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J-Modulated TROSY Experiment Extends the Limits of Homonuclear Coupling Measurements for Larger Proteins

Katalin E. Kövér*,[1] and Gyula Batta†

*Department of Inorganic and Analytical Chemistry, University of Debrecen, P.O. Box 21; and †Research Group for Antibiotics of the Hungarian Academy of Sciences, P.O. Box 70, H-4010 Debrecen, Hungary

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This paper describes the use of a TROSY experimental scheme and its variant extended with a scaled *J*-modulation spin-echo sequence for accurate and sensitive measurement of homonuclear $^3J(H_NH_\alpha)$ coupling constants in larger proteins with uniform ^{15}N labeling. Exclusive selection of the most slowly relaxing component of a ^{15}N–^1H multiplet by the TROSY approach leads to substantial improvement in resolution; this is a prerequisite for accurate measurement of couplings from the ^1H multiplets directly along the ^1H frequency dimension or from the *J*-scaled doublets along the ^{15}N frequency dimension. © 2001 Academic Press

Key Words: **isotope labeled proteins; nuclear magnetic resonance; scalar coupling constant; transverse relaxation-optimized spectroscopy; TROSY.**

INTRODUCTION

Scalar coupling constants, together with quantities like homonuclear nuclear Overhauser effects (*1*) and the recently introduced residual dipolar couplings (*2, 3–5*) and relaxation interference rates (*6–10*), provide important insight into the conformational properties of proteins in solution. In particular, the homonuclear scalar coupling constant, $^3J(H_NH_\alpha)$, being directly related to the ϕ angle via the appropriately parametrized Karplus equation (*11–14*), can be used to characterize the backbone conformation and identify secondary structural elements.

Several experiments, including three-dimensional triple-resonance techniques (*15–17*) and spin-state-selective excitation methods (*18–23*), have been proposed for the measurement of scalar coupling constants in isotopically (^{15}N and/or ^{13}C) enriched proteins. Basically, the different experimental approaches can be classified into two groups. One type of the methods are based on quantitative *J*-correlation spectroscopy (*15, 24–27*) in which the coupling information is encoded in the resonance intensity. The other group comprises experiments (*16, 20, 28–33*) in which the couplings are observed as splitting in the frequency domain spectrum. The high-resolution TROSY experiment and its *J*-modulated variant we propose here belong to the latter group and offer substantial resolution enhancement that allows accurate measurement of $^3J(H_NH_\alpha)$ couplings when traditional methods will likely fail, as in the case of proteins over 100 residues.

RESULTS AND DISCUSSION

A normal TROSY spectrum recorded with high resolution in the proton dimension, taking advantage of the TROSY effect in both frequency dimensions, allows the direct measurement of $^3J(H_NH_\alpha)$ couplings. Homonuclear coupling constants can be evaluated from the highly digitized ^1H multiplets or by comparison of the homonuclear coupled and decoupled multiplets acquired in two separate experiments. For the latter method the reference (decoupled) signals are obtained by applying a train of semi-selective-shaped inversion pulses for the band-selective decoupling (*34*) of H_α protons during acquisition. Note that an efficient suppression of bulk water magnetization is a prerequisite of H_α decoupling. To this end, selective presaturation of the water signal enhanced with gradient dephasing (G0) is applied during the relaxation delay between two transients. In addition, a selective 90° pulse on the water resonance, orthogonal with respect to the first nonselective 90° ^1H pulse, is also introduced in order to continue dephasing of the residual water signal by the subsequent gradients used for coherence selection (Fig. 1). Decoupling sidebands can be efficiently suppressed by simple averaging of experiments recorded with four time-shifted decoupling sequences (*34*).

If necessary, further resolution enhancement can be achieved by the *J*-modulated TROSY experiment proposed here. The new method, called TROSY-JM (Fig. 1), is basically a simple extension of the sensitivity enhanced, H_α-decoupled TROSY (*35–38*) experiment discussed above (*39–43*). After the TROSY sequence, an extra *J*-modulation spin-echo period is incorporated, as described recently for the *J*-multiplied HSQC (MJ-HSQC) (*44*) and HMQC (MJ-HMQC) (*50*) experiments. The synchronous incrementation of the homonuclear *J*-modulation and ^{15}N chemical shift evolution period, together with an appropriate scaling factor (n) for multiplying the effect

[1] To whom correspondence should be addressed. Fax: +36-52-489-667. E-mail: kover@tigris.klte.hu.

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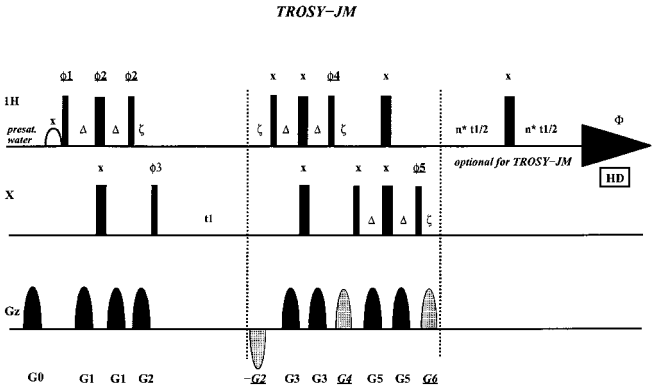

FIG. 1. Pulse scheme for 2D [^{15}N, ^{1}H]-TROSY-JM experiment using sensitivity-enhanced gradient echo/antiecho coherence selection. Narrow and wide bars designate nonselective 90° and 180° pulses, respectively. The delay $\Delta = 2.75$ ms. Echo–antiecho selection is achieved by the shaded pulsed field gradients applied along the z-axis with duration of 1 ms ($\zeta = 1.2$ ms including a recovery delay of 200 μs). For ^{1}H and ^{15}N spins the echo is obtained for $\phi_1 = y$, $\phi_2 = -x$, $\phi_4 = -y$, $\phi_5 = -y$, G2 = 35, G4 = 15, G6 = 10 G/cm; where the gradient −G2 acts on ^{15}N SQC with coherence order +1, G4 acts on heteronuclear MQC with coherence order −1 and +1 on ^{15}N and ^{1}H, respectively, and G6 acts on ^{1}H SQC with coherence order −1. In the second experiment the antiecho signal is obtained for $\phi_1 = -y$, $\phi_2 = x$, $\phi_4 = y$, $\phi_5 = y$, G2 = 40, G4 = 10, G6 = 15 G/cm; where the gradient −G2 acts on ^{15}N SQC with coherence order +1, G4 acts on heteronuclear MQC with coherence order −1 on both ^{15}N and ^{1}H, and G6 acts on ^{1}H SQC with coherence order +1. The amplitudes of other gradients are as follows: G0 = 38, G1 = 12, G3 = 14, and G5 = 27 G/cm. $\phi_3 = x - x$ and the receiver phase $\Phi = -xx$. This phase cycling scheme allows the detection of the most slowly relaxing component of the ^{15}N–^{1}H multiplet. Water suppression is achieved by selective irradiation of the water resonance during the relaxation delay followed by G0 purging gradient between two scans. Residual water magnetization is excited by a selective 90° Gaussian pulse of 2-ms duration and attenuated by the successive gradients. Except for acquisition when the ^{1}H carrier frequency is set to the middle of the amide proton region, the ^{1}H transmitter is on-resonance for the water signal. During acquisition H_α-band-selective homonuclear decoupling is applied in the decoupled TROSY and TROSY-JM experiments. The semi-selective inversion pulse of the SESAM decoupling sequence (*34*) was a hyperbolic secant pulse of 20-ms duration. Decoupling sidebands are suppressed with time-shifted decoupling (*34*). In the TROSY-JM experiment, the scaling of homonuclear coupling evolution is achieved by a spin-echo sequence inserted before acquisition.

of J-modulation, yields a ^{15}N–^{1}H correlation map where all the cross peaks appear as doublets along the ^{15}N dimension with a splitting corresponding to the relevant homonuclear couplings scaled up by n. The band-selective decoupling (*34*) applied during acquisition removes the undesired antiphase magnetization and retains only the cosine modulated ($\cos \pi n t_1 J_{H_N H_\alpha}$) component. Because homonuclear decoupling, as it is known, may cause some sensitivity loss, the use of a purging pulse prior to acquisition seems to be an attractive alternative for removal of antiphase components. However, this approach would fail here. Since the sensitivity-enhanced TROSY scheme applied in the present study relies on the simultaneous detection of orthogonal proton magnetization components (*40*), a purging pulse would have a deleterious effect in destroying one of the orthogonal components. It is noteworthy that the resolution improvement attained by the band-selective homonuclear decoupling can compensate for the inherent sensitivity loss.

Here we demonstrate that the high resolution obtained in the ^{15}N dimension as a result of the interference of CSA and dipolar interactions (TROSY principle) often ensures a clear separation of the J-scaled doublet components. If some J-splittings remain unresolved, a second experiment with unit scaling factor ($n = 1$) may be performed to obtain reference signals. Then, a simultaneous fit of the relevant multiplets in the scaled and unscaled spectra may yield the homonuclear couplings or provide an estimate for their upper limit. Moreover, since the long J-coupling evolution period is placed after TROSY selection, the experiment takes full advantage of the TROSY principle. It is expected that the effect is more pronounced for larger proteins at higher fields, closer to the TROSY optimum condition. We note that in order to enhance the relative contribution of ^{1}H CSA to the amide proton relaxation and thereby to enhance the efficiency of TROSY effect, the use of a protein with deuterium enrichment of passive spins (H_β, H_γ, etc.) would be the most desirable. In the present work inclusion of ^{15}N steady-state magnetization is applied and leads to further sensitivity enhancement. In comparison with the recently proposed MJ-HMQC (*50*) experiment, the J-modulated TROSY scheme requires fewer 90°/180° pulses, and this way signal loss due to pulse imperfections can be minimized. In the TROSY-JM experiment the ^{15}N chemical shift evolution and proton–proton J-modulation period are separate similarly to that of the MJ-HSQC (*44*) experiment, but here the slowly relaxing components are employed during the evolution periods. In addition, the band-selective homonuclear decoupling applied during acquisition can lead to further sensitivity and resolution enhancement, especially with the use of the recently proposed adiabatic inversion WURST-2 pulse (*51*). A disadvantage of the homodecoupling is that simultaneous water suppression is a must, since the water signal is in the α-proton region. This may cause some sensitivity loss for the rapidly exchanging amide protons.

The evolution of the density matrix in the J-modulated TROSY experiment can be given in a simplified form as

$$I_y^{13} + I_y^{24} > S_{34}^{+} \exp\left(-i\omega_S^{34} t_1\right) > I_{24}^{-} \exp\left(-i\omega_S^{34} t_1\right) > I_{24}^{-} \exp\left(-i\omega_S^{34} t_1\right) \cos\left(\pi J_{H_N H_\alpha} n t_1\right),$$

where I_y^{13}, I_y^{24}, and S_{34}^{+} designate the single-transition operators of ^{1}H and ^{15}N, respectively, as introduced by Pervushin *et al.* (*35*).

The first step above represents the coherence transfer from ^{1}H to ^{15}N and the ^{15}N chemical shift evolution during t_1. The second step ensures back transfer from ^{15}N to ^{1}H. Finally, the last step describes the homonuclear J-modulation of ^{1}H(^{15}N) magnetization occurring during the nt_1 incremented echo period. The inclusion of ^{15}N steady-state magnetization is not shown explicitly. The phase cycling scheme given in Fig. 1 combined with the echo–antiecho gradient coherence selection (*45*) allows

sensitivity-enhanced, phase-sensitive detection of the desired slowly relaxing ^{15}N–^{1}H multiplet component. The unwanted multiplet components are suppressed effectively by the applied phase cycling and the gradient pulses (G2, G4, and G6) used for coherence selection. Echo and antiecho signals are recorded in alternate scans with inversion of the phases ϕ_1, ϕ_2, ϕ_4, and ϕ_5 together with appropriate setting of the amplitudes of grayed gradients (G2, G4, and G6). For experimental verification of the proposed pulse schemes, a uniformly ^{15}N-enriched ubiquitin sample was used as a test case. A small region from the high resolution coupled (a) and H_α decoupled (b) TROSY spectra together with the corresponding cross-sections (c, d) taken parallel to the ^{1}H axis is depicted in Fig. 2. The well-resolved doublets allow the direct measurement of the relevant homonuclear couplings. The same region expanded from the J-modulated [^{15}N, ^{1}H]-TROSY spectrum is shown in Fig. 3 together with the corresponding cross sections taken along the ^{15}N axis. In the TROSY-JM experiment, homonuclear J-scaling with a factor of 3 was applied for resolution enhancement. The cross sections taken through the

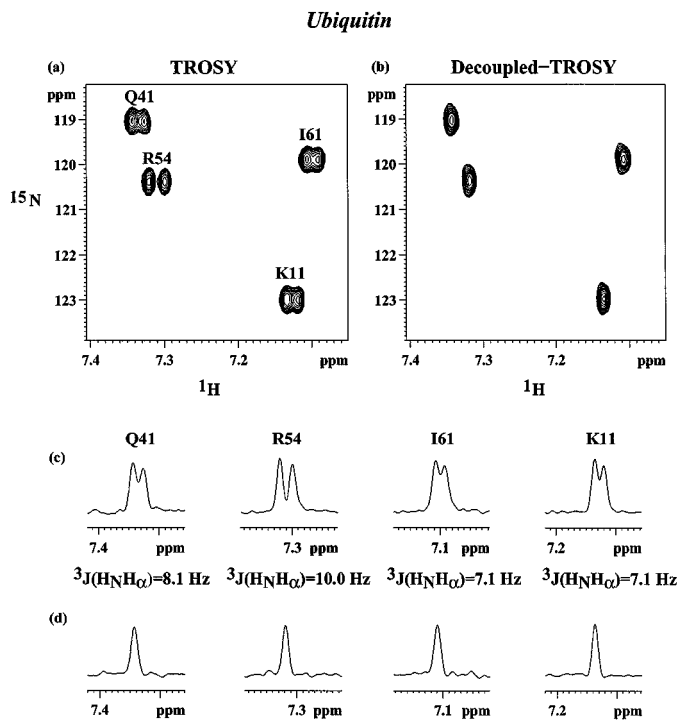

FIG. 2. Expansions of high-resolution TROSY (a) and H_α-decoupled TROSY (b) spectra recorded on ^{15}N-labeled ubiquitin and the corresponding cross sections (c, d) taken through the crosspeaks along the ^{1}H dimension. TROSY spectra were recorded with the pulse scheme of Fig. 1 without the optional J-modulation spin-echo period. For the correlation spectra 64*1K complex data points for both echo and antiecho signal were recorded with spectral widths of 2003 and 1318 Hz in F_2 and F_1, respectively, and collecting 16 transients per t_1 increment with a recycle delay of 1.5 s. The ^{1}H and ^{15}N nonselective 90° pulses were 10.2 and 26 μs, respectively. H_α decoupling was achieved with four time-shifted SESAM decoupling sequences in b. Gaussian window function in F_2, shifted cosine square in F_1, and zero-filling in both dimensions were applied prior to 2D Fourier transformation.

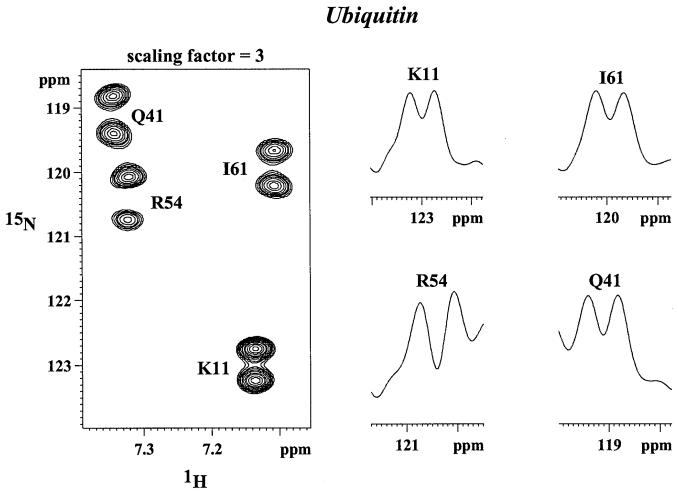

FIG. 3. Expansion of the J-modulated [^{15}N, ^{1}H]-TROSY spectrum on ubiquitin and the corresponding cross sections taken along the ^{15}N dimension. TROSY-JM correlation spectra of 100*256 complex data points for both echo and antiecho signals were recorded using the pulse scheme of Fig. 1 with spectral widths of 2003 and 1318 Hz in F_2 and F_1, respectively, and collecting 16 transients per t_1 increment with a recycle delay of 1.5 s. Homonuclear J-scaling in F_1 with a factor of 3 was applied. H_α decoupling during acquisition was achieved with SESAM decoupling. Shifted cosine square in F_2, Gaussian window function in F_1, and zero-filling in both dimensions were applied prior to 2D Fourier transformation.

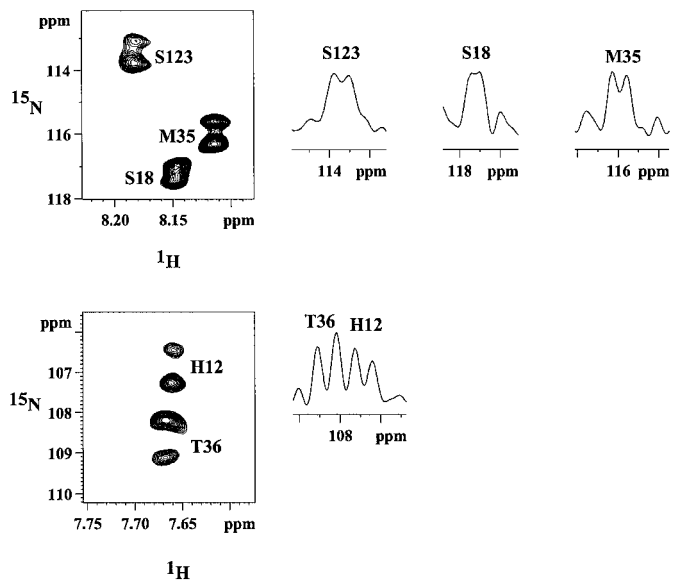

FIG. 4. Expansions of the J-modulated [^{15}N, ^{1}H]-TROSY spectrum of uniformly ^{15}N-labeled dimeric HP-RNAse together with the corresponding cross sections taken along the ^{15}N dimension. 2D J-modulated correlation spectrum of 100*256 complex data points for both echo and antiecho signal were recorded with spectral widths of 2003 and 2179 Hz in F_2 and F_1, respectively, and collecting 48 transients per t_1 increment with a recycle delay of 1.5 s. Homonuclear J-scaling with a factor of 3 was applied. H_α decoupling during acquisition was achieved with SESAM decoupling. Shifted cosine square in F_2, Gaussian window function in F_1, and zero-filling in both dimensions were applied prior to 2D Fourier transformation.

peaks of the coupled TROSY (Fig. 2) and TROSY-JM (Fig. 3) spectra all show well-resolved doublets due to the homonuclear J-coupling evolution, which verifies our expectation that both experiments perform equally well in the case of smaller proteins. The J-values measured from the splitting of doublets in the two experiments were found to be in agreement within the achieved digital resolution. However, experiments with a dimeric form of uniformly ^{15}N-labeled human pancreatic ribonuclease (HP-RNAse), a 128-residue protein (MW $= 2 \times 13.7$ kDa) (*46*), indicate that in the case of larger proteins only the resolved, J-scaled doublets of the TROSY-JM spectrum allow an accurate measurement of the desired homonuclear couplings. Figure 4 shows expanded regions of the TROSY-JM spectrum recorded on HP-RNAse using a value of $n = 3$ for homonuclear J-scaling together with the corresponding cross sections taken along the ^{15}N dimension.

CONCLUSION

In summary, it has been shown that the resolution improvement achieved by the use of the TROSY scheme in the proposed TROSY-JM experiment is useful for resolving homonuclear scalar couplings in larger proteins. The described sensitivity-enhanced gradient variant of the experiment results in high sensitivity and high resolution ^{15}N–^{1}H correlation spectra, in which the cross peaks appear as doublets along the ^{15}N dimension with a splitting of $n * {}^{3}J(\mathrm{H_N H_\alpha})$ Hz and singlets along the ^{1}H dimension due to H_α-band-selective decoupling during the acquisition. Typically, a scaling factor of 3 to 5 allows the resolution of doublets arising from homonuclear couplings between 2 and 3 Hz. Successful measurements were carried out at 11.7 T using ubiquitin (MW = 8.56 kDa), calretinin I–II fragment (*47–49*) (MW = 12.05 kDa), and dimeric HP-RNAse (MW $= 2 \times 13.7$ kDa). In the latter cases all coupling constants for residues giving well-resolved peaks in the [^{15}N, ^{1}H]-TROSY-JM spectrum were determined. The pulse sequence was also effective for resolving proton–proton couplings in ^{13}C-labeled model molecules. The applicability of the proposed method for oligosaccharides and RNA will be further investigated.

EXPERIMENTAL

All NMR experiments were carried out on a Bruker Avance DRX 500 spectrometer operating at 500 MHz ^{1}H frequency, equipped with an actively shielded ^{1}H/^{13}C/^{15}N gradient triple-resonance probe. The [^{15}N, ^{1}H]-TROSY spectra were recorded in 1.7 mM [U-^{15}N]ubiquitin (95% : 5% = $\mathrm{H_2O}$: $\mathrm{D_2O}$, pH 4.7, purchased from VLI, Southeastern, PA) and in 1.0 mM [U-^{15}N] HP-RNAse (95% : 5% = $\mathrm{H_2O}$: $\mathrm{D_2O}$) at 300 K. All other relevant experimental parameters are given in the figure legends.

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