A TROSY CPMG sequence for characterizing chemical exchange in large proteins

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Abstract

A new NMR spin relaxation experiment is described for measuring chemical exchange time constants from approximately 0.5 ms to 5 ms in ^{15}N-labeled macromolecules. The pulse sequence is based on the Carr–Purcell–Meiboom–Gill technique [Carr and Purcell (1954) *Phys. Rev.*, **94**, 630–638; Meiboom and Gill (1958) *Rev. Sci. Instrum.*, **29**, 688–691; Loria et al. (1999) *J. Am. Chem. Soc.*, **121**, 2331–2332], but implements TROSY selection [Pervushin et al. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 12366–12371] to permit measurement of exchange linebroadening contributions to the narrower component of the ^{1}H-^{15}N scalar-coupled doublet. This modification extends the size limitation imposed on relaxation measurements due to the fast decay of transverse magnetization in larger macromolecules. The new TROSY-CPMG experiment is demonstrated on a [U-98% ^{15}N] labeled sample of basic pancreatic trypsin inhibitor and a [U-83% ^{2}H, U-98% ^{15}N] labeled sample of triosephosphate isomerase, a 54 kDa homodimeric protein.

Introduction

The kinship between conformational fluctuations and function in biological macromolecules is widely recognized (Frauenfelder et al., 1991; Palmer, 1997; Kay et al., 1998). A detailed knowledge of molecular dynamics is necessary for a comprehensive understanding of biological function. Many biochemical processes, including allostery, enzyme catalysis, protein folding and ligand binding events, occur on the microsecond to millisecond timescale. Recently, significant progress has been made in the development of $R_{1\rho}$ (Deverell et al., 1970; Akke and Palmer, 1996; Felli et al., 1998) and Carr–Purcell–Meiboom–Gill (CPMG) (Loria et al., 1999a) NMR methods to quantitate protein motions on these timescales. However, transverse relaxation rates of large macromolecules limit these methods to the study of dynamic processes in relatively small (< 20 kDa) proteins.

Wüthrich and co-workers have shown, through the implementation of transverse-relaxation optimized spectroscopy (TROSY), that significantly narrower NMR signals can be obtained from cancellation of ^{1}H-^{15}N dipole–dipole and ^{15}N chemical shift anisotropy (CSA) interactions (Pervushin et al., 1997). The decay of the individual ^{15}N multiplet components during a spin-echo sequence applied to the ^{15}N spins is described by (Goldman, 1984):

$$\frac{d\langle S^+ I^\alpha\rangle(t)}{dt} = -(R_2 + \eta + R_{ex})\langle S^+ I^\alpha\rangle(t) - \mu\langle S^+ I^\beta\rangle(t) \qquad (1)$$

$$\frac{d\langle S^+ I^\beta\rangle(t)}{dt} = -(R_2 - \eta + R_{ex})\langle S^+ I^\beta\rangle(t) - \mu\langle S^+ I^\alpha\rangle(t) \qquad (2)$$

in which S = ^{15}N, I = ^{1}H, $< S^+ I^{\alpha/\beta} > (t) =$ Trace$[S^+ I^{\alpha/\beta}\sigma(t)]$, $\sigma(t)$ is the density operator, R_2

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is the average transverse relaxation rate for in-phase and antiphase ^{15}N coherences, η is the ^{1}H-^{15}N dipole-dipole/^{15}N CSA relaxation interference rate constant, R_{ex} is additional signal damping caused by chemical or conformational exchange, and μ is the rate constant for cross relaxation between the two lines of the doublet. Identical equations can be written for the $S^- I^{\alpha/\beta}$ coherences. For two sites in fast conformational or chemical exchange, R_{ex} is approximated by (Luz and Meiboom, 1963):

$$R_{ex} = \Phi_{ex}\tau_{ex}\left[1 - (2\tau_{ex}/\tau_{cp})\tanh(\tau_{cp}/2\tau_{ex})\right] \quad (3)$$

in which $\Phi_{ex} = (\omega_1 - \omega_2)^2 p_1 p_2$ and p_i and ω_i are the populations and Larmor frequencies for the nuclear spin in site i; τ_{ex} is the reduced lifetime of the exchanging sites and τ_{cp} is the delay between 180° ^{15}N pulses in the CPMG pulse train. Exact expressions, valid for all timescales, are given elsewhere (Davis et al., 1994). R_{ex} is a function of τ_{cp}, which provides the basis for characterizing exchange by varying τ_{cp}. The value of μ also depends on τ_{cp} because the average overlap of the $S^+ I^{\alpha}$ and $S^+ I^{\beta}$ operators depends on the relative phase angles acquired by the operators during the spin echo period (Palmer et al., 1992). Thus, $\mu = \mu_0 \sin(\pi J\tau_{cp})/(\pi J\tau_{cp})$ and μ_0 is the difference between the relaxation rate constants for in-phase and antiphase ^{15}N coherences. In the limit where $\tau_{cp} \to 0$, $\mu \to \mu_0$, and as $\tau_{cp} \to \infty$, $\mu \to 0$.

The present paper describes a modified proton-detected ^{15}N CPMG spin-echo pulse sequence in which the narrow multiplet component is selected during the CPMG relaxation period, the indirect labeling period, and the detection period. The TROSY-CPMG sequence facilitates the quantification of exchange linebroadening contributions in larger macromolecules because the relative conformational exchange contribution to the phenomenological relaxation rate constant during the CPMG period is enhanced and the improved resolution and sensitivity of TROSY ^{1}H-^{15}N correlation spectra are obtained. The experiment is demonstrated on basic pancreatic trypsin inhibitor (6 kDa) and triosephosphate isomerase (TIM; 54 kDa). A related pulse sequence for measuring ^{1}H chemical exchange has been developed by Ishima and co-workers (Ishima et al., 1998).

Materials and methods

The 2.6 mM [U-98% ^{15}N] BPTI (pH 5.1, T = 300 K) and 2.0 mM [U-83% ^{2}H, U-98% ^{15}N] TIM
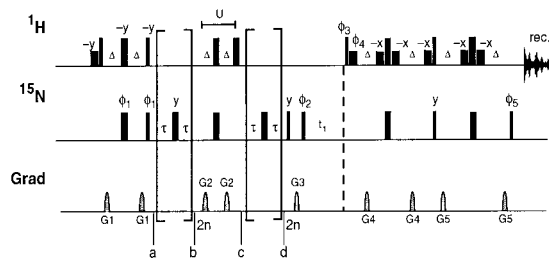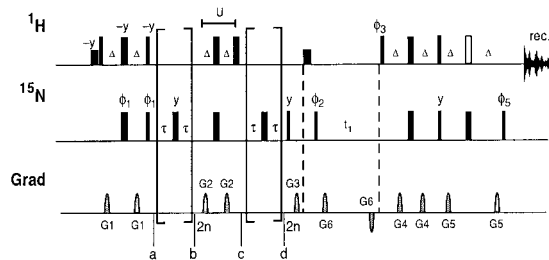

Figure 1. TROSY-CPMG pulse sequences for measuring conformational and chemical exchange in ^{15}N-labeled proteins. All pulses are x phase unless otherwise indicated. Narrow and wide bars depict 90° and 180° pulses, respectively, and short wide bars indicate selective (1 ms) 90° pulses. The 180° ^{15}N pulses in the spin-echo sequence had durations of 97 μs. Delays are $\Delta = 2.7$ ms, $\tau = 0.5$ τ_{cp}. The FIDs were acquired with the PEP scheme in which echo and antiecho FIDs are acquired for each t_1 point (Cavanagh et al., 1991; Palmer et al., 1991; Rance et al., 1999). The phase cycle for the first FID is $\phi_1 = 4(x)$, $4(-x)$; $\phi_2 = -y$, y, x, $-x$; $\phi_3 = y$; $\phi_4 = -y$; $\phi_5 = x$; receiver = x, $-x$, $-y$, y, $-x$, x, y, $-y$. The phase cycle for the second FID is $\phi_1 = 4(x)$, $4(-x)$; $\phi_2 = -y$, y, x, $-x$; $\phi_3 = -y$; $\phi_4 = y$; $\phi_5 = -x$; receiver = $-x$, x, $-y$, y, x, $-x$, y, $-y$. These phases are specific for Bruker spectrometers as described previously; for Varian spectrometers, the y and $-y$ phases must be interchanged (Rance et al., 1999). In (b) the open bar represents a WATERGATE soft -90° hard -180° soft -90° pulse element (Piotto et al., 1992) or the 3-9-19 selective pulse (Sklenár et al., 1993).The gradient pulses had the shape of the center lobe of a sine function. Gradient durations G1–G5 were 0.4 ms, 0.3 ms, 0.35 ms, 0.4 ms, 0.5 ms. Gradient amplitudes were G1$_z$ = 9 G/cm; G2$_z$ = 6.2 G/cm; G3$_z$ = 9.7 G/cm; G4$_z$ = 10.4 G/cm; G5$_{xyz}$ = 24.2 G/cm. In b, the bipolar gradient pulses (G6) should be set at a level sufficient to prevent radiation damping of the water magnetization during the t_1 period.

(pH 5.8, T = 293 K) samples were as previously described (Loria et al., 1999a; Rance et al., 1999). However, a new MALDI-TOF mass spectrometric analysis (HHMI Protein Core Facility, Columbia University) has established the level of deuterium incorporation in the TIM sample to be 83%, rather than 45% as originally reported (Rance et al., 1999). All

experiments were performed on a Bruker DRX600 spectrometer operating at a ^{15}N Larmor frequency of 60.82 MHz. TROSY-CPMG pulse sequences for measuring conformational exchange are depicted in Figure 1; relaxation-compensated CPMG experiments used the pulse sequence of Figure 1b of Loria et al. (1999a). For BPTI, spectra were acquired using (128 × 4096) complex points and spectral widths of (2500 × 12500) Hz in the (t_1 × t_2) dimensions. The recycle delay was 3 s and a total of 16 transients were recorded for each complex t_1 point. A total of 10 to 15 relaxation time points (including duplicates) were acquired for each decay curve by varying n in Figure 1. NMR data for TIM were recorded in an identical manner except that 32 transients were recorded per t_1 point. For BPTI, TROSY-CPMG relaxation decay curves were recorded for τ_{cp} = 1.0 ms, 4.5 ms, and 10.0 ms; relaxation-compensated CPMG relaxation decay curves were recorded for τ_{cp} = 1.0 ms and 10.0 ms. For TIM both TROSY-CPMG and relaxation-compensated CPMG relaxation decay curves were recorded for τ_{cp} = 0.8 ms. Intensity versus time data were fitted to a monoexponential decay equation. Uncertainties in the peak intensities were determined from duplicate experiments and uncertainties in the relaxation rate constants were obtained from jackknife simulations (Mosteller and Tukey, 1977). Backbone assignments for BPTI were taken from Glushka et al. (1989). Backbone assignments for TIM were determined using TROSY HNCO, HN(CA)CO, HN(CO)CA, HNCA, HN(CA)CB and HN(COCA)CB experiments (Loria et al., 1999b; Salzmann et al., 1999) and will be reported elsewhere.

Results and discussion

Figure 1 shows pulse sequences used for measuring ^{15}N $R_2(\tau_{cp})$ for the narrow component of the ^{1}H-^{15}N scalar-coupled doublet. The pulse sequences are designed to suppress cross-relaxation between the doublet components, which would otherwise contribute to the apparent τ_{cp}-dependence of the phenomenological relaxation rate constants. At point a, the magnetization is present as a linear combination of $S_y I^\alpha = (1/2i)(S^+ - S^-)I^\alpha$ and $S_y I^\beta = (1/2i)(S^+ - S^-)I^\beta$ coherences (representing the two multiplet components) and relaxes during the first spin echo sequence, between points a and b, according to Equations 1 and 2. The **U** period uses the S^3CT pulse sequence element (Sørensen et al., 1997) to invert the sign of

$S^+ I^\alpha$ at point c. Selective inversion of either one of the multiplet components effectively inverts the sign of μ. Relaxation continues through a second spin echo sequence between points c and d to yield the effective relaxation equations at point d:

$$\langle S^+ I^\alpha \rangle(t) = \langle S^+ I^\alpha \rangle(0) e^{-(R_2 + \eta + R_{ex})t} \qquad (4)$$

$$\langle S^+ I^\beta \rangle(t) = \langle S^+ I^\beta \rangle(0) e^{-(R_2 - \eta + R_{ex})t} \qquad (5)$$

in which $t = 4n\tau_{cp}$ and n is an integer. More complex procedures can be utilized for suppression of cross relaxation if necessary (Kroenke et al., 1998; Loria et al., 1999a). At point d in Figure 1a, the partially relaxed ^{15}N magnetization is returned to the Z-axis and residual transverse magnetization is eliminated by gradient G3. The remainder of the pulse sequence serves to frequency-label and detect the narrow component of the ^{1}H-^{15}N doublet using the TROSY approach (Pervushin et al., 1997). In Figure 1a, water suppression utilizes a combination of selective 90° and WATERGATE (Piotto et al., 1992) soft 90°-hard 180°-soft 90° pulses to maintain the water magnetization along the +Z axis following point d. In Figure 2b, water suppression utilizes the FHSQC approach (Mori et al., 1995; Rance et al., 1999). In this case, the water magnetization is rotated into the transverse plane by a selective 90° pulse prior to the t_1 period and returned to the +Z axis by the last non-selective 90° ^{1}H pulse.

The TROSY-CPMG experiment was performed on a [U-98% ^{15}N] BPTI sample (Huang et al., 1997). Figure 2a shows the increasing decay rate of the narrow multiplet component, $R_2(\tau_{cp})$, as τ_{cp} increased for the backbone amide ^{15}N spin of residue C38 in BPTI. The increase in $R_2(\tau_{cp})$ as τ_{cp} is increased is indicative of a conformational exchange contribution to relaxation of this spin. The differences, $\Delta R_2(\tau_{cp})$, between $R_2(\tau_{cp})$ measured at τ_{cp} values of 10.0 ms and 1.0 ms for all residues in BPTI are presented in Figure 2b. Values of $\Delta R_2(\tau_{cp})$ obtained using the TROSY-CPMG agreed well with data acquired under identical conditions using the relaxation-compensated CPMG experiment (Loria et al., 1999a) (data not shown). Because $\Delta R_2(\tau_{cp})$ depends only on the exchange linebroadening, this result substantiates the accuracy of the TROSY-CPMG method. For the majority of the backbone ^{15}N spins $\Delta R_2(\tau_{cp})$ is less than 0.5 s^{-1}, which confirms the robustness of the procedure incorporated into the pulse sequence for suppressing cross relaxation between the multiplet components. Larger values of $\Delta R_2(\tau_{cp})$, indicative of conformational exchange processes associated with disulfide isomerization, are
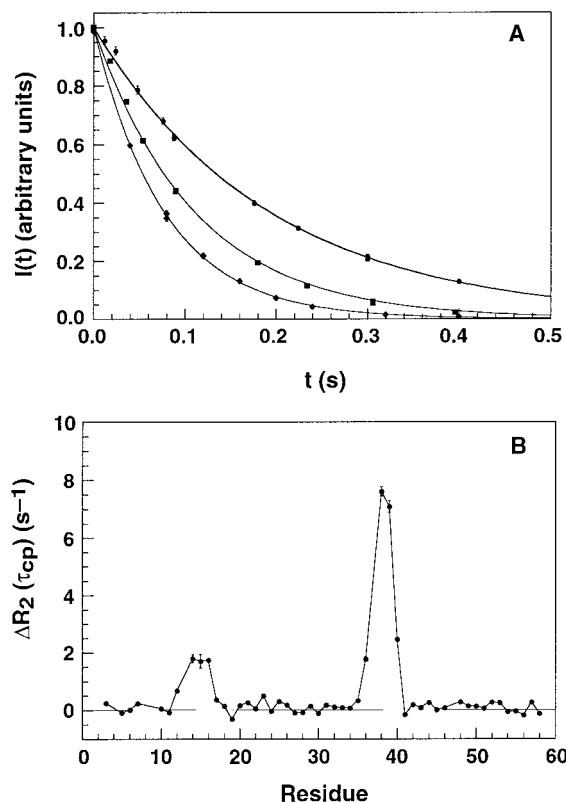
Figure 2. Conformational exchange in BPTI. (A) ^{15}N TROSY-CPMG relaxation data for C38 in BPTI. Decay curves were measured at τ_{cp} values of (●) 1 ms, (■) 4.5 ms, and (♦) 10.0 ms. Fitted $R_2(\tau_{cp})$ rates at these τ_{cp} values are 5.21 ± 0.06 s^{-1}, 9.10 ± 0.15 s^{-1} and 12.82 ± 0.18 s^{-1} respectively. (B) The differences $\Delta R_2(\tau_{cp}) = R_2(10\ ms) - R_2(1\ ms)$ are plotted versus the residue number for all assigned residues in BPTI. Uncertainties usually are smaller than the size of the plotted data points.

observed for residues in the vicinity of C14 and C38, as previously described (Szyperski et al., 1993; Loria et al., 1999a).

The usefulness of incorporating TROSY selection into the ^{15}N CPMG experiment for larger proteins was demonstrated using a [U-98% ^{15}N, U-83% ^{2}H] sample of TIM, a 54 kDa homodimer (Rance et al., 1999). The data presented in Figure 3 are for residue T172 in the active-site loop of TIM; from numerous other studies, this loop is known to move between open and closed conformations (Alber et al., 1981; Lolis et al., 1990; Lolis and Petsko, 1990; Davenport et al., 1991; Noble et al., 1991; Verlinde et al., 1991; Williams and McDermott, 1995). The $R_2(\tau_{cp})$ values for T172 are 20.39 ± 0.36 s^{-1} using TROSY selection and 41.49 ± 1.17 s^{-1} for the relaxation-compensated experiment. The average $R_2(\tau_{cp})$ at $\tau_{cp} = 0.8$ ms for all residues
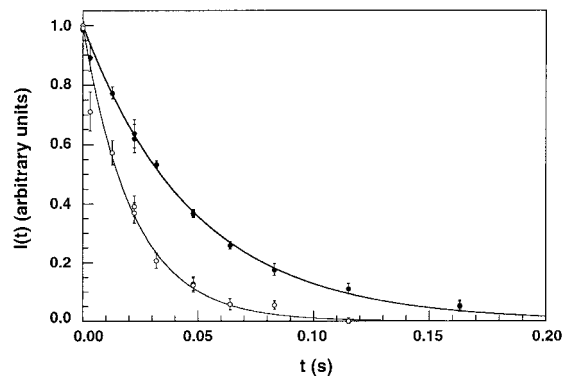

Figure 3. ^{15}N CPMG relaxation data for T172 in TIM. The relaxation decay curves are compared for (●) the TROSY-CPMG sequence and (○) the relaxation-compensated CPMG experiment (Loria et al., 1999a) with $\tau_{cp} = 0.8$ ms. The fitted $R_2(\tau_{cp})$ values are (●) 20.39 ± 0.36 s^{-1} and (○) 41.49 ± 1.17 s^{-1}.

in TIM is 16.11 ± 0.29 s^{-1} using the TROSY-CPMG experiment and 40.12 ± 2.11 s^{-1} with the relaxation-compensated experiment, a decrease of 2.5-fold in the apparent relaxation rate. Therefore, exchange broadening contributes a factor of 2.5-fold more to the TROSY-CPMG linewidth compared to the relaxation-compensated CPMG; for example, an $R_{ex} = 4$ s^{-1} represents 25% of the TROSY-CPMG linewidth, but only 10% of the relaxation-compensated CPMG. This increased contrast is the principal advantage of the TROSY-CPMG sequence.

Conclusions

In conclusion, the TROSY-CPMG pulse sequence allows quantitative measurement of conformational exchange processes on 0.5 to 5 ms timescales in proteins by recording the τ_{cp}-dependent relaxation rate constant for the narrow component of the backbone amide ^{15}N doublet. To characterize faster dynamic processes, the same pulse sequence can be employed for off-resonance $R_{1\rho}$ measurements by replacing the CPMG sequence elements with off-resonance spin-locking periods (Akke and Palmer, 1996). The decrease in relaxation rates obtained with TROSY selection allows relaxation measurements aimed at quantifying conformational exchange dynamics to be performed on larger macromolecules than is currently possible.

Acknowledgements

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