Journal of Biomolecular NMR, **26:** 181–186, 2003.
KLUWER/ESCOM

Direct measurement of the ^{15}N CSA/dipolar relaxation interference from coupled HSQC spectra

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Received 16 December 2002; Accepted 7 February 2003

Key words: chemical shift anisotropy, coupled HSQC, cross-correlation, IPAP method, relaxation interference

Abstract

Here we propose a method for the measurement of the ^{15}N CSA/dipolar relaxation interference based on direct comparison of the ^{15}N doublet components observed in a ^{1}H-coupled ^{1}H-^{15}N HSQC-type spectrum. This allows the determination of the cross-correlation rates with no need for correction factors associated with other methods. The signal overlap problem of coupled HSQC spectra is addressed here by using the IPAP scheme (Ottiger et al., 1998). The approach is applied to the B3 domain of protein G to show that the method provides accurate measurements of the ^{15}N CSA/dipolar cross-correlation rates.

Measurements of the interference effects due to relaxation arising from CSA and dipolar interactions have recently attracted attention as valuable source of information about local structure and dynamics of biomolecules (e.g., Yang et al., 1997; Brutscher, 2000; Fushman and Cowburn, 2001; Schwalbe et al., 2001). In particular, ^{15}N CSA/dipolar cross-correlation rates can be used to determine the magnitude and orientation of the ^{15}N chemical shift tensor (Tjandra et al., 1996; Fushman et al., 1998), identify residues involved in conformational exchange (Fushman and Cowburn, 1998; Kroenke et al., 1998), and in combination with the more conventional autocorrelation rates (R_1, R_2) could be used to characterize the overall and local dynamics in proteins and nucleic acids (Tjandra et al., 1996; Fushman et al., 1998; Fushman and Cowburn, 1998; Boisbouvier et al., 1999; Dayie et al., 2002).

Several experimental approaches have been suggested and implemented to measure the cross-correlation rates in proteins (Tjandra et al., 1996; Tjandra and Bax, 1997; Tessari et al., 1997a, b; Pang and Zuiderweg, 2000). Most of these methods, such as those proposed by Bax and colleagues (Tjandra et al., 1996), are based on the comparison of signals observed in two separate experiments, A and B, that select for the in-phase (experiment A) or anti-phase (experiment B) components of ^{15}N magnetization. The cross-correlation rate is derived from the ratio of signals observed in two separate experiments. To compensate for the difference in the pulse sequences for the two experiments, these spectra have to be scaled. This scaling is somewhat arbitrary and could introduce bias in the values of the cross-correlation rate. Pulse sequences have also been published (Tessari et al., 1997a,b) that utilize the A/B strategy but achieve A or B selection by re-ordering refocusing pulses and delays (in combination with purge pulses), rather than introducing additional pulses to select the anti-phase or in-phase components.

Because the relaxation interference between ^{1}H-^{15}N dipolar interaction and ^{15}N CSA results in differential line broadening of the two components of the ^{15}N spin doublet (Goldman, 1984), the relative amplitudes of the corresponding ^{15}N signals in a ^{1}H-

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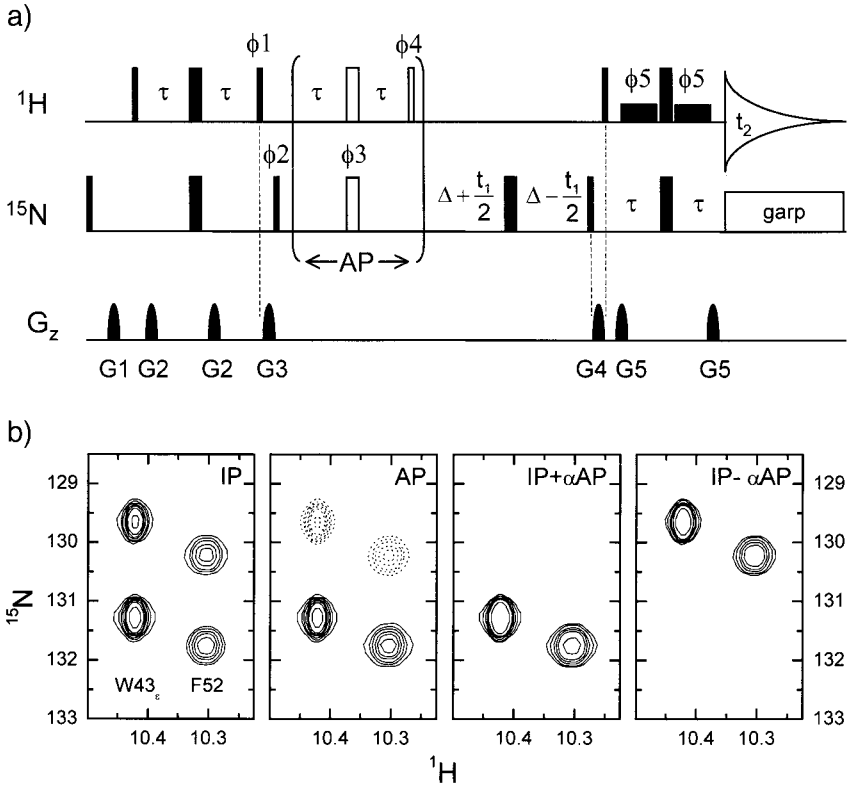

Figure 1. (a) Pulse sequences for the IP or AP experiments. The element labeled AP is omitted in the IP experiment. Narrow and wide pulses correspond to 90° and 180° flip angles, respectively. The two low power 90° pulses flanking the last nonselective 180° pulse on protons are 1 ms long. The delay τ is set to 2.5 ms, while the constant-time period 2Δ varies from one experiment to another, as described below. The phases are: $\phi1 = -y, y, \phi3 = 4\{x\},4\{y\},4\{-x\},4\{-y\}, \phi4 = 8\{x\},8\{-x\}, \phi5 = -x$; for the IP experiment $\phi2 = 2\{x\},2\{-x\}$ and receiver = x,−x,−x,x, for the AP experiment: $\phi2 = -y,-y, y, y$ and receiver = x,−x,−x, x,−x, x, x,−x. For quadrature detection, phases $\phi2$ and $\phi3$ are incremented in States-TPPI fashion. All other pulses are along x. Gradients were sine-shaped with the following strengths: G1 = 12 G cm^{-1}, G2 = 9 G cm^{-1}; G3 = 18 G cm^{-1}; G4 = 11 G cm^{-1}; G5 = 24 G cm^{-1}, their durations were 600 μs, 600 μs, 700 μs, 600 μs, and 700 μs, respectively. All experiments were performed on Bruker DRX-600 spectrometer equipped with a quadruple-resonance probe. Three IPAP measurements were performed with the delay Δ set to 31.91, 42.55, and 47.87 ms. Each IPAP experiment was run in an interleaved fashion as a pseudo-3D experiment, with the first and second 2D planes corresponding to IP and AP spectra, respectively. In addition, a separate set of eight IP-only experiments (referred to as IP experiment in Figures 2 and 3) was performed with the Δ values of 15.95, 21.28, 26.59, 31.91, 37.23, 42.55, 47.87 and 53.19 ms. The spectra were acquired with spectral widths of 7.2 kHz and 2 kHz in the ^{1}H and ^{15}N dimensions, respectively. 128 t_1 increments, each consisting of 1024 complex points, were collected for each 2D plane. The number of transients was the same for the IP and AP experiments corresponding to a given delay Δ. This number varied with Δ, from 64 for the shortest up to 160 for the longest delay, to compensate for the overall signal decay because of T_2 relaxation in order to keep the signal/noise ratio at approximately the same level. (b) Representative region illustrating IP, AP, and the simplified spectra; the solid and dashed contours represent positive and negative intensities, respectively. The scaling factor α for adding/subtracting the IP and AP spectra was optimized by least-squares minimization of the residual intensities in those positions of the resulting spectra where signal cancellation was expected. The value of α was determined from 49 non-overlapping doublets analyzed simultaneously and varied from 1.067 to 1.070 for different Δ values used here.

coupled ^{1}H-^{15}N HSQC spectrum directly yield this cross-correlation rate. As discussed previously (Tjandra and Bax, 1997), this principle is quite general for measuring cross-correlations for various types of interactions. Known examples include measurements of cross-correlation rates between H^N CSA and $H^N-^{15}N$ dipolar coupling (Tjandra and Bax, 1997) and between ^{13}CO CSA and ^{13}CO-^{13}Cα dipolar interaction (Pang and Zuiderweg, 2000). The obvious advantage of this approach is that both signals are observed in the same spectrum and, therefore, no ambiguity associated with the correction factors is involved. The application of this method to biomacromolecules, however, could be complicated by the signal overlap in the coupled 2D spectra. The problem is particularly severe in the case of H-coupled ^{1}H-^{15}N HSQC spectra for macromolecules greater than 10 kDa. Though 3D experimental schemes have been suggested to overcome the overlap

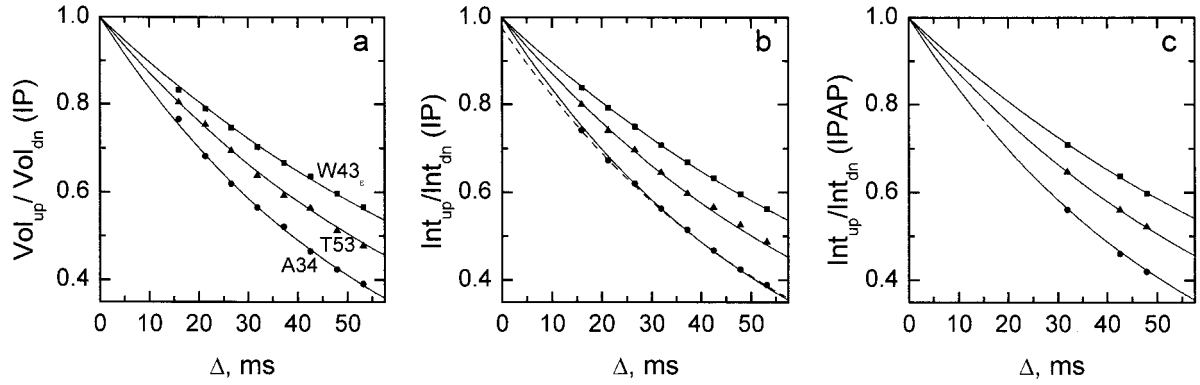

Figure 2. Representative decay curves for the ratios of (a) peak volumes and (b) peak intensities from the IP experiment and (c) of peak intensities in the IPAP experiment. Peak volume integration was performed using *peakint* program from XEASY package (Bartels et al., 1995). Shown are data for residues A34 (circles), T53 (triangles), and $W43_\varepsilon$ (squares). The error bars are comparable to the size of the symbols. The corresponding η values for these residues are $4.45 \pm 0.03\ s^{-1}$, $3.45 \pm 0.02\ s^{-1}$, and $2.71 \pm 0.01\ s^{-1}$ derived from the ratios of peak volumes and $4.50 \pm 0.01\ s^{-1}$, $3.41 \pm 0.01\ s^{-1}$, and $2.71 \pm 0.01\ s^{-1}$ from peak intensities, and $4.49 \pm 0.04\ s^{-1}$, $3.41 \pm 0.01\ s^{-1}$, $2.67 \pm 0.02\ s^{-1}$ from IPAP peak intensities. Because of possible signal truncation artifacts, we also considered a two-parameter expression: $\sigma_{up}/\sigma_{dn} = C \exp(-4\Delta\eta)$. An F statistics test (Press et al., 1992) was used to validate the necessity of an additional fitting parameter, C. The cutoff probability was set to 10^{-3}. For 4 out of 49 peak doublets in (a) and 13 out of 49 in (b) this two-parameter equation provided a better fit, with C values in the range 0.94 to 1.04. The one-parameter fit, Equation 1, was statistically better for all signals in the IPAP measurements. Of the data presented here, the two-parameter fit could be justified only for A34 in (b), as judged by the probability $P = 0.0004$ that the improvement in the fit occurred by chance. The corresponding fitting curve is shown as a dashed line in panel (b) and corresponds to $C = 0.98$ and $\eta = 4.32 \pm 0.09\ s^{-1}$.

problem, the substantial amount of experimental time needed can be prohibitive.

We therefore propose a 2D method for the measurement of the ^{15}N CSA/dipolar relaxation interference, based on direct comparison of the ^{15}N doublet components observed in a ^{1}H-coupled ^{1}H-^{15}N HSQC-type spectrum without the overlap problems of coupled spectra or associated correction factors. The signal overlap problem is addressed by using the IPAP scheme (Ottiger et al., 1998) that helps simplify coupled HSQC spectra, and the resulting data analysis avoids the need for a scaling factor. We apply this approach to the B3 domain of protein G and show that the method provides accurate measurements of the ^{15}N CSA/dipolar cross-correlation rates.

The NMR pulse sequence implemented here to measure the transverse cross-correlation rates is shown in Figure 1. The relaxation of interest takes place during the constant-time evolution period 2Δ. Protons are not decoupled during ^{15}N evolution period. This results in a ^{1}H-coupled ^{1}H-^{15}N HSQC spectrum with resolved ^{15}N spin doublet components. In the simple implementation of the experiment (called in-phase, IP, here), when the anti-phase (AP) element is absent, both components of the doublet are in-phase. The signals corresponding to the up-field ($\sigma_{up} = I_y S_z + I_y/2$) and downfield ($\sigma_{dn} = I_y S_z - I_y/2$) components of the ^{15}N spin doublet depend on the relaxation period 2Δ, and are described by the relations: $\sigma_{up} = \sigma_o \exp[-2\Delta(R_2 + \eta)]$ and $\sigma_{dn} = \sigma_o \exp[-2\Delta(R_2 - \eta)]$. Here η is the ^{15}N CSA/dipolar cross-correlation term, and R_2 is the transverse relaxation rate averaged over the in-phase and anti-phase components of the spin density. To achieve this averaging we implement the values of Δ as multiples of $1/(4J_{NH})$ (Ghose and Prestegard, 1998), where J_{NH} is the one-bond scalar ^{1}H-^{15}N coupling. The cross-correlation term, η, can then be determined directly from fitting the time dependence of the ratio of these signals to a mono-exponential decay function:

$$\sigma_{up}/\sigma_{dn} = e^{-4\eta\Delta}. \quad (1)$$

In the IPAP method two spectra are recorded with the ^{15}N doublet being in-phase (see above) and anti-phase (AP), and the two are added or subtracted to produce simplified spectra in which only one of the two components is retained while the other one is eliminated.

When the AP element is introduced into the pulse sequence (Figure 1), the corresponding signals can be written as $-f\sigma_{up}$ and $f\sigma_{dn}$, where f represents signal attenuation due to the AP element (Figure 1). To compensate for these losses and to achieve full cancellation of the unwanted signals, an empirically determined scaling factor α is applied to the AP spectrum prior to its addition to or subtraction from the IP spectrum (Ottiger et al., 1998). The ratio of the signals observed

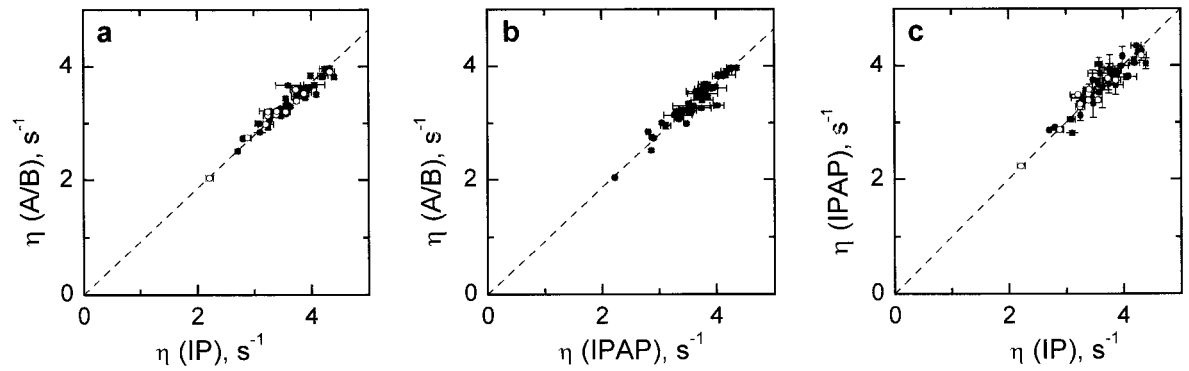

Figure 3. The agreement between the η values measured here using various experimental schemes discussed in the text: (a) IP experiment versus the A/B method (Tjandra et al., 1996), (b) IPAP scheme versus the A/B method, and (c) IP versus IPAP scheme. Only those spin systems (49 out of 56) that give isolated doublets were selected for the comparison with the IP data, panels (a) and (c). The corresponding relaxation delays (Δ) for the measurements using the A/B method were set to 31.91, 42.55, 53.19 and 63.82 ms. The data points in panels a and b fall on a straight line with the slope less than one (0.934 ± 0.020 and 0.930 ± 0.018, respectively, correlation coefficient R = 0.96), indicating a slight (7%) underestimation of the η values from the A/B method. This is a result of the difference in pulse sequences used in the experiments A and B (see text) and could be corrected by applying a uniform scaling factor of 1.07 to the η valued derived by the A/B method. Open circles in (a) and (c) indicate data points where the η values from the IP experiment were derived using the 2-parameter fit (see Figure 2).

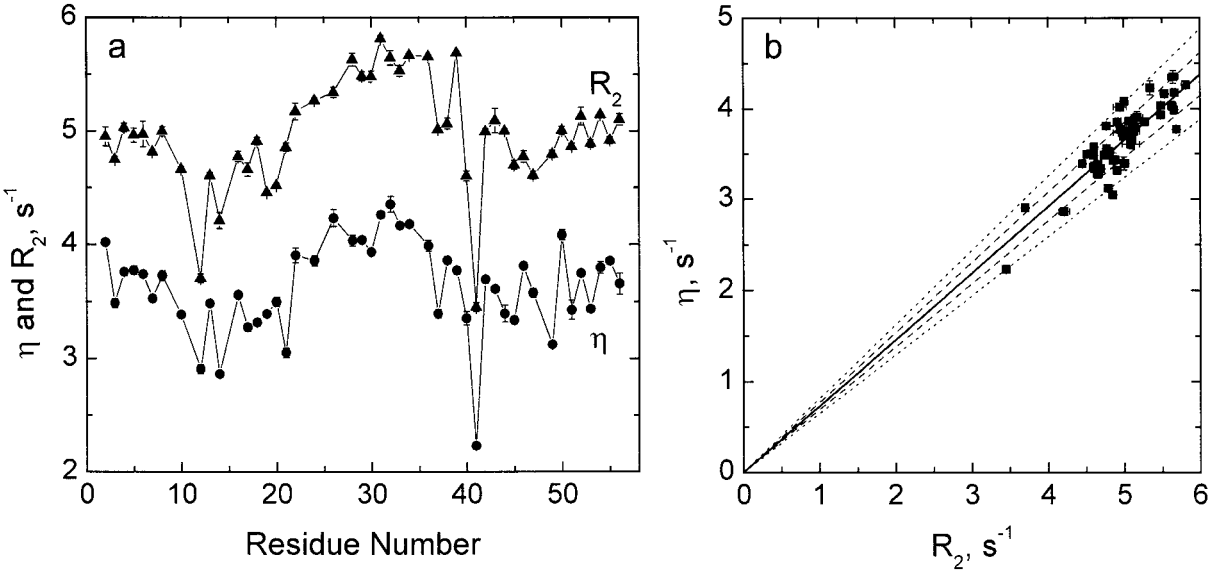

Figure 4. (a) ^{15}N transverse relaxation (R_2) and CSA/dipolar cross-correlation rates for the backbone amides in GB3 and (b) linear relationship (Fushman and Cowburn, 1998) between the η values determined here (IPAP measurement) and the ^{15}N transverse relaxation rates R_2. The solid line in b corresponds to average ^{15}N CSA of −160 ppm, the angle β between the ^{15}N CSA and ^{1}H-^{15}N dipolar tensors of 20° and the NH bond length of 1.02 A, consistent with our previous observations for ubiquitin (Fushman et al., 1998; Fushman et al., 1999). The dotted lines represent the range of ^{15}N CSA values (from −216 ppm to −125 ppm) observed in ubiquitin (here we assumed β = 20°) and dashed lines correspond to variations in β (20° ± 5°) for CSA = −160 ppm. The measurements of ^{15}N R_2 were performed using CPMG method as described earlier (Fushman et al., 1997). The R_2 values plotted here were corrected by subtracting high-frequency contributions, as outlined elsewhere (Fushman et al., 1999).

in the difference and sum spectra is then $\sigma_{diff}/\sigma_{sum} = (\sigma_{up} + \alpha f \sigma_{up}) / (\sigma_{dn} + \alpha f \sigma_{dn}) = \sigma_{up}/\sigma_{dn}$, same as in Equation 1. *This ratio of the signals derived from the IPAP experiment is independent of the factors f and α, which then eliminates the problem of possible bias by an arbitrary scaling, even if α is not exactly set to 1/f.*

Note that the signals measured using the A/B scheme could also be combined to yield the same relationship as in Equation 1. However, in this case the results will depend on the scaling factor, $\phi = A_t/A \geq 1$, correcting for additional signal attenuation in A: $\sigma_{up}/\sigma_{dn} = (B - A)/(B + A) \approx e^{-4\eta_t\Delta}$ [1+ (ϕ-1) $\sinh(4\eta_t\Delta)] \approx e^{-4(\eta_t/(\phi)\Delta}$, (assuming $\phi - 1 \ll 1$), which gives $\eta = \eta_t/\phi$. Here subscript 't' indicates 'true' values of A and η, unaffected by the additional signal attenuation in experiment A, so that $A_t/B = \tanh(2\Delta\eta_t)$ (see Tjandra et al., 1996).

Due to the constant-time spin evolution, signal evolution as a function of the incremented delay t_1 is not directly modulated by spin relaxation (Brutscher, 2000). This has the following consequences for the data analysis:

(1) The resulting line shape in F1 is no longer determined by spin relaxation, and therefore strongly depends on signal apodization in t_1. Therefore particular attention should be paid to the choice of window function applied in the t_1 dimension, to avoid 'wiggles' (base line oscillations) due to signal truncation. These artifacts could influence the intensities/volumes of the nearby peaks along the F1 dimension and could result in deviations from Equation 1. Our extensive analysis indicates that of the standard set of window functions available within XWINNMR package, squared *sinc* function provides the best results.

(2) The linewidths of the two spin doublet peaks are equal and determined by apodization rather than spin relaxation during ^{15}N evolution. Therefore the ratio of peak intensities can be used (Brutscher, 2000) as the experimental measure of σ_{up}/σ_{dn} in Equation 1 (Figure 2).

We applied this proposed method to measure the ^{15}N CSA/dipolar cross-correlation in the B3 domain of protein G. The η values determined here directly from the ^{1}H-coupled spectrum (IP experiment) are in good agreement with those derived from the A/B method (Tjandra et al., 1996) (Figure 3a). The comparison provided a scaling factor of 1.07 to correct the results of the A/B method. Note that the actual value of this factor that corrects for additional signal losses in the experiment A depends on the experimental setup (e.g., pulse calibration) and particular implementation of the original pulse sequence. For example, repeating these experiments on a cryoprobe resulted in the scaling factor of 1.06; similar measurements at 400 MHz (Bruker AM400 spectrometer, broadband probe) gave a smaller value, 1.03.

A comparison of the η values derived from the coupled spectrum (IP) with those from the simplified spectra from the IPAP experiment (Figure 3b) demonstrates that the IPAP scheme does not introduce any bias in the data. Finally, the data from IPAP measurements are in good agreement (Figure 3c) with those from the A/B method (Tjandra et al., 1996), scaled as described above. All these comparisons indicate that the suggested approach provides an accurate method for measuring the ^{15}N CSA/dipolar cross-correlation rates.

The derived values of η scale linearly with the R_2 values (Figure 4), in agreement with the theoretical prediction (Fushman and Cowburn, 1998). The average η/R_2 ratio corresponds to ^{15}N CSA of −160 ppm assuming the angle β between the unique axis of the ^{15}N CSA tensor and the NH vector of 20° and the effective NH bond length of 1.02 A. These average numbers are consistent with those reported earlier for ubiquitin (Fushman et al., 1998, 1999) and with solid-state NMR data on short peptides (e.g., Oas et al., 1987; Hiyama et al., 1988). The spread of data points around the average-slope line reflects site-specific variations in the ^{15}N CSA values and/or in the β angle. As pointed out earlier (Fushman and Cowburn, 1998), it is impossible to independently determine these parameters from single-field η/R_2 data. Their determination from multiple-field measurements is currently in progress.

In conclusion, we presented experimental approach to directly measure ^{15}N CSA/dipolar interference from the ratio of ^{15}N spin doublet components in a ^{1}H-coupled spectrum. This approach can be combined with the IPAP method to simplify the spectra. The approach provides cross-correlation rate values unbiased by the scaling factors. Similar experimental schemes could be used to measure the longitudinal ^{15}N CSA/dipolar cross-correlation term or cross-correlation between other couplings.

Acknowledgements

We thank Dr Ad Bax for providing us with the protein sample. Supported by grants from NIH (GM65334-01) and NSF (DBI-0138000) to D.F. and start-up funds from C.C.F. to K.T.D.

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