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Improved TROSY-HNCA experiment with suppression of conformational exchange induced relaxation

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Abstract

A general method for improving of the sensitivity of the TROSY-type triple resonance experiments in the presence of conformational exchange-induced (CSX) relaxation is proposed based on the use of CPMG-INEPT (Müller et al., *J. Am. Chem. Soc.*, 1995, **117**, 11043–11048) during the N–C polarization transfer periods. Significantly improved sensitivity is demonstrated for the majority of cross-peaks in the new [^{15}N,^{1}H]-TROSY-XY-HNCA experiment, measured with partially folded RNase AS-Protein, with negligible loss of sensitivity for resonances unaffected by CSX relaxation. In addition, a comparison of cross-peak amplitudes in [^{15}N,^{1}N]-TROSY-XY-HNCA and conventional [^{15}N,^{1}H]-TROSY-HNCA spectra provides a quick and sensitive estimation of the CSX relaxation contribution.

Abbreviations: TROSY, transverse relaxation-optimized spectroscopy; CPMG-INEPT, Carr Purcel Meiboom Gill-type polarization transfer; CSX, conformational exchange-induced relaxation

The presence of strong conformational exchange-induced (CSX) relaxation is frequently observed for partially folded or unfolded proteins, which can severely deteriorate NMR spectra of such proteins (Mulder et al., 1996) and impede the polarization transfer between spins in triple-resonance experiments (Mueller et al., 1995; Simorre et al., 1996; Zhang et al., 1997a,b). In addition, partially folded proteins or proteins under nonnative conditions frequently exhibit a tendency to form dynamical aggregates in solution, which increases the apparent molecular weight of the particles and further complicates NMR studies due to the strong dipole–dipole coupling (DD) and chemical shift anisotropy (CSA) relaxation of the involved spins.

The TROSY method (Pervushin et al., 1997) is based on the use of the interference between DD and CSA (Goldman, 1984; Shimizy, 1964) or between two remote CSA interactions (Kumar and Kumar, 1996) to reduce transverse relaxation in two heteronuclear spin systems such as ^{1}H-^{13}C and ^{1}H-^{15}N moieties in proteins and nucleic acids (Brutscher et al., 1998; Meissner and Sorensen, 1999; Pervushin et al., 1997, 1998, 1999; Yang and Kay, 1999). Interference effects are manifested in different relaxation rates of the components of single quantum multiplet or multiple-quantum manifolds with different quantum number. Since interference induced cross-correlated relaxation is independent of the presence of chemical shift and spin-spin scalar coupling hamiltonians (Goldman, 1984), TROSY-type optimization can be employed during polarization transfer periods under conditions where the transverse part of the coherence with the preferred relaxation properties is spin-locked by an rf-field. In turn, spin-locking rf-fields or CPMG-type sequences consisting of 180° pulses can suppress conformational exchange-induced transverse relaxation provided that the rf-field strength or the repetition rate of the CPMG pulse train exceeds the rate of the exchange process (Deverell et al., 1970; Jen, 1978). In the present work

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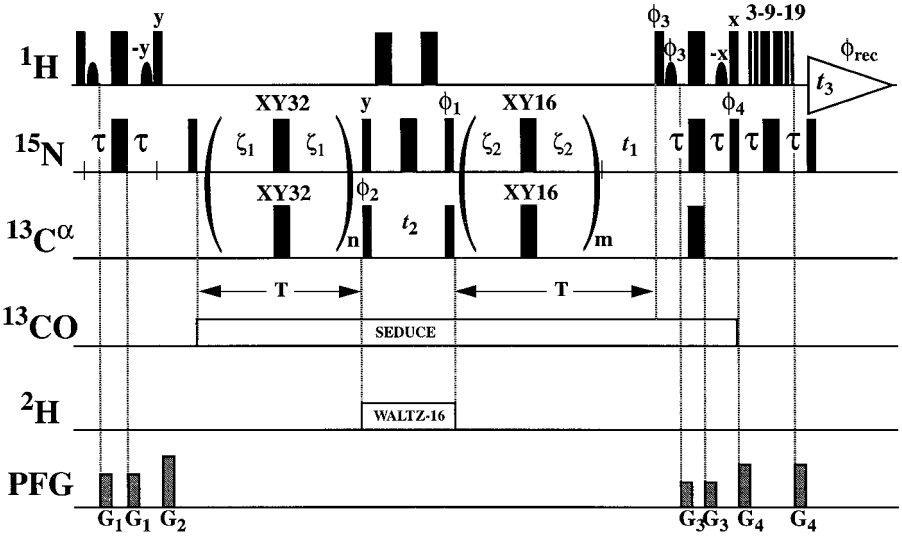

Figure 1. Experimental scheme for the [^{15}N,^{1}H]-TROSY-XY-HNCA experiment. The radio-frequency pulses on ^{1}H, ^{15}N, ^{13}C, ^{13}CO, ^{2}H and ^{1}H$^{\alpha}$ are applied at 4.7, 118, 55, 174, 3.6 and 4.7 ppm, respectively. Narrow and wide black bars indicate non-selective 90° and 180° pulses, respectively. Sine bell shapes on the line marked ^{1}H indicate water selective 90° pulses. The line marked PFG indicates the duration and strength of pulsed magnetic field gradients applied along the z-axis: G_1: 800 μs, 50 G/cm; G_2: 800 μs, 80 G/cm; G_3: 800 μs, 40 G/cm; G_4: 800 μs, 70 G/cm. The delays are τ = 2.2 ms, T = 24.7 ms, ζ_1 = 0.35 ms and ζ_2 = 0.73 ms - t_1/(2m), and the number of cycles n = 32 and m = 16. The phases of the ^{15}N and ^{13}C 180° pulses enclosed in brackets follow the XY32 and XY16 phase modulation supercyles, respectively (Gullion et al., 1990), The XY32 phases are: {xyxy yxyx $\overline{\text{xyxy}}$ $\overline{\text{yxyx}}$ $\overline{\text{xyxy}}$ $\overline{\text{yxyx}}$ xyxy yxyx} and the XY16 phase cycle contains the first 16 steps of XY32. The rest of the phase cycle is: ϕ_1 = {y, −y, −x, x}; ϕ_2 = {4x, 4(−x)}; ϕ_3 = {−y}; ϕ_4 = {−y}; ϕ_{rec} = {y, −y, −x, x, −y, y, x, −x}, {x} for all other pulses. A phase-sensitive spectrum in the ^{15}N(t_1) dimension is obtained by recording a second FID for each t_1 value, with ϕ_1 = {y, −y, x, −x} and ϕ_4 = {y}, and data processing as described by (Kay et al., 1992). Quadrature detection in the ^{13}C$^{\alpha}$(t_2) dimension is achieved by the States-TPPI method (Marion et al., 1989) applied to the phase ϕ_2. The use of water flip-back pulses (Grzesiek & Bax, 1993; Mori et al., 1995) ensures that the water magnetization stays aligned along +z axis throughout both the constant-time periods T and the data acquisition period ^{1}H(t_3). The final proton pulse is a composite pulse sequence 90°*3/13(y) -120u- 90°*9/13(y) -120u- 90°*19/13(y) -120u- 90°*19/13(−y) -120u- 90°*9/13(−y) -120- 90°*3/13(−y). For the deuterated proteins the experiments can be performed with ^{2}H-decoupling during t_2 achieved with WALTZ-16 (Shaka et al., 1983) at a field strength of γB_2 = 2.5 kHz.

we use a CPMG-type polarization transfer together with TROSY optimization to simultaneously suppress DD, CSA and CSX relaxation of ^{15}N spins during long polarization transfer delays in the HNCA experiment resulting in significantly improved sensitivity.

Figure 1 shows the experimental scheme for the proposed 3D [^{15}N,^{1}G]-TROSY-XY-HNCA pulse sequence, which is based on the TROSY-type HNCA experiment developed for partially deuterated and uniformly ^{15}N and ^{13}C labeled proteins (Eletsky et al., 2001). The key element of the new experiment is the use of XY CPMG-type sequences of 180° pulses (Gullion et al., 1990; Mueller et al., 1995; Mulder et al., 1996) during N–C polarization transfer delays. XY16 or XY32 phase alternation can effectively compensate for propagation of signal dephasing during the CPMG pulse train for all three orthogonal components of the magnetization thus resulting in simultaneous inversion and refocussing of magnetization with good offset properties suitable for efficient polarization transfer (Gullion et al., 1990; Mulder et al., 1996). In the framework of the TROSY-HNCA experiment, the application of XY sequences to J-coupled ^{15}N and ^{13}C spins during polarization transfer preserves the evolution of scalar coupling without interchanging TROSY and anti-TROSY ^{15}N multiplet components due to the longitudinal ^{1}H spin and at the same time reduces dephasing of spin coherence of the transverse ^{15}N spin.

Recording of ^{15}N chemical shifts in the TROSY-XY-HNCA experiment is combined with the C→N polarization transfer by simultaneously decreasing the delays between 180° pulses and incrementing the delay t_1 during which CSX relaxation is not suppressed (see Figure 1). Numerical simulations show that this scheme is preferable to a truncation of the XY16 supercycle within the CPMG-INEPT to allow chemical shift evolution of the transverse spin. The choice of the number of XY CPMG pulses during polarization transfer is dictated by a compromise between the sup-

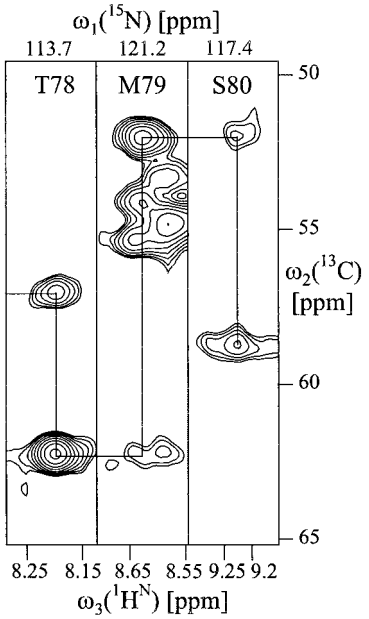

Figure 2. $^{1}H^{N}$-^{13}C Strips from a 3D [^{15}N,^{1}H]-TROSY-XY-HNCA experiment recorded with uniformly ^{15}N and ^{13}C-labeled RNase AS-Protein in $^{1}H_2O$:$^{2}H_2O$ (97:3) solution (protein concentration 1 mM, pH = 7.5, 35 °C) on a Bruker DRX-800 spectrometer using the experimental scheme of Figure 1 $36(t_1)^*32(t_2)^*1024(t_3)$ complex points were accumulated yielding t_{1max} = 13 ms, t_{2max} = 6.4 ms and t_{3max} = 51.2 ms, respectively. An interscan delay of 840 ms and 32 scans per increment were used resulting in a measuring time of 38 h. Shown are contour plots of [$\omega_2(^{13}C)$, $\omega_3(^{1}H)$] strips taken at the positions of $^{1}H^{N}$ and ^{15}N resonances of residues T78, M79 and S80. The direct peaks and their corresponding relay peaks are connected with thin lines, thus outlining the identification of sequence specific assignments.

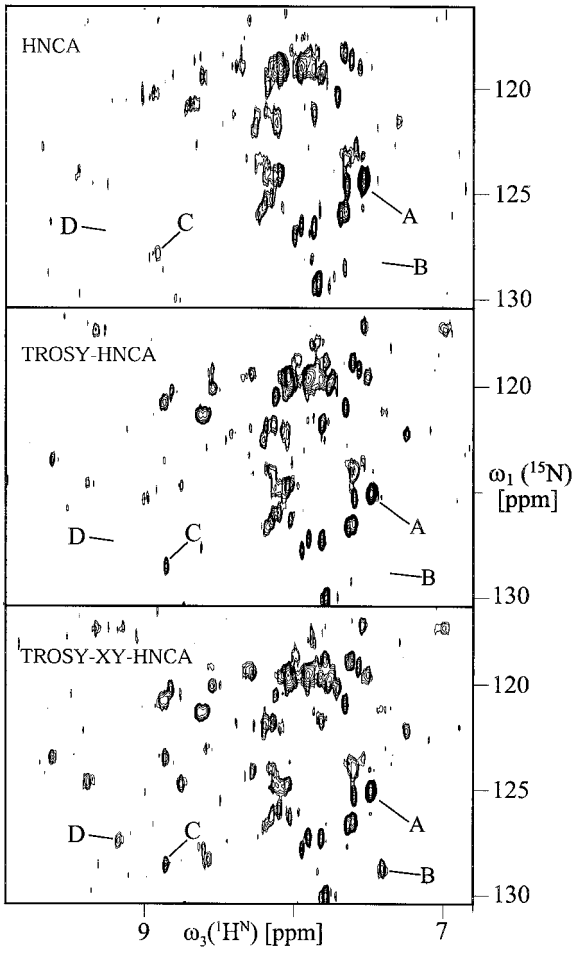

Figure 3. Comparison of the [^{15}N,^{1}H]-TROSY-XY-HNCA of Figure 1 with the [^{15}N,^{1}H]-TROSY-HNCA (Eletsky et al., 2001) and a conventional water-flip-back HNCA (Cavanagh et al., 1996; Kay et al., 1990) applied to the uniformly ^{15}N and ^{13}C-labeled RNase AS-Protein (see Figure 1 for sample details). For each of the experiments a 2D [^{15}N,^{1}H]-correlation spectrum was measured with the ^{13}C evolution delay t_2 set to 0 ms. $36(t_1)^*1024(t_3)$ complex points were accumulated yielding t_{1max} = 13 ms and t_{3max} = 51.2 ms, respectively, and a total measurement time of 35 min. The spectra were processed and plotted identically. The four cross-peaks marked from A to D are chosen for the detailed comparison shown in Figure 4.

pression of the CSX relaxation pathway and scaling down the effective J_{NC} coupling constant due to the finite length of the ^{15}N 180° pulses (Pervushin et al., 2000). With the experimental setup described in Figure 1 the J_{NC} coupling constant is expected to decrease by less than 5%.

The experimental scheme of Figure 1 was used to improve the performance of triple-resonance experiments measured with uniformly ^{15}N and ^{13}C-labeled RNase AS-Protein derived from RNase A, which is a 124 amino acid protein with a compact globular structure stabilized by four disulfide-bonds (Richards and Wyckow, 1971). RNase occurs in two-forms, A and B, that differ only in that B has an N-linked, high-mannose glycan in position N34. Conformational variants of both can be obtained by the so-called ribonuclease S system (Trombetta and Helenius, 2000). By limited proteolysis the peptide bond between A20–S21 can be selectively cleaved, resulting in a structurally only slightly altered (Kim et al., 1992), but enzymatically fully active complex. By removing the N-terminal 20 amino acid peptide, the enzymatically inactive, but folding competent so-called RNase AS-Protein is generated. While no structural data are available, the presence of the 4 disulfide bonds and far-UV CD-spectra suggest the retention of some residual structure (Richard and Wyckow, 1971; Ritter and Helenius, 2000). It has been shown recently that

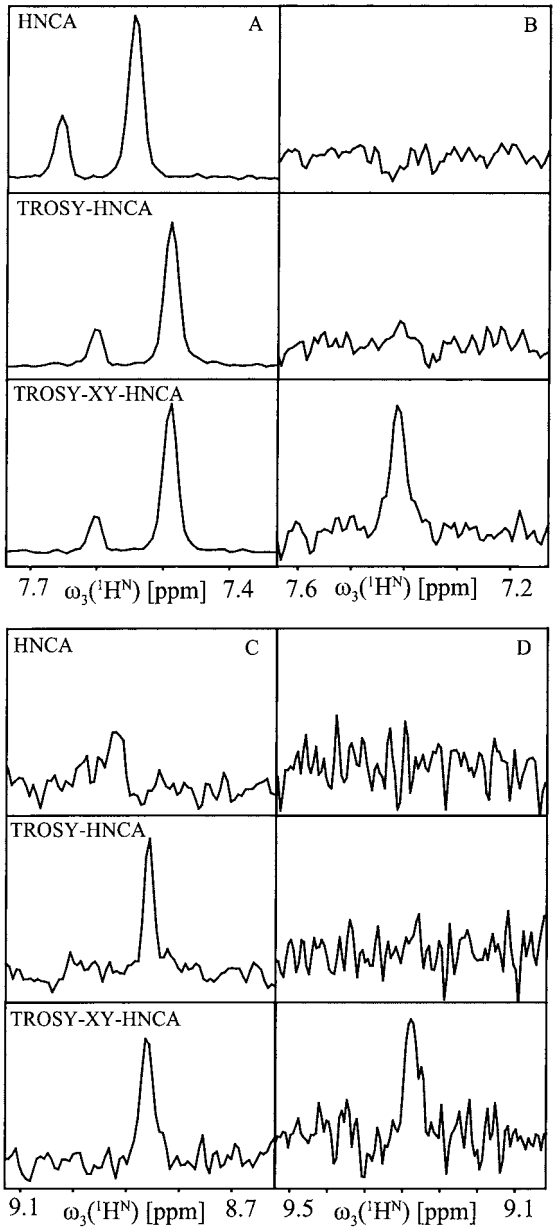

Figure 4. 1D slices along the $\omega_3(^1H^N)$ dimension taken through the positions of the four cross-peaks marked (a) A and B and (b) C and D in Figure 3. Identical vertical scaling was applied to 1D slices of the same cross-peak in the three experiments though for clarity scaling of the 1D slices within one spectrum is adjusted individually for each cross-peak. The corresponding 2D [^{15}N,^{1}H]-correlation spectra were measured with the [^{15}N,^{1}H]-TROSY-XY-HNCA experimental scheme of Figure 1, [^{15}N,^{1}H]-TROSY-HNCA (Eletsky et al., 2001) and conventional HNCA (Cavanagh et al., 1996; Kay et al., 1990) with an otherwise identical setup outlined in Figure 3.

UDP-glucose:glycoprotein glucosyltransferase, an enzyme which specifically glucosylates partially structured glycoproteins in the endoplasmic reticulum, recognizes RNase BS-Protein, where as nicked or reduced and alkylated, random-coil-like RNase B is not recognized (Ritter and Helenius, 2000; Trombetta and Helenius, 2000). These findings support the notion that RNase AS-Protein has a non-native, but partially structured conformation which might display significant flexibility.

A good quality TROSY-XY-HNCA spectrum is obtained for the RNase AS-Protein using the experimental scheme of Figure 1. Figure 2 shows a stretch of the [$\omega_2(^{13}C)$,$\omega_3(^1H)$] strips taken at the positions of $^1H^N$ and ^{15}N resonances of residues T78, M79 and S80. The use of XY CPMG-INEPT significantly improves the signal-to-noise ratio of a number of resonances throughout the HNCA correlation spectrum. This is demonstrated in Figure 3 by a comparison of the 2D [^{15}N,^{1}H-correlation planes recorded with the [^{15}N,^{1}H]-TROSY-XY-HNCA experimental scheme of Figure 1 [^{15}N,^{1}H]-TROSY-HNCA (Eletsky et al., 2001) and conventional water-flip-back HNCA (Cavanagh et al., 1996; Kay et al., 1990). Four cross-peaks with distinct spectral properties are selected, which are marked from A to D in Figure 3. 1D slices along the ω_3 ($^1H^N$) dimension taken trough their corresponding positions are shown in Figures 4a and 4b. Apparently the cross-peak A is not affected by CSX relaxation as manifested by very similar spectral intensity in the conventional and XY experiments. This comparison also demonstrates that long CPMG sequences do not results in appreciable dephasing of transverse ^{15}N magnetization or decreased polarization transfer efficiency between ^{15}N and ^{13}C spins. For the cross-peaks B and D a dramatic improvement in sensitivity is observed with the use of [^{15}N,^{1}H]-TROSY-XY-HNCA. However the lower signal-to-noise ratio observed for peak D compared to peak B implies a significantly larger contribution to CSX relaxation for peak D. In the case of peak C, most of the improvement comes from the use of TROSY optimization, which is expected for a large protein in the absence of strong CSX relaxation (Salzmann et al., 2000).

The ratio of cross-peak intensities in TROSY-XY-HNCA and TROSY-HNCA experiments with non-constant time recording of ^{15}N chemical shifts (or their simplified 2D versions where the ^{13}C evolution are removed) can provide a quick and sensitive estimation of the CSX contribution to the transverse ^{15}N

relaxation. The main advantage of that approach is the absence of the need to uniformly suppress cross-correlated relaxation in a series of the $T_{1\rho}$ (^{15}N) experiments (Cavanagh et al., 1996) measured with the variation of the delay between the 180° pulses (Orekhov et al., 1995) or with the range of the spin-locking field strengths (Szyperski et al., 1993), which might significantly complicate the interpretation of the results. To estimate the time scale of exchange processes on a per-residue basis a pair of TROSY experiments can be acquired at different polarizing magnetic field strengths (Millet et al., 2000).

The sensitivity improvement of the proposed TROSY-XY-HNCA experiment stems from the suppression of CSX relaxation during long N–C polarization transfer delays. For individual residues which are strongly affected by CSX relaxation and additional improvement in sensitivity can be expected if the ^{1}H,^{15}N-INEPTs are replaced by the ^{1}H,^{15}N-CPMG-INEPTs (Mueller et al., 1995; Mulder et al., 1996). Although this indeed resulted in a significant improvement of the signal-to-noise ratio for a few backbone resonances of RNase AS-Protein, a larger saturation of the water resonance with a concomitant decrease of cross-peak amplitudes from solvent exposed residues due to exchange between the H^N and the water protons (Stonehouse et al., 1994) recommended against the use of the ^{1}H,^{15}N-CPMG-INEPT as a general technique.

Thus, the use of CPMG-type polarization transfer together with TROSY optimization enables simultaneous suppression of the DD, CSA and CSX relaxation pathways of ^{15}N spins during long polarization transfer delays resulting in significantly improved sensitivity for TROSY-type triple resonance experiments. Although these experiments are well suited for biomolecules exhibiting a significant amount of CSX relaxation such as partially folded or unfolded proteins, they can also be expected to improve sensitivity for the spins affected by CSX relaxation in folded proteins without compromising sensitivity of unaffected spins. The XY polarization transfer element can be employed with practically all other TROSY-type triple-resonance experiments designed for the backbone resonance assignments in proteins (Salzmann et al., 1999).

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