Residual Dipolar $^1H-^1H$ Couplings of Methyl Groups in Weakly Aligned Proteins

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Residual dipolar couplings measured for weakly aligned proteins provide important restraints for molecular structure determinations by NMR[1] spectroscopy which cannot be obtained otherwise.[2] Residual dipolar couplings are usually measured by comparing multiplet splittings measured in anisotropic phase with those measured in isotropic phase.[2,3] In the absence of scalar couplings, a residual dipolar coupling between two spins in the weak-coupling limit is directly manifested in a doublet splitting, but the sign of the coupling is more difficult to determine.[4,5] Here we show that the sign and magnitude of residual dipolar couplings between the protons of a methyl group are readily measured in a single experiment. These resulting splittings are larger than those due to intra-methyl residual dipolar couplings between ^{13}C and ^1H spins, and they depend on the molecular alignment tensor in a way completely analogous to residual dipolar couplings in two-spin systems.[6] They are thus straightforward to use as structural parameters.

Dipolar couplings lead to line splittings even for isolated methyl groups.[7] The dipolar contribution to the ^1H NMR spectrum of an isolated methyl group is determined by the secular part of the dipolar Hamiltonian which can be decomposed into products of spatial and spin terms:[8]

$$H_d = \sum_{i<j=1}^{3} B_{ij} S_{ij} \tag{1}$$

where $B_{ij} = (\mu_0/4\pi)(\gamma_H^2\hbar/r_{HH}^3)^1/_2(1 - 3\cos^2\theta_{ij})$ and $S_{ij} = 3H_{zi}H_{zj} - H_iH_j$. θ_{ij} is the angle between the magnetic field and the internuclear vector connecting the nuclei i and j, r_{HH} is the internuclear distance, and γ_H is the proton magnetogyric ratio.

For fast reorientation of the methyl group around its C_3 symmetry axis, the effective part of H_d can be expressed in a symmetry-adapted fashion which includes only the fully symmetric part of the Hamiltonian:[9,10]

$$H_d^A = \frac{1}{2}\left(\frac{\mu_0}{4\pi}\right)\left(\frac{\gamma_H^2\hbar}{r_{HH}^3}\right)\frac{1}{2}(3\cos^2\theta - 1)\sum_{i<j=1}^{3} S_{ij} \tag{2}$$

where θ is the angle between the C_3 symmetry axis and the magnetic field. This Hamiltonian results in a triplet with relative line intensities of 1:2:1[7,9] and a line separation of

$$d_{HH} = \frac{3}{4}\left(\frac{\mu_0}{4\pi}\right)\left(\frac{\gamma_H^2\hbar}{r_{HH}^3}\right)(3\cos^2\theta - 1) \tag{3}$$

This is completely equivalent to the dipolar splitting between two weakly coupled protons, except for a scaling factor of $^3/_4$. For weak molecular alignment, the line separation in the triplet, D_{HH}, which depends on the axial component D_A and the rhombicity R of the alignment tensor, defined in the usual way,[11] as:

$$D_{HH} = \frac{3}{4}D_A\left\{(3\cos^2\vartheta - 1) + \frac{3}{2}R(\sin^2\vartheta\cos 2\phi)\right\} \tag{4}$$

where ϑ denotes the angle between the C_3 axis of the methyl group and the z axis of the tensor, and ϕ is the angle between the x axis of the tensor and the projection of the C_3 axis onto the $x-y$ plane.

The pulse sequence of Figure 1 was designed to measure the separation between the two outermost lines of the triplet by creating antiphase magnetization which suppresses the central resonance of the triplet, resulting in a peak separation of $2D_{HH}$. This magnetization is created via one-bond $^{13}C-^1H$ couplings, allowing the determination of the sign of D_{HH} with respect to that of the heteronuclear one-bond coupling.

Considering evolution only under the large, predominant heteronuclear one-bond couplings, and disregarding for simplicity signs and coefficients, the relevant coherence transfer pathway achieved by the pulse sequence of Figure 1 can be written as

$$\begin{aligned} H_{1z} \xrightarrow{90^0_x(H)} H_{1y} \xrightarrow{\Delta} H_{1x}C_z \xrightarrow{90^0_y(H),90^0_x(C)} H_{1z}C_y \xrightarrow{\Delta(t_1)} \\ H_{2z}H_{3z}C_x \xrightarrow{90^0_x(H),90^0_y(C)} H_{2y}H_{3y}C_z \xrightarrow{\Delta/2} H_{2x}H_{3y} + \\ H_{2y}H_{3x} \xrightarrow{90^0_x(H)} H_{2x}H_{3z} + H_{2z}H_{3x} \end{aligned} \tag{5}$$

As all three methyl protons are equivalent, a complete description starts from $H_{1z}+H_{2z}+H_{3z}$, resulting in the density matrix $\sigma_{acq} = H_{1x}H_{2z}+H_{1z}H_{2x}+H_{1x}H_{3z}+H_{1z}H_{3x}+H_{2x}H_{3z}+H_{2z}H_{3x}$. Since ^{13}C decoupling is applied during the acquisition time, the relevant terms of the Hamiltonian are:

$$H = \delta_H\sum_{i=1}^{3} H_{iz} + \left(J_{HH} + \frac{1}{3}D_{HH}\right)\sum_{i<j=1}^{3} H_iH_j - D_{HH}\sum_{i<j=1}^{3} H_{iz}H_{jz} \tag{6}$$

All of these terms commute with each other, and the second term commutes with σ_{acq}. Therefore, the evolution of σ_{acq} during the acquisition time can be interpreted as for the case of weak scalar coupling, that is the triplet assumes an antiphase multiplet fine structure in the F_2 dimension with one positive and one negative line separated by $2D_{HH}$ and vanishing intensity of the central multiplet component.[12,13] The delay Δ is tuned to $1/(2^1J_{CH})$, assuming that the scalar coupling is much larger than the residual dipolar coupling. The final terms depend on $^1J_{CH}$ as $\sin^4(\pi^1J_{CH}\Delta) \times \sin(\pi^1J_{CH}\Delta/2)\cos(\pi^1J_{CH}\Delta/2)$ and refocus during acquisition by evolution under D_{HH} as $\sin(\pi D_{HH}t_2)\cos(\pi D_{HH}t_2)$. Therefore, the sign of the cross-peak reflects the relative sign of D_{HH} and $^1J_{CH}$.

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(1) Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; C12E5, *n*-dodecyl-penta(ethylene glycol); NMR, nuclear magnetic resonance.

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(13) Homonuclear CSA/dipolar cross-correlated relaxation renders σ_{acq} observable also in the absence of dipolar splittings, but the resulting cross-peaks are relatively weak.

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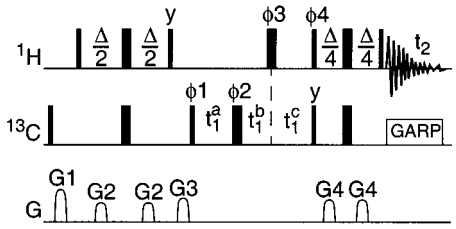

Figure 1. Pulse scheme of the DiM ("dipolar couplings in methyls") experiment. Narrow and wide bars denote 90° and 180° pulses, respectively. Pulses are applied along the *x*-axis, unless indicated otherwise. $\Delta = 1/(2\ ^1J_{CH})$. Chemical shift evolution during t_1 is achieved in a semiconstant manner, with $t_1^a = t_1^c = \Delta/2$ and $t_1^b = 0$ for the initial t_1 value. t_1^a is decremented in steps of $\Delta/(2N)$, and t_1^b and t_1^c are incremented by $(t_{1max} - \Delta)/(2N)$ and $t_{1max}/(2N)$, respectively, where N is the number of increments and t_{1max} is the maximum total evolution time chosen. N depends on the sweepwidth in hertz in the ^{13}C-dimension, SW, through $N = t_{1max}\ SW$. Phase cycle: $\phi_1 = x,-x$; $\phi_2 = x,x,y,y,-x,-x,-y,-y$; $\phi_3 = 16(x),16(-x)$; $\phi_4 = 8(x),8(-x)$; receiver $= x,-x,-x,x$. Gradient pulses were applied with a sine shape and the following durations (maximum amplitudes): $G_{1,2,3,4} = 1.0$ (25), 0.5 (5), 1.0 (12.5), 0.5 (9) ms (G/cm).

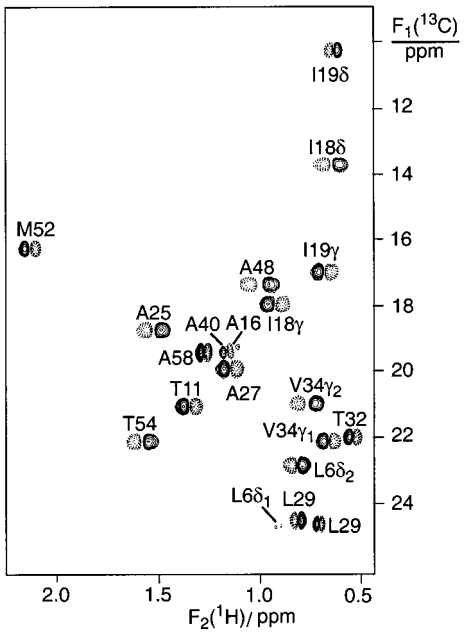

Figure 2. DiM spectrum recorded of a 10 mM solution of BPTI at natural isotopic abundance in 90% H_2O/10% D_2O containing 5% C12E5/*n*-hexanol at 30 °C, pH 4.7. The spectrum was recorded on a Bruker DMX-600 NMR spectrometer with a total recording time of 18 h. Other parameters were: $\Delta = 3.7$ ms, $t_{1max} = 20$ ms, $t_{2max} = 146$ ms. Positive and negative contour levels are distinguished by solid and dashed lines. The methyl resonances are labeled with their assignment.

For experimental verification, a spectrum was recorded for BPTI at natural isotopic abundance in the presence of a dilute liquid crystal composed of 5% C12E5/*n*-hexanol.[14] The spectrum displayed significant intensities only for cross-peaks from methyl groups (Figure 2). Cross-peaks were observed for all methyl groups with good sensitivity. Independent measurements of D_{CH} from ^{13}C-HSQC spectra with α/β-half-filter in the F_2 dimensions,[15] recorded in isotropic and liquid crystalline phase, correlated with the $2D_{HH}$ splittings as measured by the peak-to-peak separation in the antiphase multiplets (Figure 3). Mutual cancellation of signal intensities increases the apparent line splitting, an effect

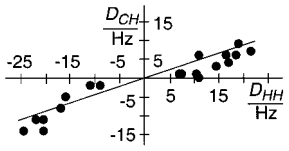

Figure 3. Plot of D_{CH} versus D_{HH} values measured for BPTI at 30 °C, pH 4.6 in the presence of 5% C12E5/*n*-hexanol. The peak-to-peak separations observed in the spectrum of Figure 2 correspond to $2D_{HH}$. No correction for cancellation effects[16] was applied. The solid line indicates the correlation $D_{HH} = 2.3D_{CH}$.

which is particularly pronounced for small couplings,[16] compromising the correlation between these two types of dipolar couplings.

Residual dipolar C−H couplings in methyl groups result in splittings, D_{CH}, that depend on the alignment tensor in a way similar to that for D_{HH}. In general, the dipolar splitting d_{CH} can be described as:

$$d_{CH} = \frac{\gamma_C\gamma_H\hbar}{r_{CH}^3}\left(\frac{\mu_o}{4\pi}\right)(1 - 3A^2\cos^2\theta - 6AB\cos\theta\sin\theta\cos\Psi - 3B^2\sin^2\theta\cos^2\Psi) \quad (7)$$

where $A = \sqrt{3r_{CH}^2 - r_{HH}^2}/\sqrt{3}r_{CH} = \cos\theta_1$, where θ_1 is the angle between the CH vector and the C_3 symmetry axis, $B = r_{HH}/\sqrt{3}r_{CH}$, θ is the angle between the C_3 axis and the magnetic field, and Ψ the rotation angle around the C_3 axis. Averaging over the rotation angle Ψ leads to

$$d_{CH} = \frac{\gamma_C\gamma_H\hbar}{r_{CH}^3}\left(\frac{\mu_o}{4\pi}\right)\frac{1}{2}(3A^2 - 1)(1 - 3\cos^2\theta) \quad (8)$$

As in the derivation of the corresponding equation for d_{HH} (eq 4),[9] this result is independent of whether the methyl-group rotation is isotropic or by exchange between three distinct rotamers. For a methyl group, $A^2 \cong 0.13$[17] and thus $D_{HH} \cong 2.3D_{CH}$, as reflected by Figure 3.

Residual dipolar ^{1}H−^{13}C couplings of methyl groups have been shown to correlate well with predictions based on the three-dimensional structure of a protein.[18] Equation 4 can be used like eq 8 in existing programs for structure refinement. For BPTI in a dilute liquid crystal, our new experiment (Figure 1) was about as sensitive as a HSQC spectrum recorded without decoupling, where the large intra-methyl D_{HH} splittings resulted in significant line broadening. The new experiment should be particularly useful in combination with isotope-labeling schemes, where the protein is perdeuterated except for the methyl groups,[19,20] as such a labeling pattern would reduce the line widths of the methyl resonances by avoiding additional scalar and residual dipolar couplings with non-methyl protons.

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Supporting Information Available: A table with the assignments of the multiplet splittings D_{HH} and D_{CH} reported in Figure 3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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