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Suppression of Diagonal Peaks in TROSY-Type ^{1}H NMR NOESY Spectra of ^{15}N-Labeled Proteins

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A novel method for suppression of diagonal peaks in the amide region of NOESY NMR spectra of ^{15}N-labeled proteins is presented. The method is particularly useful for larger proteins at high magnetic fields where interference between dipolar and chemical shift anisotropy relaxation mechanisms results in large TROSY effects, i.e., large differences in ^{1}H^N linewidths depending on the spin state of attached ^{15}N nuclei. In this limit the new TROSY NOESY method does not compromise sensitivity. It is demonstrated using a perdeuterated ^{15}N-labeled protein sample, Neural Cell Adhesion Molecule 213–308 (NCAM) from rat, in H_2O at 800 MHz. © 1999 Academic Press

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Diagonal peaks in homonuclear correlation spectra are usually a nuisance as the interesting information is manifested exclusively by the cross peaks. This is exacerbated by the typical situation of the diagonal peaks being significantly larger than the cross peaks and in some cases having a $\pi/2$ phase shift relative to cross peaks. Hence diagonal peak suppression has been a subject of continuous interest in multi-dimensional NMR spectroscopy.

Over the years several diagonal peak suppression schemes have been published of which the most popular is multiple-quantum filtration in the mixing sequence of COSY (*1, 2*). The main feature of multiple-quantum filtration is elimination of solvent signals and a general reduction of other diagonal signals by enforcing an antiphase multiplet structure as is present in the cross peaks. An additional benefit is that diagonal peaks largely acquire the same phase as the cross peaks so that long dispersive tails from the diagonal obscuring nearby cross peaks are avoided.

Other methods for diagonal peak suppression are of more specialized nature and include constant-time spectroscopy (*3*) where some diagonal peaks can be suppressed completely for favorable spin systems (*4*). The same holds true for planar mixing or combination of bilinear mixing with E.COSY-type rotation cycles (*5*). Yet another approach is based on only one of the two spins involved in a cross peak being attached to a heteronuclear spin, as for example in a system of a proton attached to an ^{15}N atom correlating with another proton without an attached ^{15}N atom. Then heteronuclear editing techniques distinguishing I and IS spin systems can be employed to eliminate the diagonal. However, this approach cannot be applied if both protons are attached to a heteronuclear spin of the same isotope.

In this Communication, we present a new method for diagonal peak suppression that is ideally suited for combination with the so-called TROSY approach of recording NMR spectra without heteronuclear decoupling (*6*). For large molecules at high fields favorable interference between dipole and chemical shift anisotropy relaxation mechanisms results in improved resolution and sensitivity. The TROSY effect is most pronounced in ^{1}H–^{15}N amide groups of the protein backbone and is further enhanced by the absence of nearby protons which is met by perdeuteration except in the amide groups. Our new technique selects the TROSY peaks in an ^{15}N-coupled two-dimensional (2D) ^{1}H–^{1}H NOESY spectrum without degrading the sensitivity in comparison with a spectrum recorded without ^{15}N decoupling throughout while also suppressing the diagonal peaks.

The idea behind our NOESY diagonal peak suppression scheme is outlined in Fig. 1. It assumes two pairs of ^{1}H–^{15}N groups with one-bond and, for the sake of clarity, also long-range NH couplings. The experiment works independent of the size of the latter. Consider only the magnetization originating from the ^{1}H(a) proton. In a first step, a Spin-State-Selective Excitation (S^{3}E) element (*7, 8*) excites only the low-frequency half of the multiplet corresponding to the attached ^{15}N(a) nucleus being in the β state and both spin states of the remote ^{15}N(b) nucleus. Then these magnetization components precess in an evolution period t_1 and are partially returned to the z axis by the first $\pi/2$ pulse of the NOESY mixing sequence. During the succeeding delay τ^{NOE} some of this longitudinal ^{1}H(a) magnetization gets transferred to ^{1}H(b) by cross relaxation while preserving the spin states of the ^{15}N spins. Then an ^{15}N π pulse is applied that interchanges ^{15}N α and β spin states, i.e., transfers magnetization within the ^{1}H multiplets. Note that this cre-

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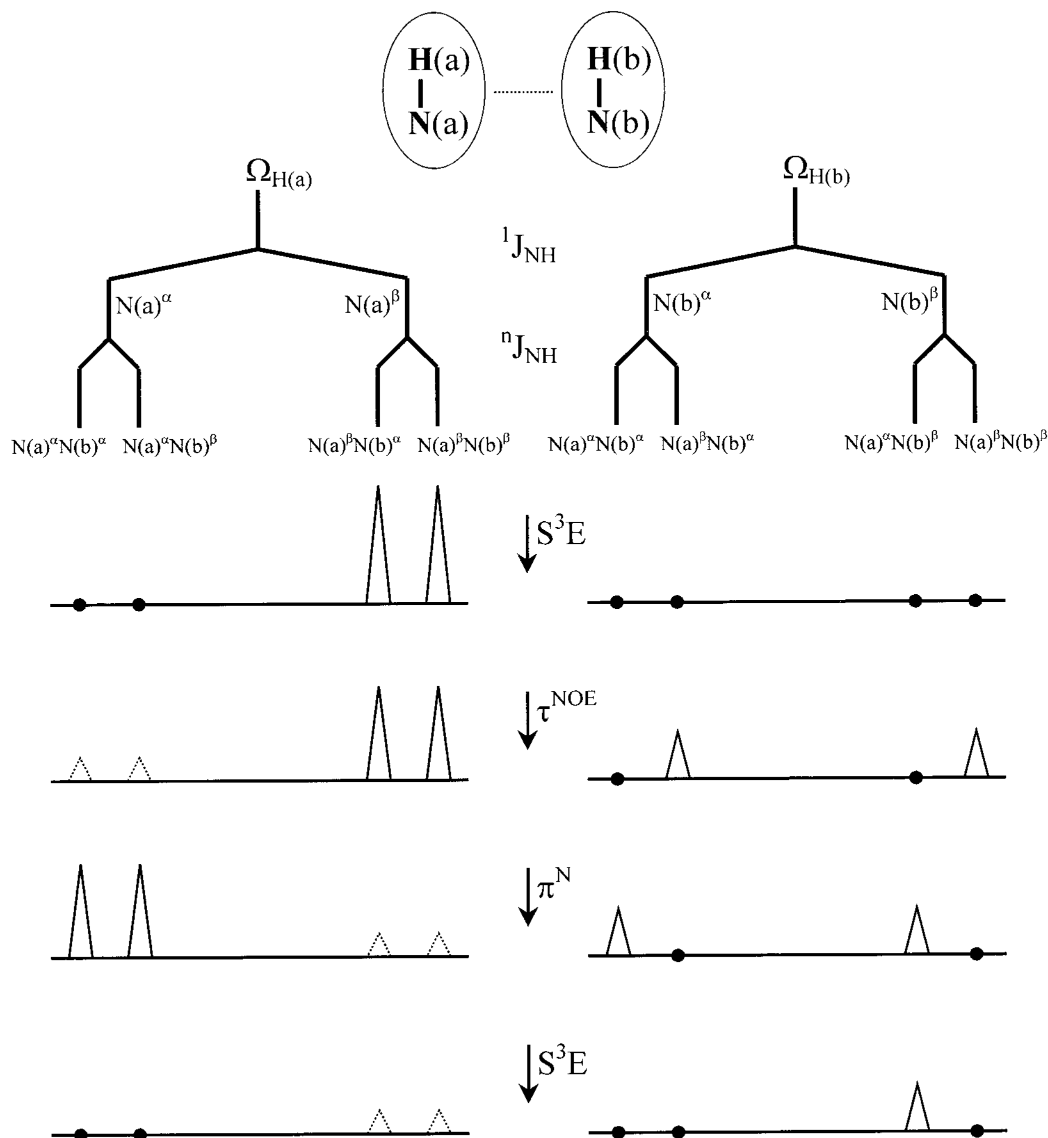

FIG. 1. Schematic illustration of the idea for diagonal peak suppression in NOESY spectra. Two protons with attached ^{15}N nuclei are close in space and for the sake of argument the stick-plot spectra each consist of four resonances corresponding to the four possible spin states of the two pertinent ^{15}N spins. Only the magnetization originating from the H(a) proton is considered. In a first step, the ^1H resonances with the attached ^{15}N in the β state are selectively excited and frequency labeling occurs in a t_1 evolution period. This is followed by a $\pi/2$(H) pulse and a NOESY mixing sequence where magnetization is transferred to H(b) with conservation of the N(a) and N(b) spin states. A π^N pulse anytime during NOESY mixing will interchange ^{15}N α and β spin states and thereby transfer magnetization within the H(a) and H(b) multiplets. The final step is equivalent to the first one, namely selective excitation of the ^1H resonances with the attached ^{15}N in the β state which ideally results in a spectrum with no magnetization remaining on the ^1H spin from which it originated, i.e., diagonal peak suppression. The dashed components in the spectra represent H(a) magnetization where the attached N(a) spin changed its state during the NOESY mixing sequence. They will appear on the diagonal in the 2D spectrum.

ates a state where the magnetization having remained on ^1H(a) (i.e., the component that would lead to a diagonal peak) is now on the high-frequency half of the multiplet. In contrast, the magnetization on the ^1H(b) spin (i.e., the component leading to the cross peak) is invariant to the π^N pulse assuming the long-range NH coupling constant is unresolved. Finally, another S^3E element exciting only the low-frequency half of the ^1H multiplets will generate magnetization of cross peaks and not excite magnetization on the diagonal.

Complete suppression of diagonal peaks cannot be expected by the above scheme because relaxation will cause a fraction of the ^{15}N spins to change their spin state during the NOESY mixing time and this fraction will pass the second S^3 filter. The dashed lines in Fig. 1 indicate the corresponding peaks occurring at the TROSY-peak positions on the diagonal. It is the

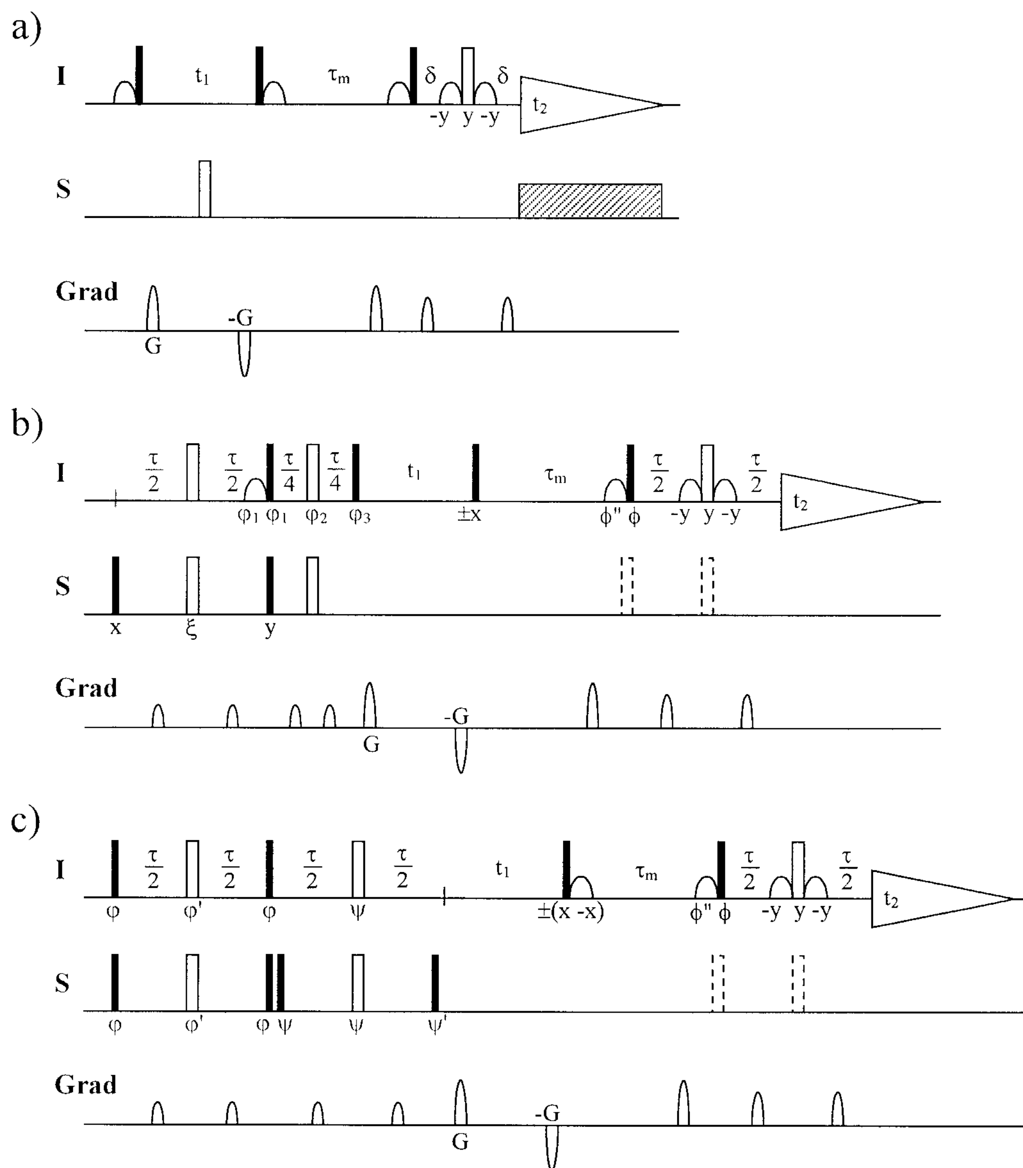

FIG. 2. 2D NOESY pulse sequences without (a) and with (b,c) diagonal peak suppression. Filled and open bars represent $\pi/2$ and π pulses, respectively. Selective $\pi/2$ water pulses are shown as open bell shapes. $\tau = (2J_{NH})^{-1}$; δ = gradient delay; τ_m = mixing time. In (a) the phase cycling scheme of Derome and Williamson (*11*) for suppression of rapid pulsing artifacts was applied. For (b) and (c) the phases are indicated below the pulses. $\psi' = \psi + \pi/2$, $\varphi' = \varphi + \pi/2$, and $\phi'' = \phi + \pi$. For all odd-numbered scans the phase ϕ is x and the left dashed π pulse on the S channel is applied while for all even-numbered scans ϕ is y and the right dashed π pulse on the S channel is applied with opposite receiver phase. The phases and selective water pulses ensure that the water magnetization is kept along the $+z$ axis. Apart from the purge gradient during τ_m the pulsed field gradients are arranged in self-compensating pairs. The gradients G and $-G$ during the t_1 periods are omitted for the first few t_1 increments until the delays are large enough to accommodate them. States–TPPI is applied on all pulses before t_1. (a) 2D NOESY pulse sequence with Watergate (*12*) and decoupling in t_1 and t_2. (b) S^3E TROSY NOESY pulse sequence. Phase settings for TROSY-line selection in F_1: $\varphi_1 = \{\pi/4, \pi/4, \pi/4, \pi/4\}$, $\varphi_2 = \{0, 0, \pi/2, \pi/2\}$, $\varphi_3 = \{0, 0, 0, 0\}$, $\phi\{0, \pi/2, 0, \pi/2\}$ and receiver $\{0, \pi, \pi, 0\}$. The prefix $\pm$ on the phase of the pulse following the t_1 period indicates an independent two-step phase cycle with opposite receiver phase. In concert with this cycle the phase of φ_3 must be shifted by π as well in order to return the water magnetization to the $+z$ axis before τ_m and the ^1H (I-spin) carrier frequency must be on-resonance for the water signal. To add the native ^{15}N (S-spin) magnetization to the TROSY line the phase ξ must be y on our Varian Unity Inova spectrometers while for our DRX-600 Bruker Instrument it must be x. (c) S^3CT TROSY NOESY pulse sequence, where the phase φ is cycled to create two data sets: A, $\{\varphi = 0, 0, \pi, \pi\}$; and B, $\{\varphi = \pi/2, \pi/2, 3\pi/2, 3\pi/2\}$ both with $\phi\{0, \pi/2, 0, \pi/2\}$ and receiver $\{0, \pi, 0, \pi\}$. Subtraction of these two data sets yields the spectrum with selection of the TROSY resonances in F_1. The phase settings in the initial INEPT transfer add the native ^{15}N (S-spin) magnetization to the TROSY line. An independent 2-step phase cycle is applied on the phase $\psi = x, -x$ with opposite φ and receiver phase.

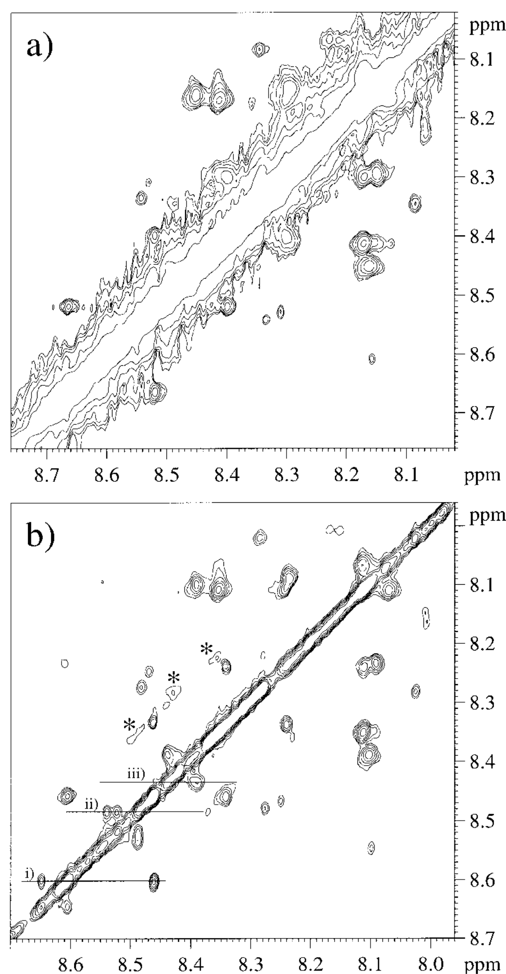

FIG. 3. Excerpts from the amide region of ^{15}N-labeled and perdeuterated rat NCAM 213–308 (90% H_2O/10%D_2O, 25°C, pH 7.32). (a) NOESY spectrum recorded with the pulse sequence in Fig. 2a without diagonal peak suppression, and (b) S^3CT TROSY NOESY spectrum recorded with the pulse sequence in Fig. 2c with diagonal peak suppression on a Varian Unity Inova 800 MHz spectrometer. Parameters: relaxation delay 1.5 s; $\tau = 5.34$ ms; $\tau_m = 125$ ms; $t_1(\max) = 64.77$ ms; 32 scans. Sinc shaped water pulses of duration 1.00 ms and Waltz-16 for ^{15}N decoupling during acquisition were applied. Data matrices of 1360 × 2048 points covering 10498.7 × 10500.1 Hz were zero-filled to 4096 × 4096 points prior to Fourier transformation and the window functions were Gaussian (lb: −1.0 gb: 0.3) in F_2 and cosine in F_1. For spectrum (b), the two subspectra were combined with a ratio of 0.501:−0.499 (A:B) which empirically resulted in the best cross talk suppression. Asterisks mark artifacts above the diagonal that arise from the second S^3E element prior to acquisition. The excerpts of the spectra are displaced by $J/2$ (48 Hz) in both dimensions to ease comparison.

same mechanism that leads to cross talk in E.COSY spectra (*9*) but fortunately, the intensity of these peaks are of the same order of magnitude as the cross peaks so that cross peaks close to the diagonal can be observed.

A crucial element of the diagonal peak suppression scheme is the quality of the S^3 filters selecting the TROSY lines and the π^N pulse interchanging the magnetization of the α and β doublet resonances. Apart from pulse imperfections the S^3 filters are sensitive to variations in the ^{1}H–^{15}N coupling constants to which pertinent delays are tuned. The simplest implementation is represented by the pulse sequence in Fig. 2b with two S^3E elements where J cross talk occurs to first order in $\Delta J = J_0 - J$, with J_0 being the coupling constant used in setting the delays. For large proteins at high fields this performance could be adequate because the undesired cross talk peaks represent the anti-TROSY resonance in one of the dimensions and hence might be broadened beyond detection. However, in the experiments at 800 MHz on our test protein (vide infra) cross talk suppression was inadequate so instead the pulse sequence in Fig. 2c was used. It employs an S^3CT element in the preparation sequence and J cross talk occurs only to second order in ΔJ. This provides a clean spectrum below the diagonal and a similar S^3 filter could be used at the end of the sequence if it were desirable to clean up the upper part of the spectrum too. But as the spectrum is symmetrical there is no need to extend the pulse sequence.

The new method for diagonal peak suppression in 2D NOESY spectra was tested on a perdeuterated ^{15}N-labeled protein sample, Neural Cell Adhesion Molecule (NCAM) (*10*), in H_2O using a Varian Unity Inova 800 MHz spectrometer. Specifically, it was a 2.5 mM sample of Ig-homology module 3 (^{15}N(99%), ^{2}H(99%)) of recombinant NCAM 213–308 from rat in PBS buffer (5mM Na phosphate, 150 mM NaCl, pH 7.32). It was produced in a yeast Pichia pastoris expression system and it has 6 extra residues on its N-terminus originating from a cloning vector. Module 3 has one site for N-linked glycosylation at Asn-222 and the recombinant protein is glycosylated as confirmed by enzymatic deglycosylation with PNGF glycosidase. The molecular weight of glycosylated module 3 as determined by MALDI mass spectroscopy was 14.3 kDa.

Figure 3 shows excerpts from the amide region of a standard NOESY spectrum with ^{15}N decoupling throughout (pulse sequence in Fig. 2a) and an S^3CT TROSY NOESY

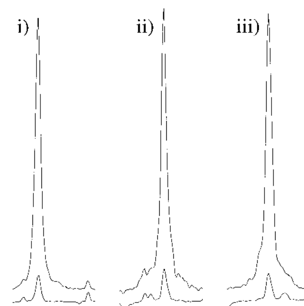

FIG. 4. Sections as indicated in Fig. 3b. Top, sections from the spectrum in Fig. 3a without suppression of diagonal peaks; bottom, sections from spectrum in Fig. 3b with diagonal suppression. The cross peaks close to the diagonal can hardly be identified much less quantitated in the upper sections in contrast to the lower ones. Corresponding sections are scaled to show similar intensities for the cross peak signals.

(pulse sequence in Fig. 2c) spectrum. Clearly, the latter shows several cross peaks close to the diagonal that are obscured in the former. That comes about for two reasons, namely the TROSY effect of narrower peaks and most importantly the diagonal peak suppression scheme that is demonstrated by the F_2 sections in Fig. 4 taken at the points indicated by lines in Fig. 3b. Asterisks in Fig. 3 mark artifacts arising from cross talk in the second S^3 filter. They can be suppressed by a higher order S^3 filter which is not considered worthwhile because it is convenient to have a Watergate element at the end and because the part below the diagonal is clean.

In conclusion, we have introduced an efficient method for diagonal peak suppression in NOESY spectra of ^{15}N-labeled proteins allowing observation of cross peaks close to the diagonal. For larger proteins at high fields there is no penalty in cross peak intensities. The scheme can easily be incorporated into three-dimensional experiments with ^{15}N resonances in one of the dimensions.

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