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# Improved sensitivity and coherence selection for [$^{15}$N,$^{1}$H]-TROSY elements in triple resonance experiments

Michael Salzmann, Gerhard Wider, Konstantin Pervushin & Kurt Wüthrich*
*Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Hönggerberg, CH-8093 Zürich, Switzerland*

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**Abstract**

In experiments with proteins of molecular weights around 100 kDa the implementation of [$^{15}$N,$^{1}$H]-TROSY-elements in [$^{15}$N]-constant-time triple resonance experiments yields sensitivity enhancements of one to two orders of magnitude. An additional gain of 10 to 20% may be obtained with the use of 'sensitivity enhancement elements'. This paper describes a novel sensitivity enhancement scheme which is based on concatenation of the $^{13}C^{\alpha} \rightarrow {}^{15}$N magnetization transfer with the ST2-PT element, and which enables proper TROSY selection of the $^{15}$N multiplet components.

*Abbreviations:* 3D, three-dimensional; ct, constant-time; INEPT, insensitive nuclei enhanced by polarization transfer; PFG, pulsed field gradient; rf, radio-frequency; ST2-PT, single transition-to-single transition polarization transfer; TPPI, time-proportional phase incrementation; TROSY, transverse relaxation-optimized spectroscopy.

For protein sizes around 100 kDa the implementation of [$^{15}$N,$^{1}$H]-TROSY elements (Pervushin et al., 1997) in [$^{15}$N]-constant-time (ct) triple-resonance experiments yields sensitivity enhancements of one to two orders of magnitude (Salzmann et al., 1998; Salzmann, 1999). This intrinsic gain is due to the optimization of the $^{15}$N transverse relaxation during the long $^{15}$N-to-$^{13}$C and $^{13}$C-to-$^{15}$N magnetization transfer periods. There is room for further improvement using 'sensitivity enhancement elements' (Palmer et al., 1991, 1992; Kay et al., 1992; Yang and Kay, 1999a), since no transverse relaxation optimization is applied during the magnetization transfer steps between $^{15}$N and $^{1}$H. In our previous implementations of TROSY into triple resonance experiments (Salzmann et al., 1998, 1999a,b), the single transition-to-single transition polarization transfer element (ST2-PT; Pervushin et al., 1998) was used to selectively transfer the narrow component of the $^{15}$N doublet to the narrow component of the $^{1}$H doublet. Similar to the sensitivity enhancement schemes (Kay et al., 1992; Palmer et al., 1992; Muhandiram and Kay, 1994) that were previously introduced into conventional triple-resonance experiments (Montelione and Wagner, 1989, 1990; Ikura et al., 1990; Grzesiek and Bax, 1992), the ST2-PT element increases the overall duration of a triple resonance experiment by 5.4 ms, so that part of the intrinsic sensitivity gain is offset by the additional relaxation losses. To overcome this limitation, an alternative signal transfer and selection element was recently proposed for triple-resonance experiments containing [$^{15}$N,$^{1}$H]-TROSY elements (Yang and Kay, 1999a,b), which is based on a two-spin order pathway in the place of the multiple-quantum coherences used in the ST2-PT element. This procedure did result in an additional sensitivity increase of about 10% due to the reduction of the overall duration of the pulse sequence, but this gain was achieved at the expense of losing proper TROSY selection of the narrow components in both the $\omega_1$($^{15}$N) and the $\omega_3$($^{1}$H) dimensions. The re-

*To whom correspondence should be addressed.

sulting spectra thus contain also a broad peak for each $^{15}$N-$^{1}$H group, which is the composite of the broad components of the $^{15}$N and $^{1}H^{N}$ doublets. These broad peaks are suppressed by rapid $^{15}$N relaxation for $^{15}$N-$^{1}$H groups located in the core of large proteins, but tend to be preserved for more flexible parts of large proteins, or when working with smaller molecules. Proper TROSY selection of the spectral components is therefore clearly desirable. Here, we describe an alternative sensitivity enhancement procedure that does not compromise the single-transition component selection by the ST2-PT element. It is based on incorporating the first part of the ST2-PT element into the $^{13}C^{\alpha} \rightarrow {}^{15}$N magnetization transfer period, which shortens the overall duration of the triple-resonance experiment by 5.4 ms, with a concomitant 10 to 20% increase in sensitivity.

The new approach to sensitivity optimization is illustrated with the sensitivity-enhanced [$^{15}$N,$^{1}$H]-TROSY-HNCA experiment for $^{2}$H,$^{13}$C,$^{15}$N-labeled proteins. The initial $^{1}$H magnetization at time point *a* (Figure 1) is transferred via $^{15}$N to $^{13}C^{\alpha}$ by two successive INEPT steps from time points *a* to *c*, and *c* to *d*, respectively. Between time points *d* and *e*, $^{13}C^{\alpha}$ evolves only with the $^{13}C^{\alpha}$ chemical shift, since $^{15}$N, $^{13}$CO and $^{2}$H are decoupled during $t_2$, and short values for $t_{2max}$ are chosen to minimize the evolution under the $^{1}J(^{13}C^{\alpha},{}^{13}C^{\beta})$ scalar couplings (Wider, 1998). During $t_2(^{13}C)$ the $^{15}$N spin-selective state with regard to $^{15}$N-$^{1}H^{N}$ is preserved, since no rf-pulses are applied on $^{1}$H (Pervushin et al., 1997). After the 90°($^{13}$C) and 90°($^{15}$N) pulses at time point *e* the $^{15}$N chemical shift is recorded during the ct $t_1(^{15}N)$ evolution period (Vuister and Bax, 1992) up to time point *f*, with $t_{1max} = T - 2\tau$. During the period from time points *e* to *g*, the $^{15}$N magnetization is refocused with regard to the scalar couplings $^{1}J(^{15}N,{}^{13}C^{\alpha})$ and $^{2}J(^{15}N,{}^{13}C^{\alpha})$. From time points *f* to *g* this refocusing period is concatenated with the first half of the ST2-PT element (Pervushin et al., 1998). After the 90°($^{1}$H) and 90°($^{15}$N) pulses at time point *g* there follows the second half of the ST2-PT element, and at time point *h* the transfer of $^{15}$N single-transition coherence to $^{1}$H single-transition coherence is completed.

The initial [$^{15}$N,$^{1}$H]-TROSY-HNCA experiment (Salzmann et al., 1998) was designed for maximum resolution in the $^{15}$N dimension by making use of both the $^{15}N \rightarrow {}^{13}C^{\alpha}$ and the $^{13}C^{\alpha} \rightarrow {}^{15}$N magnetization transfer steps for the ct $^{15}$N evolution period $t_1$. In this implementation the $^{15}$N spin-selective state stays transverse during the $^{13}$C evolution and

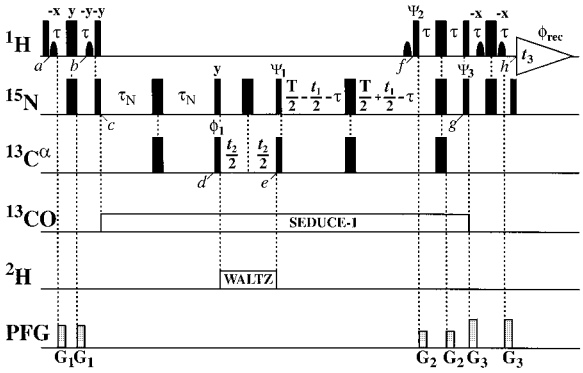


*Figure 1.* Sensitivity-optimized [$^{15}$N,$^{1}$H]-TROSY-HNCA experiment for uniformly $^{2}$H,$^{13}$C,$^{15}$N-labeled proteins in $H_2O$ solution. The narrow and wide black bars indicate non-selective 90° and 180° pulses, respectively. The radio-frequency (rf) pulses on $^{1}$H, $^{15}$N, $^{13}C^{\alpha}$, $^{13}$CO and $^{2}$H were applied at 4.8, 119, 55, 174 and 3.8 ppm, respectively. On the line marked $^{1}$H, sine bell shapes indicate selective 90° pulses with a duration of 1 ms and a Gaussian shape truncated at 5%, which are applied on the water resonance. The line marked PFG indicates Gaussian-shaped pulsed magnetic field gradients applied along the z-axis with the following durations and amplitudes: $G_1$, 800 μs and 15 G/cm; $G_2$, 800 μs and 9 G/cm; $G_3$, 800 μs and 22 G/cm. The delays are $\tau = 2.7$ ms, $\tau_N = 9$ ms, and $T = 18$ ms. The phase cycle is $\Psi_1 = \{y, -y, x, -x\}$, $\Psi_2 = \{-y\}$, $\Psi_3 = \{-y\}$, $\phi_1 = \{4x, 4(-x)\}$ and $\phi_{rec} = \{y, -y, -x, x, -y, y, x, -x\}$. For all other rf-pulses the phase is invariant throughout the experiments, either with phase x or as indicated above the pulses. In the $t_1(^{15}N)$ dimension a phase-sensitive spectrum is obtained by recording a second FID for each increment of $t_1$, with $\Psi_1 = \{y, -y, -x, x\}$, $\Psi_2 = \{y\}$, $\Psi_3 = \{y\}$, and the data is processed as described by Kay et al. (1992). Quadrature detection in the $t_2(^{13}C^{\alpha})$ dimension is achieved by the States-TPPI method (Marion et al., 1989) applied to the phase $\phi_1$. The water magnetization stays aligned along the +z-axis throughout the experiment by the use of water flip-back pulses (Grzesiek and Bax, 1993) at times *a* and *b*, and before *f*. Residual transverse water magnetization is suppressed by a WATERGATE sequence (Piotto et al., 1992) immediately before data acquisition. For $^{2}$H-decoupling, WALTZ-16 (Shaka et al., 1983) was used with a field strength of 2.5 kHz. $^{13}$CO decoupling was performed using off-resonance SEDUCE-1 (McCoy and Mueller, 1992) with a field strength of 0.83 kHz, which allows the application of rf-pulses on the $^{13}C^{\alpha}$ channel with a field strength of 20.83 kHz. The delay $\tau_N$ is optimized for maximum $^{15}N \rightarrow {}^{13}C^{\alpha}$ magnetization transfer efficiency, and T is chosen such that the desired $t_{1max}(^{15}N)$ value and thus the desired resolution in the $\omega_1(^{15}N)$ dimension is obtained.

is therefore subject to transverse $^{15}$N relaxation during the time period $t_2$. For measurements that do not require the highest possible resolution in the $\omega_1(^{15}N)$ dimension, $^{13}C^{\alpha}(t_2)$ chemical shift encoding via single-quantum coherences is preferable, since the $^{15}$N spin-selective state is then longitudinal during $t_2(^{13}C)$. This approach, which was previously used in the [$^{13}$C]-ct-[$^{15}$N,$^{1}$H]-TROSY-HNCA experiment (Salzmann et al., 1999a), is adopted in the scheme

of Figure 1. Therefore, to obtain a proper reference for assessment of the novel sensitivity enhancement scheme of Figure 1, we recorded reference spectra using the scheme of Salzmann et al. (1999a) without ct $^{13}$C-evolution. The differences between the two schemes compared in Figure 2 are located between the time points *e* and *g*, where the scheme of Figure 1 exploits two coherence pathways while recording the $^{15}$Nchemical shift during $t_1$, and the first half of the ST2-PT element is concatenated with the $^{13}C^{\alpha} \rightarrow {}^{15}$N-INEPT. The anticipated gain in sensitivity can be analyzed with the use of the magnetization transfer functions of Equations 1 and 2, where $f_A$ and $f_B$ are the transfer efficiencies (Ernst et al., 1987) for the scheme of Figure 1 and the one used to record Figure 2b, respectively.

$$f_A = \sin\left[\pi {}^1J_{NC}T\right]\cos[\pi {}^2J_{NC}T]\cdot \exp\left[-R_s^N(T-2\tau)\right]\cdot\zeta \quad (1)$$

$$f_B = \sin\left[\pi {}^1J_{NC}T\right]\cos[\pi {}^2J_{NC}T]\cdot \exp\left[-R_s^N T\right]\cdot\zeta \quad (2)$$

$^1J_{NC}$, $^2J_{NC}$ stand for $^1J(^{15}N,^{13}C^{\alpha})$ and $^2J(^{15}N,^{13}C^{\alpha})$, T is the ct-delay as defined in Figure 1, $R_s^N$ is the transverse $^{15}$N relaxation rate of the narrow component of the $^{15}$N doublet selected in TROSY (Salzmann et al., 1998), $\tau = 1/4{}^1J(^{15}N,^1H)$, and $\zeta$ accounts for all magnetization transfer steps and evolution times that are identical in the experiments used to record the spectra a and b in Figure 2. The sensitivity gain of the [$^{15}$N,$^1$H]-TROSY-HNCA experiment of Figure 1 is then given by

$$\frac{f_A}{f_B} = \exp\left[2R_s^N\tau\right] \quad (3)$$

For an effective rotational correlation time of $\tau_c$ = 70 ns, $2\tau$ = 5.4 ms, and $R_s^N$ calculated as described by Salzmann et al. (1998), which includes the contributions from dipolar relaxation with remote amide protons, gains of 10% or 15% are expected for $^{15}$N-$^1H^N$-moieties located either in a β-sheet or in an α-helix of a $^2$H,$^{13}$C,$^{15}$N-labeled protein.

The predictions from Equation 3 have been evaluated with experimental data of the type of Figure 2. Cross sections along the $\omega_3(^1H)$ dimension from 3D [$^{15}$N,$^1$H]-TROSY-HNCA spectra of the uniformly $^2$H,$^{13}$C,$^{15}$N-labeled 110 kDa protein 7,8-dihydroneopterin aldolase from *Staphylococcus aureus* (DHNA) (Hennig et al., 1998), recorded with

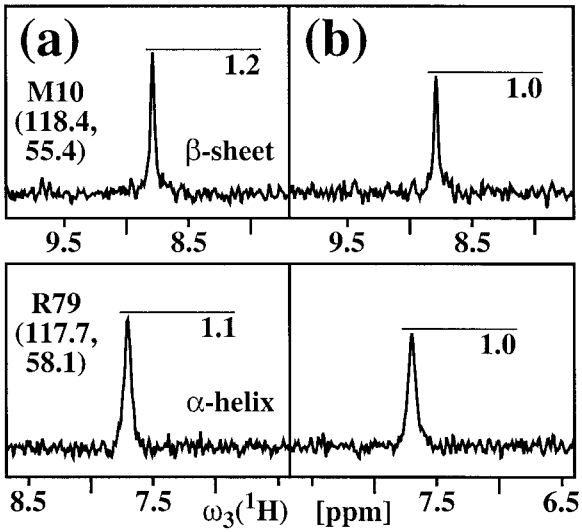


*Figure 2.* Comparison of [$^{15}$N,$^1$H]-TROSY-HNCA data obtained using the following schemes: (a) Figure 1; (b) The experiment of Salzmann et al. (1999a) without ct evolution of $^{13}C^{\alpha}$. Corresponding cross sections along the $\omega_3(^1H)$ dimension are shown. Both spectra were recorded at 20 °C with the same 0.5 mM sample of uniformly $^2$H,$^{13}$C,$^{15}$N-labeled 7,8-dihydroneopterin aldolase from *Staphylococcus aureus* (DHNA) (Hennig et al., 1998) in a mixed solvent of 95% $H_2O$/5% $D_2O$ at pH 6.5, using a Bruker DRX-750 spectrometer. Identical parameter settings were used for (a) and (b): data size 26($t_1$) × 32($t_2$) × 1024($t_3$) complex points; $t_{1max}(^{15}N)$ = 10.8, $t_{2max}(^{13}C)$ = 6.4, $t_{3max}(^1H)$ = 75.6 ms; 32 scans were acquired. In panel (a) the sequence-specific assignments are indicated by the one-letter amino acid code and the sequence number, the $^{15}$N and $^{13}C^{\alpha}$ chemical shifts are indicated in parentheses, and the secondary structure type for the residue is given. The relative peak amplitudes are indicated with a horizontal line and a number.

the experiment of Figure 1 (Figure 2a), show sensitivity improvements of 10% to 20% for individual cross peaks when compared with data acquired using the scheme of Salzmann et al. (1999a) without ct $^{13}$C evolution (Figure 2b). The data for Met10 and Arg79 (Figure 2), which are located in a β-sheet and an α-helix, respectively (Hennig et al., 1998), indicate that the predicted gain for $^{15}$N-$^1$H groups is qualitatively confirmed by the experiment.

In principle, the ct $t_1(^{15}N)$ evolution period in the [$^{15}$N,$^1$H]-TROSY-HNCA experiment of Figure 1 could be incorporated into the $^{15}N \rightarrow {}^{13}C^{\alpha}$-INEPT transfer between time points *c* and *d* rather than into the $^{13}C^{\alpha} \rightarrow {}^{15}$N INEPT period, but data recorded with this alternative scheme showed decreased sensitivity when compared to the [$^{15}$N,$^1$H]-TROSY-HNCA experiment of Figure 1 (data not shown). This is due to the presence of orthogonal transverse components of the $^{15}$N magnetization after the $t_1(^{15}N)$ chemical shift

evolution. When $t_1$ is placed between time points *c* and *d*, the two single-quantum and multiple-quantum coherences are subject to different transverse relaxation rates during the following $t_2(^{13}C)$ evolution period, which is unfavorable compared to the scheme of Figure 1, where the $^{15}N$ spin-selective state is preserved along the z-axis during $t_2(^{13}C)$.

The concatenation of the $^{13}C^{\alpha} \rightarrow {}^{15}N$ INEPT period with the ST2-PT element described here for the [$^{15}N$,$^{1}H$]-TROSY-HNCA experiment can readily be implemented in other triple resonance experiments with [$^{15}N$,$^{1}H$]-TROSY elements, such as [$^{15}N$,$^{1}H$]-TROSY-HNCO (Salzmann et al., 1998), [$^{15}N$,$^{1}H$]-TROSY-HNCACB, [$^{15}N$,$^{1}H$]-TROSY-HN(CA)CO, [$^{15}N$,$^{1}H$]-TROSY-HN(CO)CA, [$^{15}N$,$^{1}H$]-TROSY-HN(CO)CACB (Salzmann et al., 1999b) and [$^{13}C$]-ct-[$^{15}N$,$^{1}H$]-TROSY-HNCA (Salzmann et al., 1999a), with similar sensitivity gains as reported here for [$^{15}N$,$^{1}H$]-TROSY-HNCA, i.e., 10% to 20%.

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