Journal of the Korean Magnetic Resonance Society **2009**, *13* 15-26

Sensitivity Enhancement of Methyl-TROSY by Longitudinal ^{1}H Relaxation Optimization

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Received January 24, 2008

Abstract : The NMR detection of methyl groups is of keen interest because they provide the long-range distance information required to establish global folds of high molecular weight proteins. Using longitudinal ^{1}H relaxation optimization, we achieve a gain in sensitivity of approximately 1.6-fold in the methyl-TROSY and its NOESY experiments for the 38 kDa protein mitogen activated protein kinase p38 in its fully protonated and ^{13}C and ^{15}N labeled state.
Keywords : longitudinal ^{1}H relaxation optimization; methyl-TROSY; NOESY; sensitivity enhancement

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for studying biomolecules at the atomic level by accessing their 3D structures and dynamics (Wüthrich, 1986). However, the application of NMR to large molecular size over 30 kDa is hindered by the rapid transverse relaxation which causes significant line broadening and therefore poor sensitivity (Clore and Gronenborn, 1998; Kay and Gardner, 1997). Recently, a handful of methods have been developed to improve the sensitivity of NMR spectra. These methods focus on individual sub-periods in pulse schemes, such as chemical shift evolution, polarization transfer, and recycling periods. For the chemical shift evolution period, TROSY, the constructive use of the transverse relaxation interface,

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has been implemented and dramatically increases the sensitivity (Miclet et al. 2004; Pervushin et al. 1997; Tugarinov et al. 2003). Optimization of the polarization transfer has also been achieved using several different techniques (Bromek et al. 2005; Khaneja et al. 2003; Lee et al. 2005; Riek et al. 1999). Using cross-relaxation from remote protons, the recycling period may be reduced and thus one can increase the repetition rate of the experiment, resulting in sensitivity gains (longitudinal ^{1}H relaxation optimization) (Pervushin et al. 2002; Riek et al. 2002). By applying this principle, the sensitivity enhancement of NMR spectra for the amide (Pervushin et al. 2002) and aromatic (Eletsky et al. 2005) groups in relatively large proteins were shown. For methyl groups, the sensitivity gain of approximately 1.5 has been shown using small proteins (Schanda et al. 2005). Furthermore, the application of this principle to small proteins allows the study of site-resolved real-time kinetics by NMR (Schanda and Brutscher, 2005; Schanda et al. 2005). Since the longitudinal ^{1}H relaxation optimization is more favorable for large molecules than small molecules, we apply the principle to the methyl-TROSY experiment for relatively large protonated and ^{13}C and ^{15}N labeled protein using the pulse scheme, which is identical to the SOFAST-HMQC (Schanda et al. 2005) and relies on selective excitation of the methyl groups while leaving the other protons untouched such that they enhance relaxation of the methyl protons back to equilibrium via cross relaxation. It should be noted that the technique reported here can not be applied to Val, Leu, Ile(δ^1) methyl-protonated ^{15}N-, ^{13}C-, ^{2}H-labeled proteins via the methods introduced by Kay and colleagues (Goto et al. 1999) because the relaxation enhancement is not present when the methyl groups are not relaxed by other protons. In the present work, we show a gain in sensitivity of approximately 1.6-fold in the methyl-TROSY experiment for the fully protonated and uniformly ^{13}C,^{15}N- labeled 38 kDa protein mitogen activated protein (MAP) kinase p38. Particularly, the methyl groups are of keen interest because they give usually the only long-range distance information to establish global folds of high molecular weight proteins. Thus, we extend the principle to the methyl-TROSY-NOESY experiment, which gives critical distance restraints to establish global folds of large proteins, and show, here, the sensitivity enhancement by approximately 1.7-fold for the NOE cross peaks using MAP kinase p38.

MATERIALS AND METHODS

Longitudinal ^1H relaxation optimization for methyl groups is achieved using selective excitation and refocusing pulses on methyl protons, using the identical pulse scheme to the SOFAST-HMQC (Schanda et al. 2005) which we call longitudinal ^1H relaxation optimized methyl-TROSY (LROM-TROSY) to make the purpose clearer. Since the sensitivity gain in the overall methyl region is a major concern, we optimized parameters of the selective pulses and the recycling time using the 1D version of the pulse sequence by setting t_1 to 0 (Figure 1a). The validity of the 1D optimization was confirmed by comparing magnetization build-up curves using the 1D optimization with those using the 2D version (Figure 2a). Compared to an optimized recycling delay of approximately 1.2 s (Figure 2a) for the conventional methyl-TROSY, an optimal recycling delay for LROM-TROSY was ~400 ms for MAP kinase p38 (τ_c = 16 ns was measured using 1D TRACT (Lee et al. 2006)) on a 700 MHz spectrometer (Figure 2a). The result allows the number of scans for the LROM-TROSY experiment to be increased by a factor of 3 in the same experimental time, compared to the conventional methyl-TROSY experiment. By the 1D optimization, we recovered almost 90% of the initial magnetization of non-methyl protons. The detailed parameters of these optimized selective pulses are given in Figure 1. The E-Burp2 (Green and Freeman, 1991) pulse instead of PC9 (Kupce and Freeman, 1994), which was suggested by Schanda et al. (Schanda et al. 2005), for the excitation was used for the LROM-TROSY since we could not produce pure-phase spectrum of MAP kinase p38 using PC9 at least in our hands. By using Re-Burp (Green and Freeman, 1991) instead of r-SNOB (Kupce et al., 1995), we observed slight signal improvement, which may be caused by the better off-resonance performance of Re-Burp. It is noteworthy that a carrier frequency of the selective pulses has an effect on the sensitivity gain due to the perturbation of remote protons (Figure 2b) caused by imperfection of selective pulses and B_1-field inhomogeneities. At least in our experience, the sensitivity depends highly on the perturbation of remote protons (Figure 2b). The lengths of pulses have also an effect on the sensitivity due to transverse spin relaxation during the pulses and were optimized together with carrier frequency of the pulses. In the present work, the carrier frequency of the selective ^1H pulses was set to -1 ppm, which is a bit off from the center of methyl proton resonances but gives best compensation between the perturbation of remote protons and the lengths of pulses and is determined by the 1D optimization (Figure 2b).

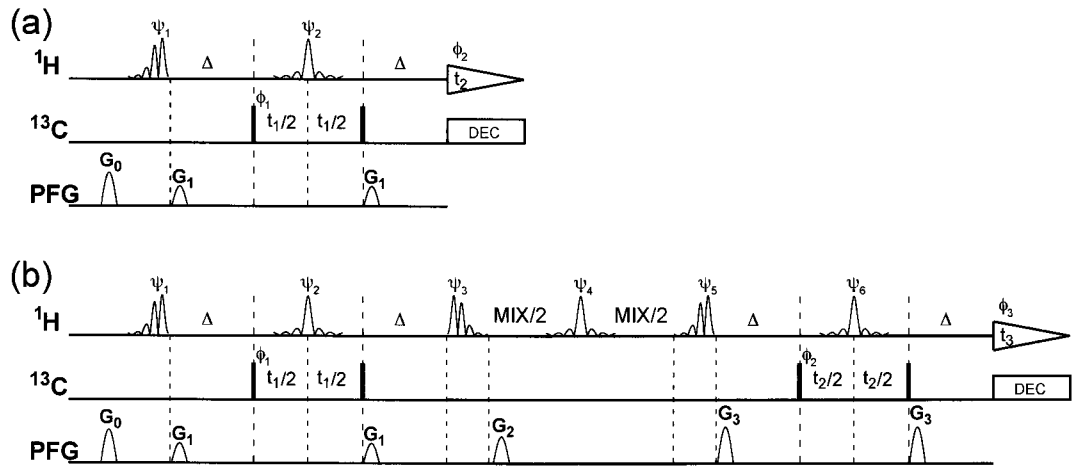

Fig. 1. Experimental scheme for (a) the 2D longitudinal ^{1}H relaxation optimized methyl-TROSY (LROM-TROSY), which is identical to the SOFAST-HMQC (Schanda, et al. 2005). In (b), pulse scheme for the 3D LROM-TROSY-NOESY experiment is shown. The radio frequency pulses on ^{1}H and ^{13}C are applied at -1 and 10 ppm, respectively. Black bars on the line marked ^{13}C indicate nonselective 90° pulses. Complex shapes on the line marked ^{1}H indicate the band-selective 1 ms excitation E-Burp2 pulses (Geen and Freeman, 1991) for ^{1}H in methyl groups with the phases ψ_1 and ψ_5 and γB_1 = 4096 Hz, identical to the pulses ψ_1 and ψ_5 but time-reversed excitation E-Burp2 pulse with the phase ψ_3 and the 1.5 ms refocusing Re-Burp pulse (Geen and Freeman, 1991) with the phases ψ_2, ψ_4, and ψ_6 and γB_1 = 4176 Hz on a 700 MHz (proton frequency) spectrometer. The line marked PFG indicates the duration and strength of pulsed field gradients applied along the z-axis. The delays Δ and NOESY mixing indicated with MIX are 1.7 ms and 100 ms, respectively. The PFGs are: G_0,1m, 40 G/cm; G_1, 500 μs, 15 G/cm; G_2, 1 m, 25 G/cm; G_3, 500 μs, 30 G/cm. The phases are φ_1 = {x,-x}, φ_2 = {-x,x}, ψ_1 = {x}, and ψ_2 = {x} for the LROM-TROSY experiment (a) and φ_1 = {x,-x},φ_2 = {x,x,-x,-x}, φ_3 = {x,-x,-x,x}, ψ_1 = {x}, ψ_2 = {x}, ψ_3 = {x}, ψ_4 = {x}, ψ_5 = {x}, and ψ_6 = {x} for the LROM-TROSY-NOESY experiment (b). Quadrature detection in the $^{13}C(t_1)$ is achieved by the States-TPPI method (Marion et al. 1989) applied to the phase φ_1 for both experiments. For the LROM-TROSY-NOESY experiment (b), quadrature detection in the $^{13}C(t_2)$ is achieved by the States-TPPI method

applied to the phase φ_2. The ^{13}C spins are decoupled by the WALTZ-16 (Shaka et al. 1983) at a field strength of γB_1 = 1562 kHz during the acquisition.

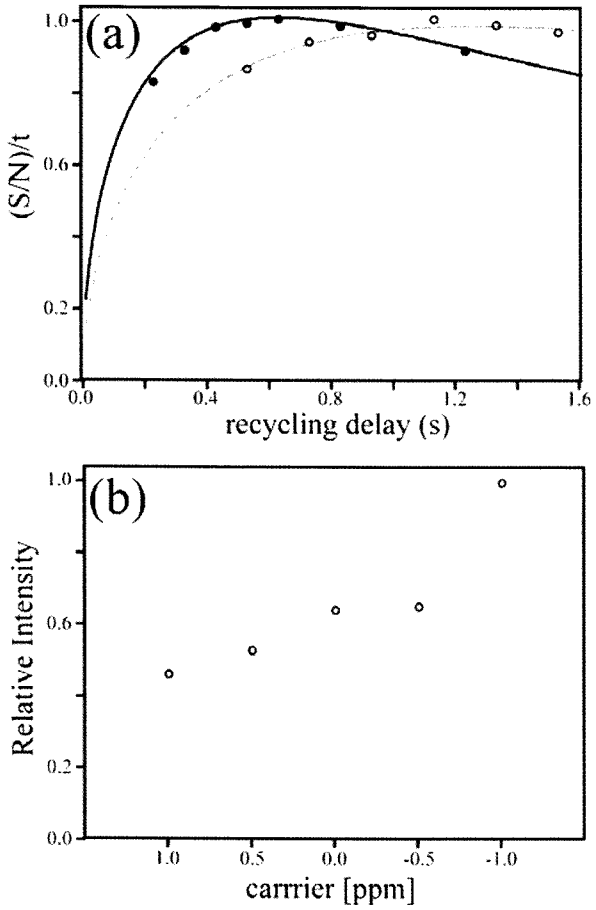

Fig. 2. (a) Signal-to-noise ratios per unit time, (S/N)/t, in a series of the 2D [^{13}C,^{1}H]-correlation spectra versus the recycling delay measured with the uniformly ^{13}C,^{15}N double-labeled 38 kDa MAP kinase p38. Open and closed symbols represent (S/N)/t of the conventional methyl-TROSY and the LROM-TROSY spectra, respectively. (b) Relative intensity versus carrier frequency of selective pulses on ^{1}H nuclei in the LROM-TROSY experiment. The lowest ppm value which can achieve all resonances of the methyl groups was -1 ppm. Acquisition parameters are identical to those described in Figure 1. The plotted

data points correspond to the sum of the intensities measured for 20 well-resolved methyl cross peaks in the 2D spectra.

RESULTS AND DISCUSSION

Sensitivity enhancement of the LROM-TROSY spectra of the MAP kinase p38, compared with the conventional methyl-TROSY, was 1.6 on average (Figure 3). Compared with the sensitivity gain (~1.5) using small proteins (Schanda et al. 2005), the value is a smaller enhancement than what we expected. This may be due to the Ernst angle (Ernst et al. 1987) of the excitation pulse. However, this value is similar to the theoretical value (1.73) due to the increase of the number of scans by reducing the recycling delay. A slightly lower sensitivity gain than the theoretical value, which was observed in some peaks, may be caused by non-fully relaxed spins during the recycling periods. However, several peaks showed more than 2-fold gain in sensitivity with the maximum gain of 3. This result was expected because of the following: even though the recycling delay in the conventional spectra is much longer (~1.2 s) than the one of the LROM-TROSY (~0.4 s), the Boltzmann steady-state magnetization of methyl protons may not be fully reached in the conventional experiment. Furthermore, the cross relaxation from non-methyl protons reduces the sensitivity of the conventional experiment. However, in the LROM-TROSY experiment, the effect is used to restore the equilibrium magnetization and results in a sensitivity gain. Thus, the protons that are surrounded by other protons will recover more quickly to the equilibrium magnetization. Protons in the hydrophobic core of the protein show better sensitivity enhancement than those on the protein surface. This was indeed observed in the spectra of $^{15}N,^{13}C$ double-labeled ubiquitin and rabphilin 3A C2A domain (data not shown).

Distance restraints derived from methyl-methyl NOEs are a critical source of structural information for the determination of global folds of large proteins in solution. Therefore, we have extended the longitudinal ^{1}H relaxation optimization to NOESY experiment (Fig. 1b). In order to suppress spin diffusion between methyl and non-methyl protons, a selective 180° pulse for methyl protons is applied in the center of the NOE mixing period (Zwahlen et al. 1994). For the MAP kinase p38 protein, the overall

sensitivity gain is on average 1.7 for the NOE cross-peaks (Fig. 4). This gain is similar to the sensitivity gain of the LROM-TROSY.

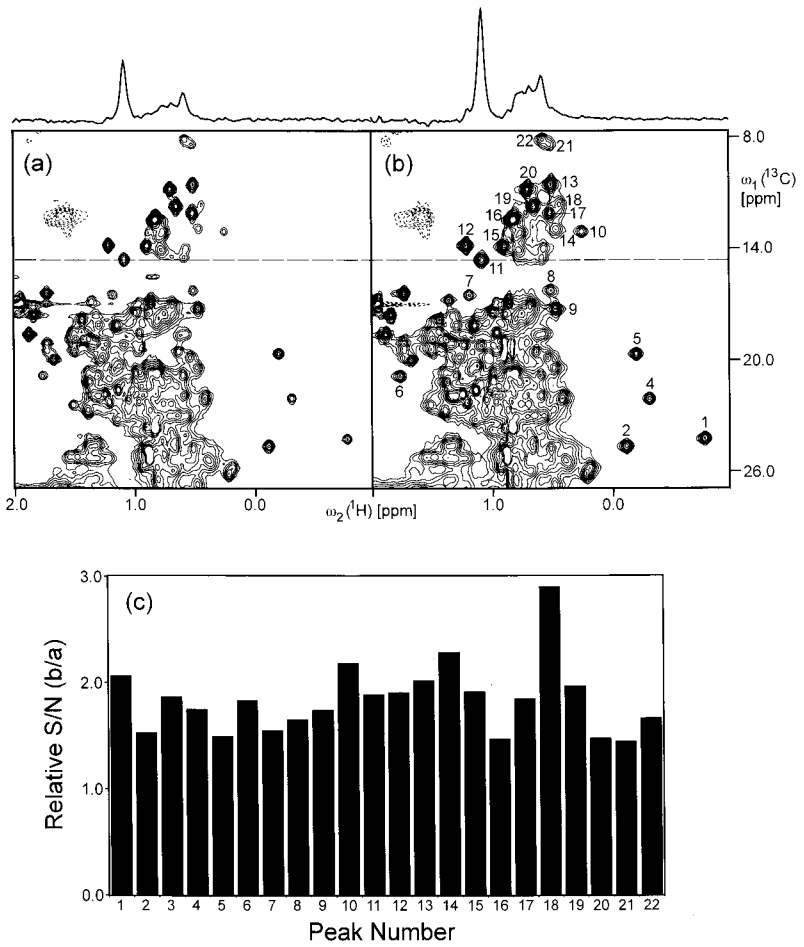

Fig. 3. Comparison of sensitivities between (a) the conventional methyl-TROSY and (b) the LROM-TROSY (Fig. 1 a)) spectra of the uniformly ^{13}C,^{15}N double-labeled 38 kDa MAP kinase p38. For both spectra, 128(t_1)*512(t_2) complex points were accumulated yielding t_{1max} = 10.6 ms and t_{2max} = 92 ms. Measurement times (~28 minutes) for each experiment were almost identical with recycling delays of 1154 ms with 4 scans and 348 ms with 12 scans for the conventional methyl-TROSY (a) and the LROM-TROSY experiments (b), respectively. Slices indicated with dashed lines in the spectra are shown on the top of the spectra. (c) Comparison of signal-to-noise ratio between the two spectra (b/a). Peak numbers are indicated in the spectra.

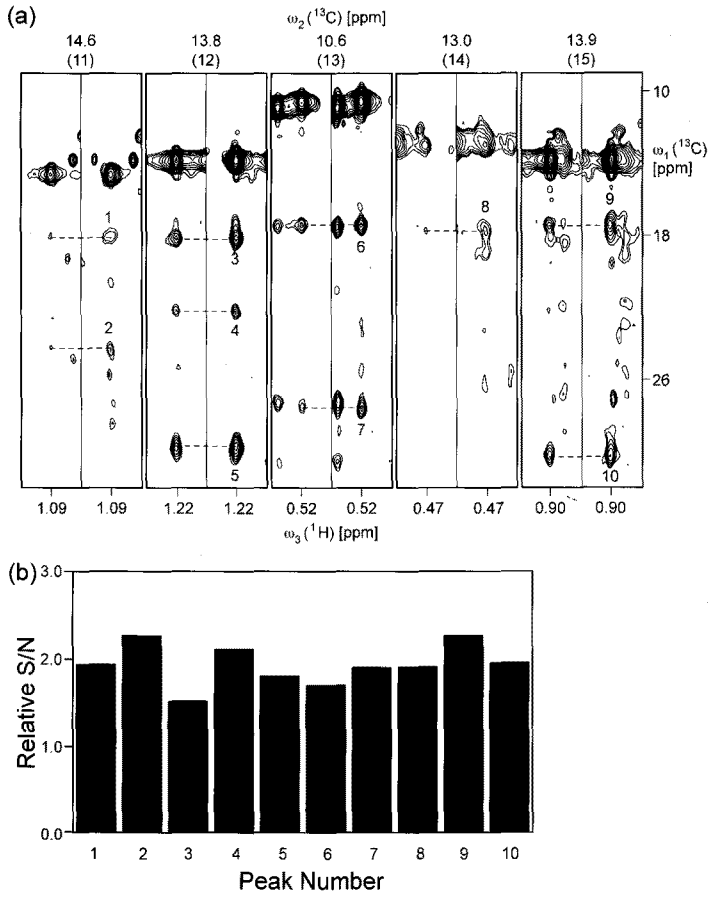

Fig. 4. Comparison of sensitivities between (a; left strips) the conventional methyl-TROSY-NOESY and (a; right strips) the LROM-TROSY-NOESY spectra of uniformly ^{13}C,^{15}N double-labeled 38 kDa MAP kinase p38. Strips taken from the peak positions of peak number 11 to 15 (indicated on the top of the strips) in Figure 2 are shown. For both spectra, $90(t_1)*32(t_2)*512(t_3)$ complex points were accumulated yielding t_{1max} = 8.6 ms, t_{2max} = 3.1 ms, and t_{3max} = 92 ms. Measurement times (~3 days, 5 hours) for each experiment were almost identical with recycling delays of 1154 ms with 20 scans and 348 ms with 52 scans for the conventional methyl-TROSY-NOESY and the LROM-TROSY-NOESY experiments, respectively. (b) Signal-to-noise ratio between NOE peaks in two spectra (LROM-TROSY-NOESY versus methyl-TROSY-NOESY). Peaks numbers are indicated in the strips.

Structure determination of large proteins is limited due to the sensitivity loss, caused by the transverse relaxation. In our study, we have improved the sensitivity of the methyl-TROSY and its NOESY experiments by a factor of approximately 2 using the longitudinal ^1H relaxation optimization. This is particularly interesting because the almost all restraints for the global fold of the large proteins are methyl-methyl NOEs. Furthermore, in the helical proteins, long-range backbone HN-HN contacts are scarce or even completely missing. It has also been proven that the methyl-methyl NOEs are crucial in this regard (Tugarinov et al. 2005). Notably, whereas dipolar couplings define the relative orientation of the domains, the positions of the domains cannot be established without distance restraints.

Overall, LROM-TROSY and LROM-TROSY-NOESY experiments are very attractive for obtaining structural information in large fully protonated biomolecules, especially in their hydrophobic core. The technique will therefore nicely complement side chain assignment techniques that have been optimized for medium size molecules (Xu et al. 2006). We also anticipate that proteins prepared with the SAIL technique will benefit from this experiment (Kainosho et al. 2006). In addition, we anticipate that the LROM-TROSY method can also be used to probe slow protein dynamics. We thus expect that the LROM-TROSY will play a key role for high-quality structure determination of large fully protonated or SAIL-proteins and for studying the dynamics of their molecular cores.

Acknowledgement

This work was supported by the MPG and the Fonds der Chemischen Industrie. We thank Dr. d'Auvergne for a critical reading of the manuscript.

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