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An Optimization of the 3D ^{1}H-^{15}N-^{1}H TOCSY-HSQC and NOESY-HSQC Experiments Using Sensitivity Enhancement with Gradient Selection

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Abstract: Proper pulse sequences and experimental optimization for the 3D ^{15}N edited TOCSY and NOESY spectra were described. Using sensitivity enhancement approach with coherent selection by pulsed field gradients described by Kay and co-workers, a considerable gain in sensitivity was achieved. The sensitivity was also improved by minimal water saturation using water flip-back pulse. Among the three types of TOCSY mixing pulse, named MLEV-17, DIPSI-2 and DIPSI-2rc, DIPSI-2rc sequence gave the most sensitive spectrum. These results suggest an appropriate pulse sequence for those 3D experiments for large proteins.

INTRODUCTION

Recent advances in NMR techniques have contributed to the recording of spectra for proteins with increased sensitivity and spectral resolution. They include heteronuclear multidimensional spectroscopy,[1,2] and sensitivity enhancement techniques described by Palmer and co-workers,[3] water suppression with minimal water saturation,[4] etc. The sensitivity enhancement technique mentioned here is to collect both cosine- and sine-modulated t_1 frequency components so that the signal-to-noise ratio is improved as much as a factor of square root of 2. Also, Kay and co-workers have incorporated the enhanced-sensitivity method into heteronuclear, multidimensional experiments which make use of pulsed field gradients to discriminate between N- and P-type coherence transfer pathways.[5,6]

The importance of minimal water saturation in a large class of experiments recorded in H_2O was recognized and demonstrated by Grzesiek and Bax.[4,7] As the T_1 of bulk H_2O can often considerably longer than the ^{1}H T_1 values in proteins, and usually the repetition rate per scan must be faster than the relaxation rate of water magnetization for the limitation of measuring time, partial saturation of water magnetization occurs. This problem is also true even if the pulsed field gradient was used for water suppression. Partial saturation of water magnetization leads to an overall decrease in the intensity of protein resonance due to saturation transfer arising from chemical exchange involving labile protons and/or cross relaxation. Grzesiek and Bax suggested that the water magnetization could be restored to the +z axis before acquisition so as to minimize its saturation or dephasing.

Meanwhile, in TOCSY type experiments, the efficiency of coherence transfer and the signal loss due to cross-relaxation depend on the mixing pulse sequences. The optimization of these mixing pulse sequences was proposed by Ernst et al.,[8] and they developed the pulses so as to maximize the transfer rate and to minimize the sensitivity to off-resonance effects or mismatch of the Hartmann-Hahn condition. In theoretical studies, in the absence of relaxation effects, they found that DIPSI-2 sequence generally has better coherence transfer efficiency than the MLEV or WALTZ sequences, at least for two-spin systems. After that, Cavanagh and Rance has reported the suppression of cross relaxation effects in TOCSY spectra via a modified DIPSI-2 mixing sequence, named DIPSI-2rc, which is the relaxation compensated version of the DIPSI-2.[9]

For the structural study of biologically interesting proteins, ^{15}N stable isotope labeling of the protein, which allows the implementation of many 2D and 3D NMR techniques, is widely used. Experiments have been based on one-bond correlation and on correlation relayed by using NOESY and TOCSY mixing schemes. 3D TOCSY-HSQC and NOESY-HSQC, experiments are particularly useful and their applications are reported in many papers.[10, 11] Here, we have made proper pulse sequences for the experiments making use of the recently developed pulsed-field gradient and sensitivity enhancement techniques, and tested them in the points of view explained above. As many NMR spectroscopists are undergoing challenge with a number of proteins whose spectra have low sensitivity due to large molecular weight or poor solubility, these results may give a useful information for using these 3D experiments.

MATERIALS AND METHOD

The ^{15}N-labeled Carboxyl-terminal fragment of the α subunit of 98 amino acid residues (αCTD) was obtained as described previously.[12] The C-terminal truncated ^{15}N-labeled ras p21 (residues 1-172, GDP form) was expressed and purified essentially in the same method as described by Kraulis et al.[13] The samples for NMR measurement comprised in 1.5 mM protein in 90% H_2O/ 10% D_2O, containing 20 mM phosphate buffer, pH 6.0, 30 mM KCl and 1 mM DTT, for αCTD, and containing 20 mM Tris-maleate, pH 7.4, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.01 % sodium azide, and 30 μM GDP for ras p21.

All NMR measurements were performed with a Bruker DMX600 spectrometer equipped with a triple resonance, pulsed field gradient probe. The pulse sequences used to record ^1H-^{15}N NOESY-HSQC and TOCSY-HSQC spectra were described in Fig.1. In 2D version of the spectra, the delays in t_1 or t_2 period are fixed at 6 and 22 μs, respectively, while the data points of the other dimension were retained at the same values for 3D spectra. All spectra were measured at 30 °C. The data were processed and analyzed using the Xwinnmr 1.3, NMRPipe,[14] and NMRPipp[15] software.

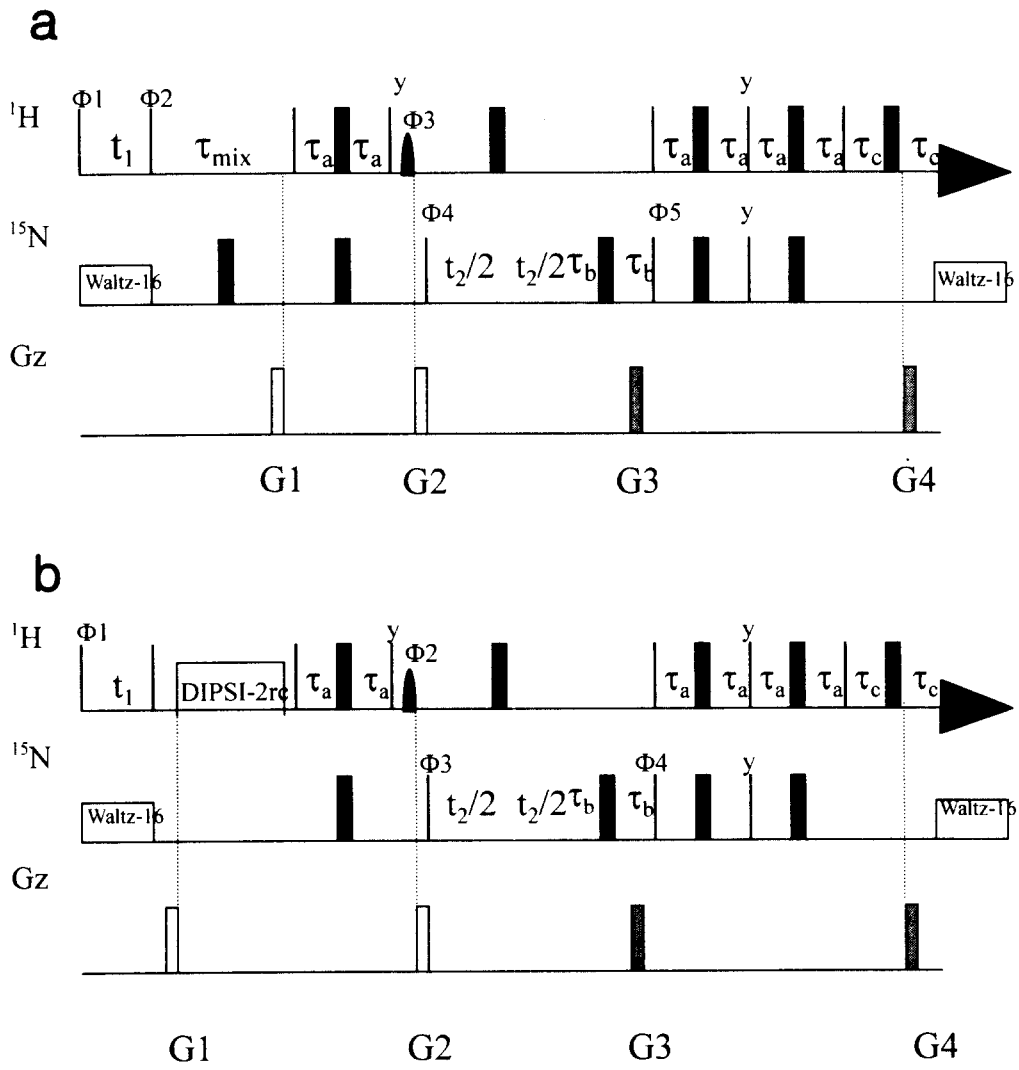

Fig. 1. Pulse sequence of the sensitivity enhanced 3D ^{1}H-^{15}N-^{1}H (a) NOESY-HSQC and (b) TOCSY-HSQC experiments. Narrow pulses represent a flip angle of 90° with wider pulses representing a flip angle of 180°. Pulses for which the phases are not indicated were applied along the x-axis. The values of τ_a, τ_b and τ_c were set to 2.3, 1.62 and 0.54 ms, respectively. Waltz decoupling was achieved using a 1 kHz field on the ^{15}N channel. The DIPSI-2rc isotropic mixing sequence was applied using 10 kHz field on the ^{1}H channel. The shaped ^{1}H 90° pulses were applied using a rSNOB shape profile (2 ms 90° pulse with 202 Hz field at peak height). The duration and strengths of the gradients were G1= (1 ms, 25 G/cm); G2= (1ms, 20 G/cm); G3= (1.2 ms, 30 G/cm); G4= (120 μs, ±30.45 G/cm). G1 and G2 gradient pulses are sinc shaped, and G3 and G4 are rectangular. All gradient pulses

were applied along the z-axis. A relaxation delay of 1.0s is used prior to the first ^{1}H pulse. The phase cycling employed were; $\phi1$ = 2(x), 2(-x); $\phi2$ = 4(x), 4(-x); $\phi3$ = -x; $\phi4$ = x, -x; $\phi5$ = x; rec = x, 2(-x), x, -x, 2(x), -x, for (a) sequence, and $\phi1$ = 2(x), 2(-x); $\phi2$ = 2(x), 2(-x); $\phi3$ = x, -x; $\phi4$ =x; rec = x, 2(-x), x, for (b) sequence. Quadrature in F_1 is obtained via States-TPPI of $\phi1$. For each value of t_2, N- and P-type coherence are obtained by recording two data sets where the sign of the gradient G4 is inverted and 180° added to the phase $\phi5$ of (a) and $\phi4$ of (b) sequence, respectively, for the second data set. The phase $\phi4$ of (a) and $\phi3$ of (b) are incremented by 180° along with the phase of the receiver for each t_2 increment.

RESULTS AND DISCUSSION

Fig. 1 describes the pulse sequences for recording the 3D ^{1}H-^{15}N-^{1}H NOESY-HSQC and TOCSY-HSQC experiments. For these pulse sequences, the sensitivity enhancement technique with heteronuclear gradient echo was employed additionally on the conventional sequence of NOESY-HSQC and TOCSY-HSQC experiments. The mechanism of magnetization transfer in the sensitivity enhancement scheme including the result of refocusing both cosine and sine-modulated ^{15}N magnetization components has been described previously.[4] Refocusing of the effects of the gradients requires that $\tau_1/\tau_2 = \pm\gamma_H G4(z)/\gamma_N G3(z)$. Therefore, 1.2ms and 120μs of the gradient pulse length for G3 and G4, respectively, were used with the strength of each gradient pulse adjusted for maximal refocusing. In the t_1 period for the proton chemical shift evolution, a broadband-decoupling scheme of waltz-16 sequence is used. For the water flip-back to the +z axis at the beginning of the acquisition, a rSNOB shaped pulse was used after the first INEPT sequence.

The effect of sensitivity enhancement was illustrated in Fig. 2. In the boxes of the upper right of each figures, the ^{1}H-^{15}N 2D projections of the sensitivity enhanced version (a) and conventional version (b) of TOCSY-HSQC experiments were shown. The 1D slices that represent the ^{15}N chemical shift of 126 ppm (shown in solid line) in the spectra were compared. In practice the full sensitivity enhancements are unlikely to be achieved due to the combined effects of the larger number of pulses in the enhanced sequences and relaxation losses that occur during the course of the various magnetization transfer steps. However, a considerable increment of sensitivity was still observed in the spectra of enhanced version.

The NH magnetization which have the chemical shift information of the correlative protons during TOCSY or NOESY mixing time is selected and transferred to amide nitrogen via an INEPT sequence. At point just before the ^{1}H shaped pulse in the sequence, the magnetization of interest is of the form I_zN_z, where I_z and N_z correspond to the longitudinal components of amide proton and nitrogen magnetization, respectively. Insertion

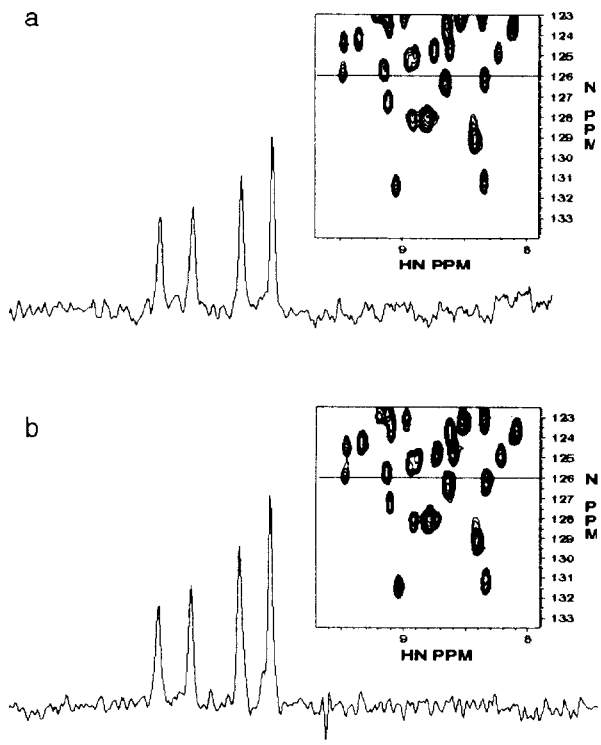

Fig. 2. 1D slices taken at ^{15}N chemical shift of 126 ppm (shown in solid line) from the ^{1}H-^{15}N 2D projections of TOCSY-HSQC spectra of ras p21; (a) unenhanced, (b) sensitivity enhanced sequence shown in Fig. 1b. In the boxes of the upper right of each figures, the ^{1}H-^{15}N 2D projections of each TOCSY-HSQC experiment was shown.

of gradient pulse at this point in sequence has no effect on the desired signal (I_zN_z) but does eliminate any potential artifacts arising from signals in the transverse plane including water signals. While the use of gradients to dephase water aid significantly in water suppression, the intensity of labile protein protons decreases due to chemical exchange with dephased water protons. The cross relaxation with proximate water molecules results in a further attenuation of protein signals. Thus, it is desirable to restore the water magnetization to the +z axis prior to the acquisition time. The effect of water flip-back pulse was illustrated in Fig. 3. In this experiment, ^{15}N labeled ras p21 is dissolved in the buffer at pH 7.4, and measured in 1D version of TOCSY-HSQC spectra. The additional gain of the sensitivity by the water flip-back pulse was observed (Fig. 3b). As the chemical exchange rate of labile

proton increase in high pH, the effect of water flip-back pulse may be more useful in neutral or high pH, which is not a preferred condition in conventional NMR experiments.

Presented in Fig. 4 are the spectra of 1D version of TOCSY-HSQC experiments, recorded with MLEV-17 (Fig. 4a), DIPSI-2 (Fig. 4b) and DIPSI-2rc (Fig. 4c) mixing sequences. Comparison of the MLEV-17 and DIPSI-2 spectra clearly indicates the superior performance of the DIPSI-2 mixing scheme which can be attributed to the improved coherence-transfer efficiency of the DIPSI-2 sequence. The relaxation compensated version of DIPSI-2 (named DIPSI-2rc or "clean" DIPSI-2) also improved the intensity of the spectrum (Fig. 4c) due to the suppression of cross-relaxation rates. By reducing the destructive interference caused by cross-relaxation, an additional gain of sensitivity can be possible.

When we compared the mixing times of the DIPSI-2rc sequence, between the 30 ms (Fig. 5a), 50 ms (Fig. 5b) and 70 ms (Fig. 5c), the longer mixing time gives the more cross peaks which is attributed to the long range transfer to the side chain protons. However, the sensitivity was reduced in the longer mixing sequence, due to the relaxation loss of the signal. Therefore, for the sequential assignment of the large molecular weight or less-soluble

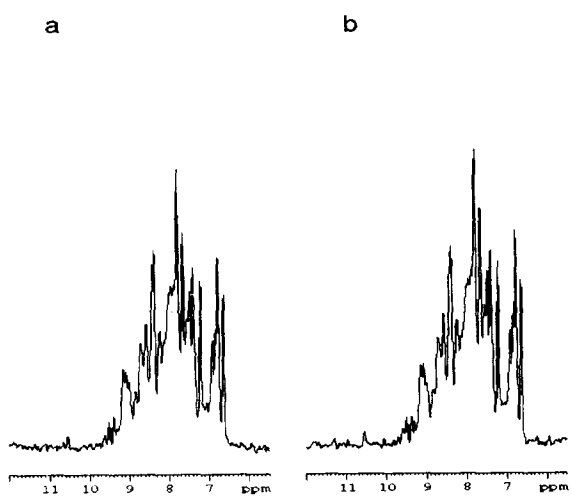

Fig. 3. ^{1}H 1D spectra of the TOCSY-HSQC experiment for ras p21 with water flip-back pulse (b), and without (a). The 1D version of the TOCSY-HSQC experiment was essentially the same as shown in Fig. 1b, except the t_1 and t_2 chemical shift evolution time which are fixed at 6 and 22 μs, respectively.

protein, the shorter mixing time would be desirable to get the connectivity. If the sensitivity is not a critical factor, the longer mixing time can give some additional information.

Fig. 6 shows the 2D slices taken at ^{15}N chemical shift of 122.73 ppm from the 3D NOESY-HSQC and TOCSY-HSQC spectra obtained with the sequence in Fig. 1 as an example. These spectra were recorded on the C-terminal truncated ras p21, a protein fragment of 172 amino acids using 600 MHz Bruker DMX spectrometer equipped with a triple-resonance gradient probe. A 100 ms of NOESY and 48 ms of TOCSY mixing time were employed. The spectrum was obtained from $64 \times 32 \times 512$ complex matrix and sixteen scans were recorded with a repetition delay of 1.0s, giving rise to a total acquisition time of approximately 58 hours.

It is clear that we can improve the 3D spectra using sensitivity enhancement and water flip-back techniques. Also the proper TOCSY mixing sequence can be an important choice to obtain a high quality spectrum. However, still the sensitivity of the TOCSY spectrum is not high enough to observe all protons of aliphatic side chain. It is due to the insufficient magnetization transfer and relaxation-derived signal loss during TOCSY mixing pulse. Thus, challenge to find the optimal condition for maximal transfer efficiency and minimal signal loss due to relaxation for each experiment would be needed.

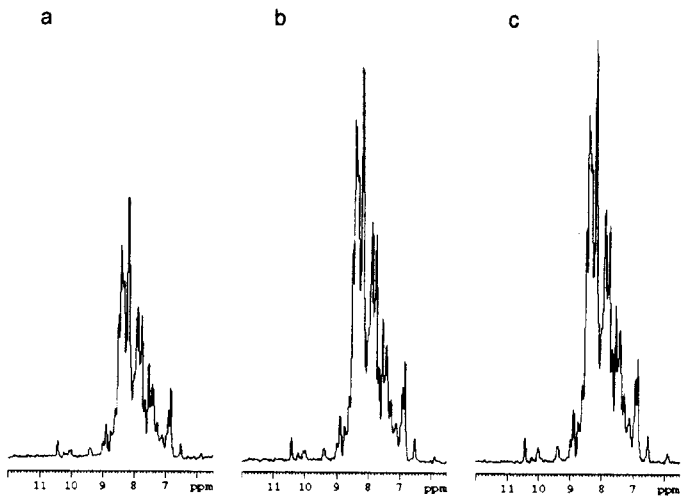

Fig. 4. ^{1}H 1D spectra of the TOCSY-HSQC experiment for αCTD recorded with (a) MLEV-17, (b) DIPSI-2, (c) DIPSI-2rc sequences. For all spectra the TOCSY mixing period was ~30 ms, and the RF field strength of 10 kHz was employed. The delays in the DIPSI-2rc sequence was the length of ^{1}H 143.87° pulse in TOCSY mixing scheme (~36 μs).

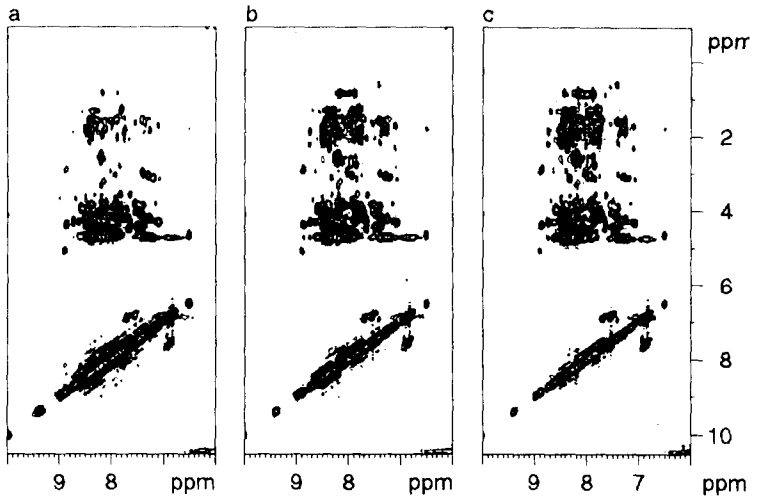

Fig. 5. ^{1}H-^{1}H 2D projection of the TOCSY-HSQC spectra of αCTD with DIPSI-2rc isotropic mixing time of (a) 30.4 ms, (b) 52.2 ms , and (c) 69.6 ms, respectively. The 2D version of the TOCSY-HSQC experiment was essentially the same as shown in Fig. 1b, except the t_2 chemical shift evolution time which are fixed at 22 μs.

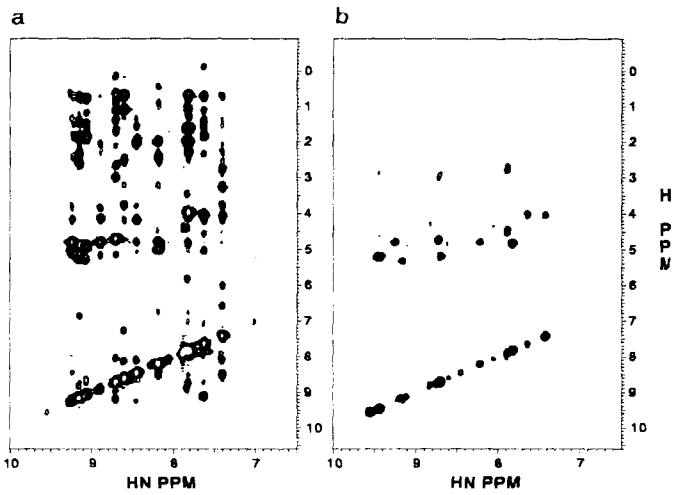

Fig. 6. 2D Slices taken at ^{15}N chemical shift of 122.73 ppm from (a) the 3D NOESY-HSQC and (b) TOCSY-HSQC spectra of ras p21. Mixing time employed are 100 ms in NOESY and 48 ms in TOCSY sequence.

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