distribution of charge density within the amino acids does not contribute to asymmetry of the electric field gradient. As the temperature of measurement is increased the quadrupolar line shape can be further narrowed by additional motion of the methyl group, leading in some cases to quite distinct axially asymmetric line shapes, as observed in studies of deutero-Leu in proteins.[35,36] For example, Torchia and coworkers have measured ^{2}H spectra of deutero-Leu in collagen with large asymmetry parameters and interpreted this in terms of (χ^1,χ^2) side chain rotamer exchange.[36] The effects of such dynamics will not influence the results reported here since contributions from motions distinct from rapid rotation about the $C^{methyl}-C$ bond are factored out by taking the ratio of ν_Q to $^1D_{CC}$ (i.e., these additional motions affect ν_Q and $^1D_{CC}$ in the same manner, see eq 9). As a check we have excluded Leu residues from the analysis described above and found that this changes the average e^2qQ/h value by only a very small amount (increase of <1%). Note also that the residues that deviate from the best fit line of Figure 4 are not restricted to a particular amino acid type.

Equation 10 shows that the ratio between quadrupolar and dipolar couplings is a function of the angle β between the principal axis of the electric field gradient and the rotation axis, assumed in the present paper to be collinear with the one-bond $^{13}C^{methyl}-^{13}C$ axis. The angular dependence is rather steep in the vicinity of $\beta \approx 109.5°$. For example, a 2° increase in β translates into an 11% decrease in $P_2(\cos\beta)$. Per-residue variations in β by 2° or less can account for nearly all deviations from the best-fit line in Figure 4.

In a recent paper Ottiger and Bax discussed how deviations of the methyl rotation axis from the $^{13}C^{methyl}-^{13}C$ axis can affect the ratio of one-bond methyl $^{13}C-^{13}C$ and $^{13}C-^1H$ dipolar couplings.[5] On the basis of the deviations from linearity observed in the correlation between these dipolar couplings they concluded that these axes were at most 0.8° removed from collinearity. In principle, differences in methyl rotation and $^{13}C^{methyl}-^{13}C$ axes could also introduce the discrepancies between ν_Q and $^1D_{CC}$ observed in Figure 4, and we have performed simulations to establish whether this is the case. It is straightforward to show that residues with $^{13}C^{methyl}-^{13}C$ axes at ∼45° to the *z*-axis of the alignment frame will be most sensitive to this effect and in this case deviations of 1° can change ν_Q values by as much as 11% (assuming an axially symmetric alignment frame). Using the alignment tensor parameters obtained from $^{15}N-^1HN$ dipolar coupling data (see Material and Methods) and the NMR structure of the wild-type drkN SH3 domain simulations establish that for most of the outlying residues in Figure 4 off-axis averaging can account for some, but not all, of the discrepancy. For example, the $^{13}C^{methyl}-^{13}C$ axis of Leu 28 δ1 lies in the $x-y$ plane of the alignment frame and ν_Q for this residue is thus very insensitive to small changes in the orientation of the averaging axis relative to the $^{13}C^{\delta}-^{13}C^{\gamma}$ bond. In contrast, in the case of Ala 39 the deviation from the line can be accounted for by a model which assumes a 1° difference between the averaging direction and the $^{13}C^{\alpha}-^{13}C^{\beta}$ bond.

Finally, we have explored the effects that neighboring charges make on values for e^2qQ/h using density functional theory calculations.[37] Values for e^2qQ/h for a methyl deuteron of Ala 11 were calculated assuming that the charges of the proximal amino acids, Asp 14, Asp 15, and Glu 16 were all either −1 or 0. Quadrupole coupling values varied by less than 1.5% between the two sets of calculations, suggesting that the effects of electrostatics are small.

Concluding Remarks

Measurement of methyl ^{2}H quadrupolar and $^{13}C^{methyl}-^{13}C$ dipolar couplings on a per-residue basis in an ^{15}N,^{13}C, fractionally ^{2}H labeled, partially aligned protein has allowed an evaluation of the product, $(e^2qQ/h) \times P_2(\cos\beta)$, where e^2qQ/h is

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the ^2H quadrupolar coupling constant and β is the angle between the unique axis of the assumed axially symmetric electric field gradient tensor and the methyl averaging axis. Assuming $\beta = 109.5°$, a value of 167 ± 1.5 kHz is obtained from the slope of ν_Q vs $^1D_{CC}$, in excellent agreement with values measured on small organic molecules.[17] The strong linear correlation between ν_Q vs $^1D_{CC}$, indicates that accurate dynamics parameters can be extracted from ^2H-based relaxation data using a constant value for e^2qQ/h of 167 kHz and $\beta = 109.5°$.

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