Residual Dipolar $^1$H—$^1$H 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 spectroscopy which cannot be obtained otherwise. Residual dipolar couplings are usually measured by comparing multiplet splittings measured in anisotropic phase with those measured in isotropic phase. In the absence of scalar couplings, a residual dipolar coupling between two spins in the weak-coupling limit is directly manifested in a single experiment. These resulting splittings are larger than those between the protons of a methyl group are readily measured in a two-spin system. They are thus straightforward to use as structural parameters.

Dipolar couplings lead to line splittings even for isolated methyl groups. The dipolar contribution to the $^1$H 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:

$$H_d = \sum_{i \neq j}^3 B_i S_{ij}$$

where $B_i = (\mu_s/4\pi)(y_i^2/I_i^{1H})/2(1 - 3 \cos^2 \theta_i)$ and $S_{ij} = 3H_iH_j - H_iH_j$, $\theta_i$ is the angle between the magnetic field and the internuclear vector connecting the nuclei $i$ and $j$, and $r_{ij}$ is the internuclear distance, and $y_i^1$ is the proton magnetogyric ratio.

For fast reorientation of the methyl group around its 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:

$$H_d = \frac{1}{2} \sum_{i \neq j}^3 \left[ B_i S_{ij} \right] \left( \cos \theta - 1 \right)$$

where $\theta$ is the angle between the $C_i$ symmetry axis and the magnetic field. This Hamiltonian results in a triplet with relative line intensities of 1:2:1 and a line separation of

$$d_{1H} = \frac{3}{4} \frac{\mu_s}{4\pi r_{1H}^3} \left( \left( \frac{y_i^1 H_i}{I_i^{1H}} \right)^2 \right) (3 \cos^2 \theta - 1)$$

This is completely equivalent to the dipolar splitting between two weakly coupled protons, except for a scaling factor of $1/3$.

For weak molecular alignment, the line separation in the triplet, $D_{1H}$, which depends on the axial component $D_x$ and the rhombicity $R$ of the alignment tensor, defined in the usual way, as:

$$D_{1H} = \frac{2}{4} \frac{\mu_s}{4\pi r_{1H}^3} \left( (3 \cos^2 \theta - 1) + \frac{3}{2} \mu_s (\sin^2 \theta \cos 2\phi) \right)$$

where $\theta$ denotes the angle between the $C_i$ axis of the methyl group and the $z$ axis of the tensor, and $\phi$ is the angle between the $x$ axis of the tensor and the projection of the $C_i$ 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 $2D_{1H}$. This magnetization is created via one-bond $^{13}$C—$^1$H couplings, allowing the determination of the sign of $D_{1H}$ 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

$$H_1 \rightarrow J_{1C} \rightarrow H_{12} \rightarrow H_{13} \rightarrow H_{1C}$$

As all three methyl protons are equivalent, a complete description starts from $H_{1x}+H_{1y}+H_{1z}$, resulting in the density matrix $\sigma_{aq}$ with $H_{aq} = H_{1x}H_{2x}+H_{1y}H_{2y}+H_{1z}H_{2z}$, and $H_{31}$. Since $^{13}$C decoupling is applied during the acquisition time, the relevant terms of the Hamiltonian are:

$$H = \delta (\sum_{i=1}^3 H_{1i} + J_{1C} \sum_{i=1}^2 H_{1i}) - D_{1H} \sum_{i=1}^3 H_iH_j$$

where $\delta$ is the evolution time.

For weak alignment, the equations can be simplified as:

$$H = \delta (\sum_{i=1}^3 H_{1i} + J_{1C} \sum_{i=1}^2 H_{1i}) - D_{1H} \sum_{i=1}^3 H_iH_j$$

All terms of these equations commute with $\sigma_{aq}$. Therefore, the evolution of $\sigma_{aq}$ 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 $F_3$ dimension with one positive and one negative line separated by $2D_{1H}$ and vanishing intensity of the central multiplet component. The final terms depend on $J_{1C} \sin (\pi/2)$ and $\cos (\pi/2)$ and reductiof during acquisition by evolution under $D_{1H}$ as $\sin (\pi/2)$ and $\cos (\pi/2)$. The sign of the cross-peak reflects the relative sign of $D_{1H}$ and $J_{1C}$.

(1) Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; C12E5, n-dodecyl-pentyl(ethylene glycol); NMR, nuclear magnetic resonance.
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. $\Delta = 1/(2 \tau_{t_{CH}})$. Chemical shift evolution during $t_1$ is achieved in a semiconstant manner, with $\tau_t^0 \approx \Delta/2(2N)$ and $\tau_t^0 = 0$ for the initial $t_1$ value. $\tau_t^0$ is decremented in steps of $\Delta/2(N)$, and $\tau_t^0$ are incremented by $(t_{\text{max}} - \Delta)/(2N)$ and $t_{\text{max}}/(2N)$, respectively, where $N$ is the number of increments and $t_{\text{max}}$ is the maximum total evolution time chosen. $N$ depends on the sweepwidth in hertz in the $^1$H-dimension, $SW$, through $N = t_{\text{max}}/SW$. Phase cycle: $\phi_1 = x,-x; \phi_2 = x,y,y,-x,-x,\ldots,y,-y; \phi_3 = 16(\Diamond)$. 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$_2$O/10% D$_2$O 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, $t_{\text{max}} = 20$ ms, $t_{\text{max}} = 146$ 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. 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 $D_{\text{CH}}$ from $^1$C-HSQC spectra with a/β-half-filter in the $F_2$ dimensions, recorded in isotropic and liquid crystalline phase, correlated with the $2D_{\text{HH}}$ 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 which is particularly pronounced for small couplings, compromising the correlation between these two types of dipolar couplings.

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

$$d_{\text{CH}} = \frac{\gamma_C \gamma_H^* H^0}{\gamma_{CH}^3} (1 - 3A^2 \cos^2 \theta)$$

where $A = \sqrt{\frac{3}{2} C_{\text{CH}} - \frac{2}{3} \frac{t_{\text{HF}}/\sqrt{3}}{C_{\text{CH}}}} \cos \theta_1$, where $\theta_1$ is the angle between the CH vector and the $C_3$ symmetry axis, $B = r_{\text{HF}}/\sqrt{3C_{\text{CH}}}$, $\theta$ is the angle between the $C_3$ axis and the magnetic field, and $\Psi$ the rotation angle around the $C_3$ axis. Averaging over the rotation angle $\Psi$ leads to

$$d_{\text{CH}} = \frac{\gamma_C \gamma_H^* H^0}{\gamma_{CH}^3} \frac{1}{4\pi} \frac{1}{2} (3A^2 - 1)(1 - 3 \cos^2 \theta)$$

As in the derivation of the corresponding equation for $d_{\text{HH}}$ (eq 4), this result is independent of whether the methyl-group rotation is isotropic or by exchange between three distinct rotamers. For a methyl group, $A^2 \approx 0.1317$ and thus $D_{\text{HH}} \approx 2.3D_{\text{CH}}$, as reflected by Figure 3.

Residual dipolar $^1$H—$^1$C couplings of methyl groups have been shown to correlate well with predictions based on the three-dimensional structure of a protein. 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, whereas the large intra-methyl $D_{\text{HH}}$ 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, 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 $D_{\text{HH}}$ and $D_{\text{CH}}$ reported in Figure 3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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