Magic-Angle-Pulse Driven Separation of Degenerate $^1$H Transitions in Methyl Groups of Proteins: Application to Studies of Methyl Axis Dynamics

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Single-transition operator description of the pulse-schemes in Figures 2A-B. Prior to the application of the pulse with flip-angle $\alpha$, the magnetization of a $^{13}$CH$_3$ methyl spin system, $2I_xC_y$, can be represented as a linear combination of fast-relaxing (outer; manifold $I = 3/2$; Figure 1, main text) and slow-relaxing (inner, manifold $I = 3/2$, and the two manifolds $I = 1/2$; Figure 1) coherences,

$$2I_xC_y = 2I_x^{3/2,F}C_y + 2I_x^{3/2,S}C_y + 2I_x^{1/2}C_y$$  \hspace{1cm} (S1.1)

where $A_Q$ is the $Q \in \{X, Y, Z\}$ component of spin operator $A$, the superscripts ‘3/2’ and ‘1/2’ indicate that the coherence derives from the $I = 3/2$ or 1/2 manifold, respectively, and the superscripts ‘F’ or ‘S’ indicate the fast and slowly relaxing coherences, respectively. Written in terms of individual transitions, with eigenfunctions $|j>$ defined as in Figure 1, the operators $I_Q$ are given by,

$$I_x^{3/2,F} = \frac{\sqrt{3}}{2}(|1><2| + |2><1| + |3><4| + |4><3|)$$  
$$I_x^{3/2,S} = 1(|2><3| + |3><2|)$$  \hspace{1cm} (S1.2)  
$$I_x^{1/2} = \frac{1}{2}(|5><6| + |6><5| + |7><8| + |8><7|)$$

Depending on the state in which magnetization is prepared, either the fast-(‘F’) or the slow-(‘S’)-relaxing transitions, or both, are present prior to the $^1$H$_\alpha$ pulse with flip-angle $\alpha$. For the purpose of analysis of the transformation properties of the magnetization in Eq. (S1) under the effect of radio-frequency (RF) pulses of arbitrary angles, the fast- and the slow-relaxing parts can be separated and treated independently of each other.

A formal derivation of the state of the density matrix of $^1$H magnetization for an arbitrary flip-angle $\alpha$ of the $^1$H$_\alpha$ pulse applied when the system is prepared in a state where only the slow-relaxing coherences are selected for (the second and third terms on the right-hand side of Eq. (S1.1), has been provided in the Supplementary Information of our previous publication related to the $I = 1/2$ manifold selection,$^1$ and is reproduced here in a concise form for the readers’ convenience. We first separate the density matrix onto the parts corresponding to the $I = 3/2$ and $I = 1/2$ manifolds (they evolve independently of each other under the effect of RF field), and define a $^1$H$_\alpha$ pulse as,
\[
\begin{pmatrix}
0 & -\sqrt{3}/2 & 0 & 0 \\
\sqrt{3}/2 & 0 & -1 & 0 \\
0 & 1 & 0 & -\sqrt{3}/2 \\
0 & 0 & \sqrt{3}/2 & 0 \\
\end{pmatrix}
\]

for the \( I = 3/2 \) manifold, and

\[
\begin{pmatrix}
0 & -1/2 & 0 & 0 \\
1/2 & 0 & 0 & 0 \\
0 & 0 & 0 & -1/2 \\
0 & 0 & 1/2 & 0 \\
\end{pmatrix}
\]

for the two \( I = 1/2 \) manifolds, operating on the column-vectors of eigenfunctions \([|1>, |2>, |3>, |4>|^T \) and \([|5>, |6>, |7>, |8>|^T \), respectively, where the eigenfunctions are defined in the energy level diagram of Figure 1, and the superscript ‘T’ denotes transposition. The forms of the density matrices describing the states of the \(^1\)H magnetization prior to the application of the \(^1\)H, pulse with flip angle \( \alpha \), are,

\[
\rho_s^{3/2} = \begin{pmatrix}
0 & 0 & 0 & 0 \\
0 & 0 & 1 & 0 \\
0 & 1 & 0 & 0 \\
0 & 0 & 0 & 0 \\
\end{pmatrix}
\]

for the \( I = 3/2 \) manifold, and

\[
\rho_s^{1/2} = \frac{1}{2} \begin{pmatrix}
0 & 1 & 0 & 0 \\
1 & 0 & 0 & 0 \\
0 & 0 & 1 & 0 \\
0 & 0 & 0 & 1 \\
\end{pmatrix}
\]

for the two \( I = 1/2 \) manifolds. The form of the density matrix of each manifold after the \(^1\)H, pulse as a function of angle \( \alpha \), is given by,\[1\]
\[
\rho^{S/2}_S(\alpha) = \frac{1}{4} \begin{bmatrix}
-3\sin^2\alpha & -3\sqrt{3}\cos\alpha(\cos^2\alpha-1) & \sqrt{3}\sin\alpha(3\sin^2\alpha-2) & 3\cos\alpha(\cos^2\alpha-1) \\
-3\sqrt{3}\cos\alpha(\cos^2\alpha-1) & 9\sin^3\alpha-8\sin\alpha & \cos\alpha(9\cos^2\alpha-5) & -\sqrt{3}\sin\alpha(3\sin^2\alpha-2) \\
\sqrt{3}\sin\alpha(3\sin^2\alpha-2) & \cos\alpha(9\cos^2\alpha-5) & 8\sin\alpha-9\sin^3\alpha & -3\sqrt{3}\cos\alpha(\cos^2\alpha-1) \\
3\cos\alpha(\cos^2\alpha-1) & -\sqrt{3}\sin\alpha(3\sin^2\alpha-2) & -3\sqrt{3}\cos\alpha(\cos^2\alpha-1) & 3\sin^3\alpha
\end{bmatrix}
\]

The corresponding density matrix of the two \(I = 1/2\) manifolds is,

\[
\rho^{1/2}_S(\alpha) = \frac{1}{2} \begin{bmatrix}
-\sin\alpha & \cos\alpha & 0 & 0 \\
\cos\alpha & \sin\alpha & 0 & 0 \\
0 & 0 & -\sin\alpha & \cos\alpha \\
0 & 0 & \cos\alpha & \sin\alpha
\end{bmatrix}
\]

Using Eqs. (S6) and (S7), it is straightforward to show that when the angle \(\alpha\) is equal to the ‘magic’ angle, \(\cos^{-1}(1/\sqrt{3}) = 54.73^\circ\), the elements [2,3] of the matrix in Eq. (S6) and the elements [1,2] or [3,4] of the matrix in Eq. (S7) are equal by absolute magnitude but opposite in sign: \((1/4)\cos\alpha(9\cos^2\alpha - 5) = -(1/2)\cos\alpha\). The cycling of the phase of the \(^1\text{H}\) pulse with flip-angle \(\alpha\) along \pm y together with concomitant retention of the receiver phase eliminates all the \(^1\text{H}\) coherences of even order (zero-quantum, ZQ, diagonal elements of the matrix; and double-quantum, DQ, elements [1,3], [2,4] and [3,1], [4,2]), as the inversion of the phase of this pulse changes the signs of only these latter terms, while preserving those of the \(^1\text{H}\) coherences of odd order. The density matrix after the application of the \(^1\text{H}\) pulse with flip-angle \(\alpha = \cos^{-1}(1/\sqrt{3})\) with phase cycle \pm y, can be written as,

\[
\rho_S = -(\sqrt{3}/6)I_{X}^{3/2,S}C_y + (\sqrt{3}/3)I_{X}^{1/2}C_y + (1/2)I_{X}^{3/2,F}C_y - (\sqrt{3}/6)I_{Y}^{3/2}C_y
\]

where \(I_{X}^{3/2,F}, I_{X}^{3/2,S}, I_{X}^{1/2,S}\) are defined in Eq. (S1.2), and the triple-quantum (‘TQ’) \(^1\text{H}\) transitions \(I_{TQ}^{3/2} = (|1><4| + |4><1|)\) (cf. Figure 2C of the main text).

Starting from the fast-relaxing (outer) \(^1\text{H}\) transitions only,

\[
\rho_{F}^{3/2} = \begin{bmatrix}
0 & \sqrt{3}/2 & 0 & 0 \\
\sqrt{3}/2 & 0 & 0 & 0 \\
0 & 0 & 0 & \sqrt{3}/2 \\
0 & 0 & \sqrt{3}/2 & 0
\end{bmatrix}
\]
and using the same procedures of analytical matrix exponentiation as described previously,\textsuperscript{11} we obtain the following form of the density matrix after the $^1$H pulse with flip-angle $\alpha$,

$$
\rho_y^{3/2}(\alpha) = \frac{1}{4} \begin{bmatrix}
-3\sin (\cos \alpha (\cos^2 \alpha + 1)) & \sqrt{3} \cos \alpha (3 \cos^2 \alpha - 1) & \sqrt{3} \sin \alpha (3 \cos^2 \alpha - 1) & -3 \cos \alpha (\cos^2 \alpha - 1) \\
\sqrt{3} \cos \alpha (3 \cos^2 \alpha - 1) & 3 \sin \alpha (3 \cos^2 \alpha - 1) & -9 \cos \alpha (\cos^2 \alpha - 1) & -\sqrt{3} \sin \alpha (3 \cos^2 \alpha - 1) \\
\sqrt{3} \sin \alpha (3 \cos^2 \alpha - 1) & -9 \cos \alpha (\cos^2 \alpha - 1) & -3 \sin \alpha (3 \cos^2 \alpha - 1) & \sqrt{3} \cos \alpha (3 \cos^2 \alpha - 1) \\
-3 \cos \alpha (\cos^2 \alpha - 1) & -\sqrt{3} \sin \alpha (3 \cos^2 \alpha - 1) & \sqrt{3} \cos \alpha (3 \cos^2 \alpha - 1) & 3 \sin \alpha (\cos^2 \alpha + 1)
\end{bmatrix}
$$

(S10)

For the angle $\alpha$ equal to the ‘magic’ angle, 54.73°, and after the cycling of the phase of the $^1$H pulse along $\pm y$, as above, the form of the density matrix is simplified to,

$$
\rho_F = (\sqrt{3}/2)I_X^{3/2}C_y + (\sqrt{3}/6)I_{10}^{3/2}C_y
$$

(S11)

(cf. Figure 2C, main text).

In the experiment of Figure 2A, the fast-relaxing transitions (‘F’) are isolated (selected for) prior to the magic-angle $^1$H pulse. Therefore, Eq. (S10) applies for the description of the density matrix after the $^1$H pulse with arbitrary angle $\alpha$, and Eq. (S11) for $\alpha = 54.7^\circ$ with the phase cycled along $\pm y$. The second term in Eq. (S11) deriving from the triple-quantum $^1$H magnetization is unobservable during the $t_1$ acquisition period, and does not lead to observable magnetization at the end of the experiment (during $t_2$). Thus, efficient selection of the slow-relaxing (inner) $^1$H transitions of the $I = 3/2$ manifold (the first term in Eq. (S11)) is achieved.

In the experiment of Figure 2B, both the fast-(‘F’) and slow-(‘S’)-relaxing $^1$H transitions are present prior to the $^1$H pulse with flip-angle $\alpha$. The description of the density matrix after the pulse is therefore given by the sum of the matrices in Eqs. (S6) and (S10) for an arbitrary angle $\alpha$. For $\alpha$ set to 54.7° and the phase of the pulse cycled along $\pm y$, this description simplifies to the sum of Eqs. (S8) and (S11) given by,

$$
\rho_{SF} = -(\sqrt{3}/6)I_X^{3/2}C_y + (\sqrt{3}/3)I_X^{1/2}C_y + (1/2)I_X^{3/2}C_y + (\sqrt{3}/2)I_X^{3/2}C_y
$$

(S12)

As the expectation value of the $^1$H magnetization of the first two operators in Eq. (S12) at the end of the experiment is zero, $Tr \left[ H - \left( (\sqrt{3}/3)I_X^{1/2} - (\sqrt{3}/6)I_X^{3/2} \right) \right] = 0$, where $H$ is the observation operator, and ‘$Tr$’ denotes the trace of the matrix, complete cancellation of the observed signal originating from the
slow-relaxing part of $^1$H magnetization is achieved at the end of the pulse-scheme. The fast-relaxing coherences created by the magic-angle $^1$H$_y$ pulse (the third term in Eq. (S12)) are eliminated by the subsequent $2\tau_b$ element in the experiment of Figure 2B, and only the last term, corresponding to the slow-relaxing (inner) $^1$H transitions (originating from the fast-relaxing coherences prior to the $^1$H$_y$ pulse), is observed, with the same selection efficiency as in the scheme of Figure 2A. Due to small differences in relaxation rates of the inner transitions of the $I = 3/2$ manifold and those of the $I = 1/2$ manifolds due to dipolar interactions with external proton spins,[2] partial ‘restoration’ of the magnetization of the latter transitions ($S^{1/2}$) is expected during the periods $4\tau_a$, $2\tau_b$, and the acquisition periods $t_1$ and $t_2$ of the scheme in Figure 2B (see the discussion of Figure S2 below).

**Sensitivity of the pulse-scheme in Figure 3A.** In the absence of relaxation, the measurement of $R^F_{2,\text{H}}$ using the scheme in Figure 3A is predicted to be more sensitive by a factor of $4/\sqrt{3} = 2.31$ compared to the experiment developed by Tugarinov and Kay[3] (see Figure 4b in reference [3]). In this latter pulse-scheme, the slow-relaxing magnetization ($S$) is selected first, evolved during the $t_1$ period, and later converted to the fast-relaxing magnetization ($F$) for $R^F_{2,\text{H}}$ measurements. Subsequently, the magnetization $F$ is converted back to $S$ for observation. Each conversion step, which is achieved by $90^\circ$ $^1$H pulses applied phase-coherently with the phase of the magnetization in question, incurs a loss of 50% of the magnetization[3] (a total factor of 4). By contrast, the scheme in Figure 3A, starts with $^1$H coherences $F$ and converts them by the application of a single magic-angle $^1$H pulse to the magnetization $S$ for observation. It can be shown that even this single conversion step by the magic-angle pulse is slightly ($2/\sqrt{3} = 1.155$) more efficient than the conversion using a phase-coherent $90^\circ$ $^1$H pulse. The fact that only a single (and slightly more efficient) ‘fast-to-slow’ conversion is employed in the scheme of Figure 3A, leads to the sensitivity gain of a factor of $4/\sqrt{3}$ in the absence of relaxation. However, this gain in sensitivity is expected to be eliminated for larger proteins, since in the scheme of Figure 3A, $^1$H relaxation with fast ($R^F_{2,\text{H}}$) rates is operative during the initial dephasing period of $2\tau_a$ duration, while it occurs with slow ($R^S_{2,\text{H}}$) rates during the same period in the earlier scheme of Tugarinov and Kay.[3] Based on these observations, we predict that the scheme of Figure 3A will have sensitivity advantages for $R^F_{2,\text{H}}$ measurements for protein molecules with rotational correlation times $\tau_C$ of up to ~45-50 ns (corresponding to a molecular weight of ~80-90 kDa at 37 °C in D$_2$O). Beyond this molecular size limit, the pulse scheme in Figure 3A is expected to become less sensitive.
Figure S1. Correlation plots comparing transverse spin relaxation rates of the inner $I = 3/2$ manifold transitions ($S^{3/2}$), $R^S_{2,H}$, obtained using the extension of the scheme in Figure 2A of the main text (y-axes) and the phase-cycling based $I = 3/2$ manifold selection experiment\(^\text{[2]}\) (x-axes) for ILV-\(^{13}\text{CH}_3\)-labeled and otherwise deuterated ubiquitin at (A) 25 °C (30 methyl cross-peaks), and (B) 10 °C (28 cross-peaks) (500 MHz). Pearson linear correlation coefficients $R$ for the set of $n$ methyl correlations and the parameters of linear regression are indicated at the top left corner and the bottom of the plots, respectively.
Figure S2. Simulated absolute errors in $S_{\text{axis}}^2$ (Δ$S_{\text{axis}}^2$, y-axes) obtained with the scheme of Figure 3A of the main text, plotted as a function of $S_{\text{axis}}^2$ (x-axes) for several distances to a proton spin external to the methyl group of interest ($r_{\text{hhext}}$, Å), for a global molecular correlation time $\tau_c$ of (A) 9 ns, and (B) 24 ns.

Simulations of methyl $^1$H relaxation rates $R_{2,H}^E$ and $R_{2,H}^S$ for calculations of $S_{\text{axis}}^2$ in Figure S2 according to Eq. (1) of the main text, were performed with the full relaxation matrix (including spectral densities $J(\omega_i)$ at all relevant frequencies $\omega_i$, where $i \in \{\text{H, C}\}$) using a basis set that separates the $^1$H transitions of the $I = 3/2$ and $I = 1/2$ manifolds, $[L_1, L_2, L_3, L_4]^T$, where T denotes transposition:

\[
L_1 = \frac{1}{\sqrt{2}} (|1\rangle\langle2|) \\
L_2 = \frac{1}{\sqrt{2}} (|2\rangle\langle3|) \\
L_3 = \frac{1}{2} (|5\rangle\langle6|+|7\rangle\langle8|) \\
L_4 = \frac{1}{\sqrt{2}} (|3\rangle\langle4|)
\]

and the notation of Figure 1 of the main text is used. The transverse $^1$H spin relaxation rates due to intramethyl $^1$H-$^1$H dipolar interactions is modeled as,
where \( k_{HH} = \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\hbar^2 \gamma_H^4 \tau_c}{J_{HH}^6} \), \( \mu_0 \) is the vacuum permittivity constant, \( \gamma_H \) the gyromagnetic ratio of a proton spin, and \( r_{HH} \) the distance between pairs of methyl protons, while \(^1H\) spin relaxation rates due to intra-methyl \(^{13}C-{^1H}\) dipolar interactions are represented by.
The simplest form of the methyl spectral density function,

\[ J_{CH}(0) = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 5/3 & -2\sqrt{3}/3 & 0 \\ 0 & -2\sqrt{3}/3 & 7/3 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} + \begin{bmatrix} 3/2 & 0 & 0 & 0 \\ 0 & 5/2 & -\sqrt{2} & 0 \\ 0 & -\sqrt{2} & 7/2 & 0 \\ 0 & 0 & 0 & 3/2 \end{bmatrix} + \begin{bmatrix} 9/4 & -\sqrt{3}/2 & -\sqrt{6}/4 & 0 \\ -\sqrt{3}/2 & 9/4 & 0 & -\sqrt{3}/2 \\ -\sqrt{6}/4 & 0 & 9/4 & -\sqrt{6}/4 \\ 0 & -\sqrt{3}/2 & -\sqrt{6}/4 & 9/4 \end{bmatrix} \]

\[ \frac{d}{dt} L_n = -k_{CH} J_{CH}(\omega_n) \]

\[ J_{CH}(\omega_n) = \begin{bmatrix} 3/4 & -\sqrt{3}/6 & -\sqrt{6}/12 & 0 \\ -\sqrt{3}/6 & 3/4 & 0 & -\sqrt{3}/6 \\ -\sqrt{6}/12 & 0 & 3/4 & -\sqrt{6}/12 \\ 0 & -\sqrt{3}/6 & -\sqrt{6}/12 & 3/4 \end{bmatrix} \]

\[ -k_{CH} J_{CH}(\omega_n) = \begin{bmatrix} 0 & 0 & 0 & 0 \\ 0 & -2/3 & 2\sqrt{3}/3 & 0 \\ 0 & 2\sqrt{3}/3 & -4/3 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} + \begin{bmatrix} 3/2 & -\sqrt{3} & \sqrt{6}/4 & 0 \\ -\sqrt{3} & 3 & 0 & -\sqrt{3} \\ \sqrt{6}/4 & 0 & -3/2 & \sqrt{6}/4 \\ 0 & -\sqrt{3} & \sqrt{6}/4 & 3/2 \end{bmatrix} + \begin{bmatrix} 1/2 & -\sqrt{3}/3 & \sqrt{6}/12 & 0 \\ -\sqrt{3}/3 & 1 & 0 & -\sqrt{3}/3 \\ \sqrt{6}/12 & 0 & -1/2 & \sqrt{6}/12 \\ 0 & -\sqrt{3}/3 & \sqrt{6}/12 & 1/2 \end{bmatrix} \]

where \( k_{CH} = \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{r_{CH}^6} \), \( \gamma_C \) the gyromagnetic ratio of a \(^{13}\)C spin, and \( r_{CH} \) the \(^{13}\)C-\(^{1}\)H internuclear distance in a methyl group. The simplest form of the methyl spectral density function,\(^{10} \) was used in all calculations,

\[ J_{\mu\nu}(\omega) = \frac{2}{5} \left\{ S_{axis}^2 S_{axis,\mu} S_{axis,\nu} \frac{\tau_C}{1 + (\omega \tau_C)^2} + \left[ P_2(\cos \theta_{\mu\nu}) - S_{axis}^2 S_{axis,\mu} S_{axis,\nu} \right] \frac{\tau_c}{1 + (\omega \tau_c)^2} \right\} \]

where the superscripts ‘auto’ and ‘cross’ denote the auto- and cross-correlated relaxation spectral density functions, the indices \( \mu \) and \( \nu \) denote the type of interaction (\( \mu = \nu \) for auto- and \( \mu \neq \nu \) for cross-correlations), \( S_{axis,\mu} = P_2(\theta_{axis,\mu}) = (1/2)(3\cos^2(\theta_{axis,\mu}) - 1) \), \( \theta_{axis,\mu} \) is the angle between the methyl symmetry
axis and a vector \( a \) connecting a pair of spins (for \(^{13}\text{C}-^{1}\text{H} \) interactions, \( S_{\text{axi},a} = -1/3 \); for \(^{1}\text{H}-^{1}\text{H} \) interactions \( S_{\text{axi},a} = -1/2 \)), \( (\tau_s)_{-1} = (\tau_c)^{-1} + (\tau_f)^{-1} \), and the correlation time of fast local methyl motions \( \tau_f \) set to 40 ps.

Spin relaxation due to dipolar interactions with external \(^{1}\text{H} \) spins in the same basis is modeled as,

\[
\frac{d}{dt} \begin{bmatrix} L_1 \\ L_2 \\ L_3 \\ L_4 \end{bmatrix} = -k_{\text{HH}}^{\text{ext}} \begin{bmatrix} 9 & -2\sqrt{3} & 0 & 0 \\ -2\sqrt{3} & 11 & 0 & -2\sqrt{3} \\ 0 & 0 & 5 & 0 \\ 0 & -2\sqrt{3} & 0 & 9 \end{bmatrix} \begin{bmatrix} L_1 \\ L_2 \\ L_3 \\ L_4 \end{bmatrix}
\]

(S17)

where \( k_{\text{HH}}^{\text{ext}} = \sum_{\text{ext}} (1/20)\left(\frac{\gamma_{1H}^{2} r_{1H}^{6} \gamma_{1C}^{6}}{r_{\text{HH}c}^{6}}\right) \), \( r_{\text{HH}c} \) is the distance between a methyl proton and a proton external to the methyl group in question, and the summation runs over all external proton spins in the protein structure. Note that the order parameter of all external interactions is implicitly assumed to be equal to 1 in this treatment. A single external \(^{1}\text{H} \) spin placed at a distance \( r_{\text{HH}c} \) from the methyl group in question was considered in all calculations. Propagation of the relaxation matrices in Eqs. (S14-15) and (S17) was performed with the starting conditions \([1,0,0,1]^T \) and \([0,1,0,0]^T \) for the calculation of \( R_{2,1}^F \) and \( R_{2,1}^S \) relaxation rates, respectively. The resulting decay curves were best-fit to single-exponential functions for extraction of \( R_{2,1}^F \) and \( R_{2,1}^S \).

The simulations of \( R_{2,1}^F \) and \( R_{2,1}^S \) and their differences described above show that the dominant contribution to the absolute errors \( \Delta S_{\text{axi}}^2 \) in Figure S2 arises from relaxation due to dipolar interactions with external \(^{1}\text{H} \) spins. In the macromolecular limit, the inner coherences of the \( J = 3/2 \) manifold (\( S_{3/2}^0 \) and \( L_2 \) in the basis adopted above) and the outer coherences (\( F; L_1+L_4 \) in the basis used above) auto-relax due to external \(^{1}\text{H} \) spins with the rates of \( 11k_{\text{HH}}^{\text{ext}} \) and \( 9k_{\text{HH}}^{\text{ext}} \) s\(^{-1} \), respectively,\(^{[2]} \) Eq. (S17). The expression in Eq. (1) of the main text does not take this difference into account. In principle, this difference \( (2k_{\text{HH}}^{\text{ext}}) \) can be corrected for, given that it constitutes 1/3 of the differences in relaxation rates of the \(^{1}\text{H} \) coherences \( S_{3/2}^0 \) (\( L_2 \)) and \( S_{1/2}^0 \) (\( L_3 \)) - \( 6k_{\text{HH}}^{\text{ext}} \),\(^{[2]} \) Eq. (S17), which can be estimated experimentally for each methyl site. In practice, we have chosen not to correct experimental differences \( (R_{2,1}^F - R_{2,1}^S) \) for these small effects. Simple numerical estimates show that such a correction would translate into a very small decrease in the derived \( S_{\text{axi}}^2 \) \((< -0.02)\) for most methyl sites, which is well within the experimental errors of \( S_{\text{axi}}^2 \) determination. We note that the plots in Figure S2 yet again underscore the importance of as complete deuteration of protein molecules as possible for \(^{1}\text{H} \) relaxation measurements (including that of the isopropyl moieties of Val and Leu residues\(^{[4]} \)), so that the targeted protons of \({^{13}\text{CH}_3}\)-labeled methyls are ‘immersed’ in a fully deuterated background, save for other \({^{13}\text{CH}_3}\)-methyl moieties in the protein.
structure. For example, in a fully protonated system, the distance \( r_{\text{hext}} \) would be close to 2.0 Å, leading to substantial errors in the derived \( S^2_{\text{axis}} \) values.

In the case of ‘forbidden’ methyl experiments for estimation of \( S^2_{\text{axis}} \), \( ^1\text{H} \) spin relaxation of the fast-relaxing coherences \( (F; L_1 + L_4 \text{ in the basis above}) \) occurs \textit{in the presence} of the slow-relaxing coherences \( S^{3/2} \) and \( S^{1/2} \) together \( (L_2 + L_3) \) during the relaxation period. The two sets of coherences, \( F \) and \( (S^{3/2} + S^{1/2}) \), relax identically due to interactions with external proton spins \((9k_{\text{HH}})\) and the main source of errors in \( S^2_{\text{axis}} \) arises from the coupling of the \( F \) and \( (S^{3/2} + S^{1/2}) \) coherences due to interactions with external proton spins, Eq. (S17), as \( F \) and \( (S^{3/2} + S^{1/2}) \) are \textit{not separated} before the relaxation period. This ‘cross-talk’ between the two types of \(^1\text{H} \) transitions can be taken into account, however, during analysis of the build-up of ‘forbidden’ magnetization. \( ^{[8,9]} \) Simulations of errors expected in \( S^2_{\text{axis}} \) extracted from ‘forbidden’ experiments provide results that are slightly superior to those in Figure S2 for the experiment in Figure 3A of the main text. Nevertheless, we note that good agreements between the \( S^2_{\text{axis}} \) derived from the two types of approaches in Figures 3C and 4C (main text), imply that these small differences cannot be ‘picked up’ by experiment.
Figure S3. (A) Pulse scheme for the measurement of methyl $^1$H relaxation rates $R_{2,H}^F$ and $R_{2,H}^S$ in $^{13}$CH$_3$ methyl groups based on the $I = 3/2$ manifold transitions selection of the experiment in Figure 2B. All narrow and wide rectangular pulses are applied with flip angles of 90° and 180°, respectively, along the x-axis unless indicated otherwise. The $^1$H pulse shown in dashed green is applied with flip angle $\alpha = \cos^{-1}(1/\sqrt{3}) = 54.7^\circ$. This pulse is applied for the measurement of $R_{2,H}^F$ rates and omitted (without changes in the phase-cycle of the rest of the scheme) for the measurements of $R_{2,H}^S$. The $^1$H and $^{13}$C carrier frequencies are positioned in the center of the Ile81-Leu-Val methyl region at 0.5 and 20 ppm, respectively. All $^1$H and $^{13}$C pulses are applied with the highest possible power, while $^{13}$C WALTZ-16 decoupling is achieved using a 2-kHz field. Delays are: $\tau_a = 1/(4J_{HC}) = 2.0$ ms; $\tau_b = 1/(8J_{HC}) = 1.0$ ms. The durations and strengths of pulsed-field gradients in units of (ms; G/cm) are: $g_1 = (1; 25)$, $g_2 = (0.5; 15)$, $g_3 = (0.4; 10)$. The phase cycle is: $\phi_1 = 2(x),2(-x); \phi_2 = x,-x; \phi_3 = 2(y),2(-y); \phi_4 = x; \phi_4 = x,-x$. Quadrature detection in $t_4$ is achieved via States incrementation of $\phi_4$. (B-C) Correlation plots comparing the values of $S_{axis}^2$ obtained for ubiquitin at 10 °C ($T_c = 9$ ns in D$_2$O; 500 MHz) using the experiment in (A) (y-axis), and $S_{axis}^2$ derived from $^2$H relaxation measurements (B) (x-axis), and 3Q-filtered ‘forbidden’ experiments (C) (x-axis). Pearson linear correlation coefficients $R$ for the set of n methyl cross-peaks and the parameters of linear regression are indicated at the top left corner and the bottom of the plots, respectively.

In the scheme of Figure S3A, the relaxation rates of $^1$H coherences $F$ ($R_{2,H}^F$) and $S = S_{3/2}^{3/2} + \frac{1}{2} S_{1/2}^{3/2}$ ($R_{2,H}^S$) are effectively measured (see Figure 2C for definitions). As opposed to the experiment in Figure 3A (main text), these magnetization modes are not isolated prior to the relaxation period $T$ and relax in the
presence of each other. Simulations of methyl $^1$H relaxation rates using the same basis set as detailed in the Supplementary Information of Tugarinov and Kay$^3$ under conditions when both the fast- and slow-relaxing parts are present (and therefore cross-relax), show that the cross-talk between these two types of magnetization constitutes only a small contribution to the main source of errors in derived $S^2_{\text{axis}}$ values. Since the coherences $S^{3/2}$ and $S^{1/2}$ relax differently due to dipolar interactions with external proton spins$^2$ (see discussion of Figure S2 above), partial ‘restoration’ of the $S^{1/2}$ magnetization is expected during the periods $4\tau_a$, $2\tau_b$, and the acquisition periods $t_1$ and $t_2$ of the scheme in Figure S3. Calculations using typical acquisition parameters used in the present work, show that this effect is strongly dependent on the molecular size of the studied system. For small proteins, the amount of ‘restored’ $S^{1/2}$ coherences remains small (on the order of 2-3% of the total signal intensity for relaxation delay $T = 0$ but increasing with larger $T$). Indeed, even in the case of ubiquitin at 10 °C ($\tau_C = 9$ ns in D$_2$O), although the correlation in Figure S3B is only marginally inferior to that of Figure 3B (main text), a noticeable underestimation of higher $S^2_{\text{axis}}$ values is apparent in the correlation plot of Figure S3C. For larger proteins, we estimate that up to 20 % of the total signal may derive from the ‘restored’ $S^{1/2}$ coherences in ΔST-DNAJB6b ($\tau_C = 24$ ns for the JD domain in D$_2$O; 25 °C) for $T = 0$. As the ‘restored’ $S^{1/2}$ coherences derive from the slow-relaxing part of magnetization ($S$) during the relaxation delay $T$, this has deleterious effects on the rates $R^{F}_{2,H}$ measured with the scheme of Figure S3A, leading to large underestimations of $R^{F}_{2,H}$ and, as a consequence, underestimation of the $S^2_{\text{axis}}$ values. Considering that as observed in multiple previous studies and confirmed in the present one, the correlations between $^1$H-relaxation derived $S^2_{\text{axis}}$ values and those derived from $^2$H-relaxation in either $^{13}$CH$_2$D or $^{13}$CHD$_2$ methyl isotopomers, tend to ‘break-down’ for proteins with lower $\tau_C$ values ($< ~7$-8 ns),$^{3,9,10}$ we predict that the scheme in Figure S3A would provide reliable values of $R^{F}_{2,H}$ and $S^2_{\text{axis}}$ in a narrow range of molecular correlation times: $~7 < \tau_C < ~12$ ns.
Materials and Methods

**NMR Samples.** Samples of \{U-[^15]N,^2]H; Ileδ1-[^13]CH₃; Leu,Val-[^13]CH₃,^12CD₃\}-labeled human ubiquitin (8.5 kDa) were prepared as described in detail previously using U-[^2]H-D-glucose as the main carbon source and the appropriate α-keto-acid precursors for selective methyl labeling.\[^{11}\] Sample conditions were: 1.3 mM protein, 99.9% D₂O, 25 mM sodium phosphate, pH 6.7 (uncorrected). The sample of \{U-[^15]N,^2]H; Ileδ1-[^13]CH₃; Leu,Val-[^13]CH₃,^12CD₃\}-labeled ΔST-DNAJB6b was prepared as described previously.\[^{12}\] Sample conditions were: 200 μM ΔST-DNAJB6b, 99.9% D₂O, 20 mM sodium phosphate, pH 7.0 (uncorrected) and 50 mM NaCl.

**NMR Spectroscopy.** NMR measurements on human ubiquitin (at 10 and 25 °C) were performed at 500 MHz (^1H frequency) on a Bruker Avance spectrometer equipped with a room-temperature triple-resonance x,y,z-gradient probe, while the experiments on ΔST-DNAJB6b were carried out at 600 MHz, 25 °C, using a Bruker Avance HD 600 MHz spectrometer with a triple-resonance z-gradient cryoprobe.

**NMR acquisition parameters for the schemes in Figures.** 2A-B. All narrow and wide rectangular pulses are applied with flip angles of 90° and 180°, respectively, along the x-axis unless indicated otherwise. The ^1H pulse shown in green is applied with flip angle \(\alpha = \cos^{-1}(1/\sqrt{3}) = 54.7°\). The ^1H and ^13C carrier frequencies are positioned in the center of the Ileδ1-Leu-Val methyl region - 0.5 and 20 ppm, respectively. All ^1H and ^13C pulses are applied with the highest possible power, while ^13C WALTZ-16 decoupling\[^{5}\] is achieved using a 2-kHz field. Delays are: \(\tau_a = 1/(4J_{HC}) = 2.0\ ms; \tau_b = 1/(8J_{HC}) = 1.0\ ms\). The durations and strengths of pulsed-field gradients in units of (ms; G/cm) are: \(g1 = (1; 25), g2 = (0.5; 15), g3 = (0.4; 10), g4 = (0.4; 12), and g5 = (0.4; 20)\) for the scheme in Figure 2A. The phase cycle is: \(\phi_1 = x,-x; \phi_2 = 2(x),2(-x); \phi_3 = 2(y),2(-y); \phi_4 = x;\) receiver = 2(x,-x) for the scheme in Figure 2A, and \(\phi_1 = 2(y),2(-y); \phi_2 = x,-x; \phi_3 = 2(y),2(-y); \phi_4 = x;\) receiver = 2(x,-x) for the scheme in Figure 2B. Quadrature detection in \(t_1\) is achieved via States incrementation\[^{6}\] of \(\phi_4\). NMR experiments acquired with the pulse schemes shown in Figures 2A-B (main text) on ubiquitin and ΔST-DNAJB6b samples were typically obtained with 8 and 16 scans/FID, respectively, (512; 128) complex points in \((t_2; t_1)\), and an inter-scan recovery delay of 1 s, resulting in net acquisition time of ~40 and ~80 min, respectively.

**NMR acquisition parameters for the scheme in Figure 3A.** All the parameters are as listed for Figure 2A above. The ^1H pulse shown with a dashed rectangle is applied with flip angle \(\alpha = 54.7°\) before the relaxation period \(T\) for the measurement of \(R^S_{2,\text{H}}\) (red) and following the relaxation period \(T\) for the
measurement of $R^{F}_{2,1H}$ (blue). The phase cycle is: \(\phi_1 = x,-x; \phi_2 = 2(x),2(-x); \phi_3 = 4(x),4(-x); \phi_4 = 2(y),2(-y); \phi_5 = 2(x),2(-x); \) receiver = \(x,-x,-x,x,-x,x,-x\). Quadrature detection in \(t_1\) is achieved via States-TPPI\(^{113}\) of \(\phi_5\). The following relaxation delays \(T\) were used for the measurement of $R^{F}_{2,1H}$ relaxation rates: 0.4, 4, 6, 8, 10, and 12 ms for \(\Delta ST\)-DNAJB6b, and 0.4, 5, 10, 12, 15, 20, 22, and 25 ms for ubiquitin (10 °C), while those for the determination of $R^{S}_{2,1H}$ were set to 0.4, 10, 30, 50, 60, and 80 ms for \(\Delta ST\)-DNAJB6b, and 1, 10, 20, 30, 50, 70, 80, and 90 ms for ubiquitin (10 °C). NMR experiments acquired with the pulse scheme of Figure 3A on ubiquitin and \(\Delta ST\)-DNAJB6b samples were typically obtained with 8 and 32 scans/FID, respectively, (512; 128) complex points in \((t_2; t_1)\), and an inter-scan recovery delay of 1 s, resulting in net acquisition times of \(~40\) and \(~160\) min, respectively, per experiment. All NMR spectra were processed and analyzed using the NMRPipe/NMRDraw suite of programs and associated software.\(^{114}\)

**Data Analysis.** $R^{F}_{2,1H}/R^{S}_{2,1H}$ relaxation rates were obtained by fitting peak heights to monoexponential decay functions, \(A_0e^{-RT}\), where \(R\) is the relaxation rate, \(T\) is relaxation period, and \(A_0\) is initial intensity (at \(T = 0\)). Errors in the fitted relaxation rates were estimated via a Monte-Carlo analysis\(^{115}\) using random noise in the spectra as an estimate of experimental uncertainties in peak heights. Subsequently, the errors in $R^{F}_{2,1H}/R^{S}_{2,1H}$ relaxation rates were propagated to obtain the relative errors in \((R^{F}_{2,1H} - R^{S}_{2,1H})\) differences, which were in turn translated to the relative errors in $S^2_{\text{axis}}$. The same inter-nuclear distances and angles between vectors connecting pairs of spins that enter into Eq. (1) of the main text as in the previous publications,\(^{13,8}\) were used throughout this work.

**Estimation of apparent rotational correlation time, \(\tau_c\), of the JD and CTD domains of \(\Delta ST\)-DNAJB6b.** The \(\tau_c\) values of 12 and 8 ns for the JD and CTD domains, respectively, were derived from $^{15}$N-relaxation measurements using a sample of 60 \(\mu\)M \(\Delta ST\)-DNAJB6b (25 °C) in our earlier study.\(^{112}\) To account for higher viscosity of D$_2$O that was used as a solvent in the present work, these values were scaled by the ratio of D$_2$O and H$_2$O viscosities at 25 °C (1.236)\(^{116}\) to obtain \(\tau_c\) values of 14.8 and 9.9 ns for the JD and CTD domains, respectively. Further, from our previous $^{15}$N-relaxation measurements,\(^{112}\) we estimate that the fractional population of higher order oligomeric species (consisting of 35 monomeric units) is ~2 % for a 200 \(\mu\)M sample of \(\Delta ST\)-DNAJB6b used in the present work, leading to effective \(\tau_c\) estimates of 24.9 ns and 16.6 ns for the JD and CTD domains, respectively. To ascertain the validity of these estimates we performed the experiment of Figure 3A using a 30 \(\mu\)M sample of \(\Delta ST\)-DNAJB6b (at this concentration, the presence of oligomeric species in solution can be ruled out). To determine the effective \(\tau_c\) for the 200 \(\mu\)M sample, a least-squares analysis was performed to minimize the difference between $S^2_{\text{axis}}$ derived from
the 30 μM and 200 μM samples. This yielded $\tau_C$ values of 24 ns and 16 ns for the JD and CTD domains, respectively, which were used in the final analysis. The same $\tau_C$ of 9 ns as in the previous studies$^{[7,8]}$ was used for ubiquitin in D$_2$O (10 °C).

Supplementary References