Protein Backbone Dynamics and $^{15}$N Chemical Shift Anisotropy from Quantitative Measurement of Relaxation Interference Effects

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Abstract: Cross-correlation between $^{15}$N–$^1$H dipolar interactions and $^{15}$N chemical shift anisotropy (CSA) gives rise to different relaxation rates of the doublet components of $^{15}$N–$^1$H peptide backbone amides. A simple scheme for quantitative measurement of this effect is described which yields information on the magnitude of the CSA from the relative intensities of $^1$H–$^{15}$N correlations obtained with two slightly different pulse schemes. The method is applied to a sample of uniformly $^{15}$N-enriched ubiquitin and measurements conducted at two field strengths (8.5 and 14 T) yield identical results. The degree of relaxation interference correlates with the isotropic $^{15}$N chemical shift and results indicate that the sum of the most shielded principal components of the CSA tensor is nearly invariant to structural differences in the polypeptide backbone. The relaxation interference is directly proportional to the generalized order parameter, $S_2$, of the peptide backbone amides, and this relation can be utilized to obtain approximate values for these order parameters.

There is a renewed interest in understanding the relation between protein structure and $^{13}$C and $^{15}$N chemical shifts.1–5 Indeed, recent ab initio calculations show considerable promise for providing an accurate correlation between chemical shift and the structure of the peptide backbone. In proteins, the results of calculations could only be compared with the value of the isotropic chemical shift, as accurate values for the individual chemical shift anisotropy (CSA) tensor elements in proteins are not easily measured. Here we demonstrate that a measure for the magnitude of the CSA of individual peptide backbone $^{15}$N nuclei can be obtained from quantitative measurement of interference effects between the CSA and dipolar relaxation mechanisms. The magnitude of the interference effect is expected to be directly proportional to the generalized order parameter, $S_2$, and this correlation is confirmed experimentally. Inversely, the simple relaxation interference measurement can be used to obtain the relative $S_2$ values of the backbone amides.

Relaxation of peptide backbone $^{15}$N nuclei in proteins is dominated by CSA and by the dipolar interaction between $^{15}$N and its directly attached proton. Based on solid-state NMR studies of model compounds containing peptide bonds, the $^{15}$N CSA tensor is nearly axially symmetric and its unique axis makes a relatively small angle of ca. 20–24° with the N–H bond vector.6–9 As a result, for a $^{15}$N nucleus attached to a proton in the $|\beta\rangle$ spin state the sum of the dipolar and CSA tensors is much smaller than for $^{15}$N nuclei attached to a $^1$H in the $|\alpha\rangle$ spin state, and the two types of $^{15}$N nuclei relax at very different rates. This differential relaxation is commonly referred to as a cross-correlation or relaxation interference effect10–15 and a simple treatment of this effect, directly applicable to the case of peptide $^{15}$N–$^1$H amide pairs, has been presented by Goldman.13 Although cross correlation forms the basis of several elegant heteronuclear magnetization transfer experiments,16,17 more often it is considered a nuisance as it can alter the outcome of relaxation measurements if no precautions are taken to eliminate the effect.18–20 Here we demonstrate that the effect can be used advantageously to obtain information on the $^{15}$N CSA tensor and on the internal dynamics of the peptide backbone. The method is demonstrated for human ubiquitin, a small globular protein of 76 residues which is well-characterized by both X-ray crystallography21 and numerous NMR studies.22–27

Experimental Section

All NMR experiments were carried out at 27 °C on a sample of commercially obtained (U-15 N)-ubiquitin (VLI Research, Southeastern, PA), 1.4 mM, pH 4.7, 10 mM NaCl. Experiments were carried out on Bruker AMX-360 and AMX-600 NMR spectrometers operating at 1 H resonance frequencies of 360 and 600 MHz, respectively. Both spectrometers were equipped with pulsed field gradient 1 H/15 N probeheads, optimized for 1 H detection.

Data matrices acquired at both 360 and 600 MHz consisted of $128^\times128$ data points, with acquisition times of 64 (1 ms) and 132 ms at 360 and 600 MHz, respectively. A total of 32 scans per complex was acquired. Data sets were processed using 45° shifted sine-bell apodization and zero filling in both dimensions to yield a digital resolution of 2.3, 3.9 Hz (F1) and 2.7, 4.5 Hz (F2) for 360 and 600 MHz data respectively. All data sets were processed using mnmPipe and analyzed with the program PIPP. 

Resonance intensities were obtained from peak heights using three-data-point interpolation, and resonance assignments are taken from Wang et al.

Results and Discussion

Assuming an axially symmetric 15 N chemical shift tensor with an angle $\theta$ between the orientation of its unique axis and the N–H bond vector, the 15 N transverse relaxation rates for the two doublet components of an isolated 15 N–1 H spin pair are given by:

$$ R_2 = \lambda \pm \eta $$

(1a)

where the + sign applies to the upfield 15 N doublet component ($\delta_{15 N} < 0$) and $\lambda$ and $\eta$ are given by:

$$ \eta = 2\alpha d(4J^{dd}(0) + 3J^{ab} + 3J^{aa}\cos(\theta) + 6\delta^{dd}(\omega_N, \omega_H)) $$

(1b)

where $d = \gamma^2/\gamma^2 h^2(80\pi^2 r_{\text{N}}^3/\gamma(A_1, \omega_1))$, $\alpha = -4\pi/3B_d(\omega_1 - \omega_0)[h\gamma_1, \gamma]$, and $r_{\text{H}}$ is the 15 N–1 H internuclear distance, assumed to be 1.02 Å, $J^{dd}(\omega)$, $J^{ab}(\omega)$, and $J^{aa}(\omega)$ are the spectral densities for dipolar autocorrelation, CSA autocorrelation, and dipolar-CSA cross correlation, respectively. For an axially symmetric CSA tensor, these spectral densities are given by:

$$ J^{dd}(\omega) = \int_0^\infty \left[P_2(\mu_d(t))\cos(\omega t)\right] dt $$

(2)

where $\mu_d(t)$ is the unit vector describing the orientation of the axially symmetric interaction $p$ at time $t$, and $P_d(x) = (3x^2 - 1)/2$. Assuming isotropic rotational diffusion of a rigid body, one has:

$$ J^{dd}(\omega) = \int_0^\infty \left[P_2(\mu_d(t))\cos(\omega t)\right] dt $$

(3)

where $\theta$ is the angle between the unique axes of the CSA and dipolar tensors, i.e., $\theta = \cos^{-1}(\mu_d(0)\mu_d(t))$. Although eq 3 is no longer rigorous in the presence of internal motion, results of calculations shown in the Appendix indicate it remains a very good approximation provided $\theta$ is small. Therefore, the superscripts in the spectral density function may be dropped and eq 1c is then rewritten as:

$$ \eta = 2\alpha d(4J^{dd}(0) + 3J^{ab}) $$

(4)

For the case of isotropic rotational diffusion and additional rapid internal motions, occurring on a time scale $t_c$ and described, in the model-free approach of Lipari and Szabo, by a generalized order parameter $S^2$, the spectral density function is defined as:

$$ J(\omega) = S^2\tau_c(1 + \omega^2\tau_c^{-2}) + (1 - S^2)\tau_c(1 + \omega^2\tau_c^{-2}) $$

(5)

with $\tau_c^{-1} = \tau_c^{-1} + \tau_c^{-1}$. The time constant $\tau_c$ is the rotational correlation time, assuming isotropic diffusion. However, rotational diffusion of ubiquitin is slightly anisotropic, and to a good approximation is described by an axially symmetric diffusion tensor, $D$, with $D_{11}/D_{\perp} = 1.17$.\footnote{Barbato, G.; Ikura, M.; Kay, L. E.; Pastor, R. W.; Bax, A. Biochemistry 1992, 31, 5269–5278.} In this case, calculation of the cross-correlation term becomes considerably more complex,\footnote{Woessner, D. E. J. Chem. Phys. 1962, 3, 647–654.} unless $\theta = 0$. For $\theta = 0$, one may simply use eq 1 in combination with the spectral density function applicable for axially symmetric rotational diffusion with internal motion:\footnote{Barbato, G.; Ikura, M.; Kay, L. E.; Pastor, R. W.; Bax, A. Biochemistry 1992, 31, 5269–5278.}

$$ J(\omega) = S^2\sum_k A_k\tau_k(1 + \omega^2\tau_k^{-2}) + (1 - S^2)\tau_k(1 + \omega^2\tau_k^{-2}) $$

(6)

with $k = 1, 2, 3$ and $A_1 = 0.75 \sin^4 \beta, A_2 = 3 \sin^2 \beta \cos^2 \beta, A_3 = (1.5 \cos^2 \beta - 0.5)^2$, where $\beta$ is the angle between the N–H bond vector and the unique axis of the rotational diffusion tensor; $\tau_1 = (4D_{11} + 2D_{\perp})^{-1}, \tau_2 = (D_{11} + 5D_{\perp})^{-1}$, $\tau_3 = (6D_{\perp})^{-1}$, and $\tau^{-1} = \tau_c^{-1} + (2D_{11} + 4D_{\perp})^{-1}$, where $D_{11}$ and $D_{\perp}$ are the rotational diffusion constants parallel and perpendicular to the unique axis of the diffusion tensor. For peptide 15 N nuclei $\theta$ is small (20–24°), and use of eqs 1 and 6 provides a reasonable approximation.


Measurement of Cross Correlation. Figure 1 shows the pulse scheme used for measuring the cross correlation effect quantitatively. The pulse scheme is essentially an HSQC \(^{1}H\)–\(^{15}N\) correlation experiment,\(^{24}\) with a relaxation period \(2\Delta\) inserted before the \(^{15}N\) evolution period. The experiment is performed twice, once with applying the shaded \(^{1}H\) 90° and composite \(^{1}H\) 180° pulses, and once without applying these pulses. In the following these experiments will be referred to as A and B, respectively.

In both versions of the pulse scheme, \(^{1}H\) magnetization (represented by S) is transferred to \(^{15}N\) (represented by I) at time point \(a\) in Figure 1, and antiphase transverse \(^{15}N\) magnetization, described by the operator product \(2I_SI_c\), is generated by the first 90° \(^{15}N\) pulse. The two \(^{15}N\) doublet components are described by \(I_S\pm I/2\). The downfield doublet component, \(I_S-I/2\), relaxes at a rate \(\lambda-\eta\), whereas the upfield component relaxes at \(\lambda+\eta\) \((\text{cf. eq 1})\). Thus, after the dephasing delay \(2\Delta\) (time point \(b\) in Figure 1), the transverse magnetization is given by

\[
\sigma_b = (I_S\rho - I/2) \exp[-2\Delta(\lambda-\eta)] + (I_S\rho + I/2) \times \exp[-2\Delta(\lambda+\eta)] = I_S\rho (e^+ + e^-) + I_S\rho (e^+ - e^-)/2 \tag{7}
\]

where \(e^\pm = \exp[-2\Delta(\lambda \pm \eta)]\). At time point \(b\) the \(^{15}N\) magnetization is returned to the z axis. In scheme A, the antiphase fraction of the \(^{15}N\) z magnetization, \(I_S(e^+ + e^-)\), is destroyed by the sequential application of a 90° \(^{1}H\) pulse and pulsed field gradient G3. The in-phase component, \(I_S(e^+ - e^-)/2\), is converted back into antiphase magnetization during the subsequent delay, \(2\Delta\), prior to \(t_1\) evolution and transfer of \(^{15}N\) magnetization to protons by the final reverse INEPT part of the pulse scheme. In scheme B, the 90° \(^{1}H\) pulse is not applied and the \(I_S\) \(^{15}N\) magnetization is converted back into antiphase transverse \(^{15}N\) magnetization at time point \(c\). This \(-I_S(e^+ + e^-)\) term remains antiphase at the end of the delay \(2\Delta\), as the composite \(^{1}H\) pulse is not applied in scheme B. After the \(t_1\) evolution period, the \(^{15}N\) magnetization is returned to observable proton magnetization, in a manner identical to that of scheme A. In the absence of the composite \(^{1}H\) 180° pulse, the in-phase \(^{15}N\) magnetization, \(I_S(e^+ - e^-)/2\), remains in phase at the end of the \(t_1\) evolution period and therefore does not contribute to the signal detected in scheme B. Thus, the ratio of the signal intensities obtained with schemes A and B equals

\[
I_A/I_B = (e^- + e^+)/(e^- + e^+ + e^- + e^+) = \tanh(2\Delta\eta) \tag{8}
\]

Note that the losses caused by relaxation are essentially identical in schemes A and B. The faster relaxation of the \(I_S\) term between time points \(b\) and \(c\) in scheme A, relative to the decay of \(I_S\) in scheme B, produces negligible relaxation loss because the duration of this delay (2 ms) is about two orders of magnitude shorter than the inverse of the difference in relaxation rates of \(I_S\) and \(I_c\). In addition to the very small relaxation loss between time points \(b\) and \(c\) in scheme B, there is also a loss of signal which occurs in scheme A and not in B, caused by the imperfection of the composite 180° \(^{1}H\) pulse. As a test, adding a composite pulse between time points \(b\) and \(c\) in scheme B is found to change the average intensity of the \(^{15}N\)–\(^{1}H\) correlations by a factor of \(-0.986\), indicating that 99.3% of the protons are inverted by the composite pulse and the imperfection in this pulse therefore may be safely ignored. The \(I_A/I_B\) ratio divided by the uncertainty in this ratio caused by thermal noise is highest when \(2\Delta \approx \lambda^{-1}\), i.e., when the duration of \(2\Delta\) is approximately equal to \(T_2\). Thus, the random uncertainty in

\[^{34}\text{Bodenhausen, G.; Ruben, D. J. Chem. Phys. Lett. 1980, 69, 185–189.}\]


Correlation between CSA and Isotropic Shift. The isotropic $^{15}$N shift, $\delta$, equals minus one-third of the trace of the chemical shift tensor: $\delta = -(\sigma_{11} + \sigma_{22} + \sigma_{33})/3$. The least shielded component, $\sigma_{11}$, is the one oriented in the peptide plane and nearly orthogonal to the C–N bond. The $\sigma_{22}$ and $\sigma_{33}$ components are most shielded and are of comparable magnitude in peptide bonds. For $\theta = 0^\circ$, the magnitude of the cross-correlation effect is determined by $\sigma_{11} - (\sigma_{22} + \sigma_{33})/2$. Although this relation is only valid when $\theta = 0^\circ$, the assumption of an axially symmetric tensor with $\sigma_{0} = \sigma_{z} = \sigma_{11} - (\sigma_{22} + \sigma_{33})/2$ yields nearly the same magnitude for the cross-correlation term, provided $\theta < 25^\circ$.

If the $\sigma_{11}$, $\sigma_{22}$, and $\sigma_{33}$ values of the $^{15}$N shielding tensors in ubiquitin are normally distributed, with standard deviations $\Delta_{1}$, $\Delta_{2}$, and $\Delta_{3}$, respectively, the standard deviation for the isotropic shift is expected to be $1/3(\Delta_{1}^2 + \Delta_{2}^2 + \Delta_{3}^2)^{1/2}$, provided variations in $\sigma_{11}$, $\sigma_{22}$, and $\sigma_{33}$ are uncorrelated. A decrease in $\sigma_{22}$ or $\sigma_{33}$ (i.e., deshielding) gives rise to a smaller chemical shift anisotropy and a smaller value of the cross-correlation term, in addition to a downfield change of the isotropic shift. In contrast, a decrease in $\sigma_{11}$ will increase the chemical shift anisotropy, but also give rise to a downfield change of the isotropic shift.

As shown in Figure 5, experimentally we observe that a downfield isotropic shift is correlated with an increase in the CSA. Therefore, this means that variations in $\sigma_{11}$ are larger than those in $(\sigma_{22} + \sigma_{33})/2$. The slope of the correlation observed in Figure 5 is $-0.30$, which is close to the value of $-1/3$, expected if $(\sigma_{22} + \sigma_{33})/2$ were invariant to structural differences. Thus, variations in $\sigma_{22}$ and $\sigma_{33}$ must be highly correlated and opposite in sign. This agrees with the remarkable observation by Hiyama et al. for the $^{1}N$ CSA tensor of a tripeptide where $\sigma_{22}$ and $\sigma_{33}$ differ by 47 ppm in the triclinic crystal form, but by only 7 ppm in the monoclinic crystal form, with the isotropic shift and $\sigma_{11}$ remaining unchanged.

Considering that fluctuations in $(\sigma_{22} + \sigma_{33})/2$ do not contribute significantly to variations in $\sigma_{11} - (\sigma_{22} + \sigma_{33})/2$, the scatter in the correlation shown in Figure 5 may originate from one or more of the following five causes. First, it is possible that all three principally vary together, i.e., without changing the chemical shift anisotropy. This assumption is implicit when plotting the deviation from random coil chemical shift, $\Delta\delta$, instead of the isotropic shift, $\delta$, itself. $^{15}$N chemical shift tensors reported by Oas et al. indicate that the change in isotropic shift between a glycine and a tyrosine residue is approximately the same as the change in the $(\sigma_{22} + \sigma_{33})/2$ values of these two tensors, and therefore provide some justification for using the $\Delta\delta$ instead of $\delta$. Second, there is considerable uncertainty in the random coil $^{15}$N shift values, which can differentially affect the $\Delta\delta$ values. For example, the pairwise rmsd between random coil shifts reported by Glushka et al. and Wishart et al. is 2.8 ppm. Third, random error in the measurement of $I_{x}/I_{0}$ results in an uncertainty in CSA$^{\text{red}}$, which is further amplified when CSA$^{\text{red}}$ is divided by $S^{2} \approx 0.85$, and by the uncertainty in $S^{2}$, estimated at 1%. The random uncertainty in CSA$^{\text{red}}$ is estimated by comparing values derived from data measured at $2 \Delta = 46.7$ and 68 ms. The pairwise rmsd is 1.4 ppm, indicating a random error of 0.7 ppm in the average, used for deriving the values shown in Figure 5. Thus, the total random error in the CSA$^{\text{red}}/S^{2}$ values is estimated to be ca. 1 ppm. Fourth, variations in the angle $\theta$ can have a large effect on CSA$^{\text{red}}$.

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Even a small increase in $\theta$ from, for example, 20 to 22° decreases the expected CSA$^{\text{red}}$/$S^2$ value by $\sim$6 ppm. It is therefore likely that variations in $\theta$ are also a significant source of the scatter observed in Figure 5. Finally, if the orientation of the $\sigma_{11}$ tensor component is not collinear with the N–H bond vector, the deviation from axial symmetry of the $^{15}$N CSA tensor provides an additional contribution to cross correlation and thereby affects the value derived for CSA$^{\text{red}}$. Following Chung et al., calculations for the case of isotropic tumbling and an asymmetric $^{15}$N CSA tensor indicate that the typical values of the $^{15}$N CSA asymmetry observed in peptide bond amides affect CSA$^{\text{red}}$ by not more than a few percent.

**Correlation between CSA$^{\text{red}}$ and $S^2$.** The magnitude of the relaxation interference effect, $\eta$, is directly proportional to the generalized order parameter, $S^2$, and the variability in $S^2$ is considerably larger than the variation in $\eta$. Therefore, the CSA$^{\text{red}}$ values provide information on the relative values of the order parameters of the backbone $^{15}$N nuclei. Figure 6 shows the correlation between the order parameters calculated previously from $^{15}$N $T_1$ and $T_2$ values and the CSA$^{\text{red}}$ values calculated using eqs 4 and 8, using an isotropic rotational correlation time of 4.1 ns. The solid line corresponds to the relation between CSA$^{\text{red}}$ and $S^2$, calculated using $\sigma_{0} - \sigma_{1} = -170$ ppm, and $\theta = 20^\circ$. The previously measured $S^2$ values deviate from the solid line with a rmsd of 0.045.

![Figure 6](image_url)  
Figure 6. Correlation between the CSA$^{\text{red}}$ values in human ubiquitin and the previously measured order parameters. CSA$^{\text{red}}$ values are calculated from data recorded at 600 MHz, using $2\Delta = 46.7$ ms, assuming isotropic diffusion with $\tau_c = 4.1$ ns. The solid line corresponds to the correlation expected if $\sigma_{0} - \sigma_{1} = -170$ ppm and $\theta = 20^\circ$. The previously measured $S^2$ values deviate from the solid line with a rmsd of 0.045.

Concluding Remarks

We have demonstrated that cross correlation between dipolar and CSA relaxation mechanisms can be quantitatively measured from two simple two-dimensional NMR experiments. For $^{15}$N, the experiments offer good sensitivity, approximately a factor of 5 lower than a regular $^{1}$H–$^{15}$N HSQC experiment, and yield information on the magnitude of the chemical shift anisotropy, provided order parameters for the backbone amides have been derived from $^{15}$N relaxation measurements.

The average $^{15}$N CSA, measured in this manner, is $\sim$170 ppm, assuming $\theta = 20^\circ$. This value is about 10 ppm larger than the width of the $^{15}$N powder pattern observed in solid-state NMR spectra of small model compounds, but this difference may be caused by the fact that the solid-state NMR powder pattern width is reduced by rapid internal fluctuations of the amide. For example, for an $S^2$ value of 0.9, the width of the powder pattern reduces by 5% relative to the static value. The correlation observed between the CSA and the isotropic shift indicates that the sum of the most shielded CSA tensor components is largely invariant to structural changes.

Provided the rotational correlation time of the protein is known, the measurement of cross correlation yields information on the generalized order parameter $S^2$, i.e., on the amplitude of the rapid internal motions. In contrast to the most commonly used approach, where this number is derived from $T_1$, $T_2$, and NOE values, the present method is not affected by line broadening caused by slow conformational exchange. However, as a result of the inherent variations in $\theta$ and the magnitude of the $^{15}$N chemical shift anisotropy, the precision of the order parameter obtained from the experiments proposed in this paper is limited by ca. 5%. The $S^2$ values derived in this manner show improved agreement with order parameters obtained from conventional $^{15}$N relaxation measurements when the previously derived axially symmetric diffusion tensor is used instead of isotropic tumbling.

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Appendix

In the presence of internal motions, there is no rigorous, generally valid relationship between cross- and auto-correlation functions. However, this Appendix shows that if the rotational diffusion of the macromolecule is isotropic and the internal motions can be described as independent, small, equal amplitude, restricted rotations about three mutually orthogonal axes, $\langle P_2(\theta_{pq} )\rangle = P_2(\cos(\theta_{pq})); i = p, q,$ just as is the case in the absence of internal motions. Moreover, for a more general class of physically plausible small amplitude internal motions, the above is a good approximation if $\theta_{pq}$ is sufficiently small.

Unlike auto-correlation functions, cross-correlation functions are not always positive and can even increase with time. Since NMR cross-correlation functions are identical to those that determine the fluorescence depolarization of probes whose
emission and absorption dipoles are not coaxial, they have been extensively studied in the fluorescence literature.\textsuperscript{31}

For an isotropically reorienting macromolecule, in the presence of internal motions that are uncorrelated with the overall motion,

\[
\langle p_\text{2}(\mu_p(0)\cdot\mu_q(t)) \rangle = \exp(-\nu \tau_c) \langle p_\text{2}(\mu_p(0)\cdot\mu_q(t)) \rangle \text{internal} \equiv \exp(-\nu \tau_c) C_{pq}(t) \quad (A.1)
\]

In general, \( C_{pq}(0) = P_2(\cos \theta_{pq}) \). The simplest approximation for \( C_{pq}(t) \), which is exact at \( t = 0 \) and \( \infty \), is

\[
\begin{align*}
C_{pq}(t) & = C_{pq}(\infty) + [P_2(\cos \theta_{pq}) - C_{pq}(\infty)] \exp(-\nu \tau_c) \\
& \text{where \( \theta_p \) and \( \theta_q \) are the polar angles of \( \mu_p \) and \( \mu_q \), \( \phi_{pq} \) is the difference in their azimuthal angle, and \( d_{m(2)} \) are reduced Wigner rotation matrices. It follows from eq A.3 that} \\
C_{pq}(\infty) & = \sum_{m=-2}^{2} |\langle \exp[(im\gamma(0) - \gamma(t))]d_{m(2)}(\theta_p)d_{m(2)}(\theta_q)\rangle| \cos(m\phi_{pq}) \quad (A.3)
\end{align*}
\]

For free rotations, such as apply to methyl groups, \( C_{pq}(\infty) = P_2(\cos \theta_{pq})P_2(\cos \theta_{pq}) \), and \( C_{pq}(\infty) = P_2(\cos \theta_{pq})^2 \), \( i = p, q \). For backbone amides the amplitude of rotations is restricted and one has \( |\langle \exp[im\gamma(t)]\rangle|^2 = 1 - m^2 \langle \langle \delta\gamma^2 \rangle \rangle \), where \( \langle \langle \delta\gamma^2 \rangle \rangle \) is the mean square of the rotation angle. After some algebra, one finds

\[
\begin{align*}
C_{pq}(\infty) & = P_2(\cos \theta_{pq}) - 3\langle \delta\gamma^2 \rangle \sin \theta_p \sin \theta_q \times \\
& \quad \cos \theta_p \cos \theta_q \cos \phi_{pq} + \sin \theta_p \sin \theta_q \cos 2\phi_{pq} \quad (A.5)
\end{align*}
\]

When \( p = q \), this reduces to

\[
\begin{align*}
C_{qq}(\infty) & = 1 - 3\langle \delta\gamma^2 \rangle \sin^2 \theta_q \quad (A.6)
\end{align*}
\]

Equation A.5 shows that, depending on geometry, \( C_{pq}(\infty) \) can be either smaller or greater than \( P_2(\cos \theta_{pq}) \).

Equation A.5 can be generalized to include independent, small-amplitude, restricted rotations about one or more additional axes. To lowest order, all one has to do is add analogous terms proportional to \( \langle \delta\gamma^2 \rangle \) with coefficients determined by the polar and azimuthal angles with axis I taken as the \( z \) axis. To illustrate this, suppose \( \mu_p \) and \( \mu_q \) lie in the \( yz \) plane, with the \( z \) axis bisecting the angle between \( \mu_p \) and \( \mu_q \). If the angle between \( \mu_p \) and the \( z \) axis is \( \pm \theta/2 \), then \( P_2(\cos \theta_{pq}) = P_2(\cos \theta) \). Suppose one has independent rotations with mean-square amplitude \( \langle \delta\gamma^2 \rangle \), \( I = x, y, z \), about the three axes, then

\[
\begin{align*}
C_{pq}(\infty) & = P_2(\cos \theta) + 3\langle \delta\gamma^2 \rangle [\sin^2(\theta/2) \cos \theta] - \\
& \quad 3\langle \delta\gamma^2 \rangle [\cos^2(\theta/2) \cos \theta] - 3\langle \delta\gamma^2 \rangle [\cos 2\theta] \quad (A.7)
\end{align*}
\]

since for rotations about the \( z \) axis \( \theta_p = \theta_q = \theta/2 \) and \( \phi_{pq} = \pi \), about the \( x \) axis \( \theta_p = \theta_q = \pi/2 \) and \( \phi_{pq} = 2\theta \), and about the \( y \) axis \( \theta_p = \pi/2 + \theta/2 \), \( \theta_q = \pi/2 - \theta/2 \), and \( \phi_{pq} = 0 \). Similarly \( C_{pp}(\infty) = C_{qq}(\infty) = 1 - 3\langle \delta\gamma^2 \rangle \sin^2(\theta/2) - 3\langle \delta\gamma^2 \rangle - 3\langle \delta\gamma^2 \rangle \cos^2(\theta/2) \quad (A.8)\)

When \( \langle \langle \delta\gamma^2 \rangle \rangle = 0 \), \( \langle \langle \delta\gamma^2 \rangle \rangle = 0 \), \( \langle \langle \delta\gamma' \rangle \rangle = 0 \), and

\[
C_{pq}(\infty) = P_2(\cos \theta)[1 - 6\langle \langle \delta\gamma^2 \rangle \rangle] \quad (A.9)
\]

and

\[
C_{qq}(\infty) = 1 - 6\langle \langle \delta\gamma^2 \rangle \rangle \quad (A.10)
\]

It then follows from eq A.2 that

\[
C_{pq}(t) = P_2(\cos \theta)C_{pq}(t) \quad (A.11)
\]

as claimed in the beginning of the Appendix. Note, however, that eq A.11 is not exact for arbitrary types of internal motion and applies only to isotropic overall motion. However, provided \( \theta \) is small, eq A.11 remains a good approximation for other physically reasonable types of internal motion of peptide amides. For example, if \( \langle \langle \delta\gamma^2 \rangle \rangle = 0 \) and \( \langle \langle \delta\gamma' \rangle \rangle = \langle \langle \delta\gamma'' \rangle \rangle = \langle \langle \delta\gamma'' \rangle \rangle \),

\[
\begin{align*}
C_{pq}(\infty) & = C_{qq}(\infty) = 1 - 3\langle \delta\gamma^2 \rangle [1 + \cos^2(\theta/2)] = \\
& = 1 - 6\langle \delta\gamma^2 \rangle (1 - \theta^2/8 + ...) \quad (A.12)
\end{align*}
\]

and

\[
\begin{align*}
P_2(\cos \theta) & = P_2(\cos \theta) \{1 - 3\langle \delta\gamma^2 \rangle \cos^2(\theta/2) \cos \theta + \\
& \quad 2\theta[\cos(2\theta)]\cos(2\theta) \}
\end{align*}
\]

Comparison of eqs A.12 and A.13 confirms that eq A.11 remains a good approximation provided \( \theta \) is small.

When the rotational diffusion is anisotropic, expressions for the correlation functions become considerably more complex. For example, for axially symmetric rotational diffusion, in the absence of internal motions, one has\textsuperscript{30,31}

\[
\begin{align*}
\langle p_\text{2}(\mu_p(0)\cdot\mu_q(t)) \rangle & = \exp[-6D_1t]P_2(\cos \theta_2)P_2(\cos \theta_2) + \\
& \exp[-(5D_1 + D_2)t](3/4) \sin(2\theta_2) \sin(2\theta_2) \cos(\phi_{pq}) + \\
& \exp[-(2D_1 + 4D_2)t](3/4) \sin^2(\theta_2) \sin^2(\theta_2) \cos(2\phi_{pq}) \quad (A.14)
\end{align*}
\]

where now \( \theta_2 \) and \( \phi_2 \) are the spherical angles describing the orientation of \( \mu_p \) and \( \mu_q \) relative to the unique axis of the rotational diffusion tensor. The auto-correlation function is

\[
\begin{align*}
\langle p_\text{2}(\mu_p(0)\cdot\mu_p(t)) \rangle & = \exp[-6D_1t]P_2(\cos \theta_2)^2 + \\
& \exp[-(5D_1 + D_2)t](3/4) \sin^2(\theta_2) + \\
& \exp[-(2D_1 + 4D_2)t](3/4) \sin^2(\theta_2) \quad i = p, q \quad (A.15)
\end{align*}
\]

and thus in general \( \langle p_\text{2}(\mu_p(0)\cdot\mu_q(t)) \rangle \neq \langle p_\text{2}(\mu_p(0)\cdot\mu_q(t)) \rangle \neq \langle p_\text{2}(\mu_p(0)\cdot\mu_q(t)) \rangle \). Thus, the approach used in the text to treat anisotropic overall motion is far from rigorous, although it is expected to be a reasonable approximation when \( \theta_{pq} \) is small.

**Supporting Information Available:** Table with CSA\textsuperscript{END} values, derived from data measured at 360 and 600 MHz \textsuperscript{1}H frequency for three different durations of the dephasing delay, 2A (3 pages). See any current masthead page for ordering and Internet access instructions.