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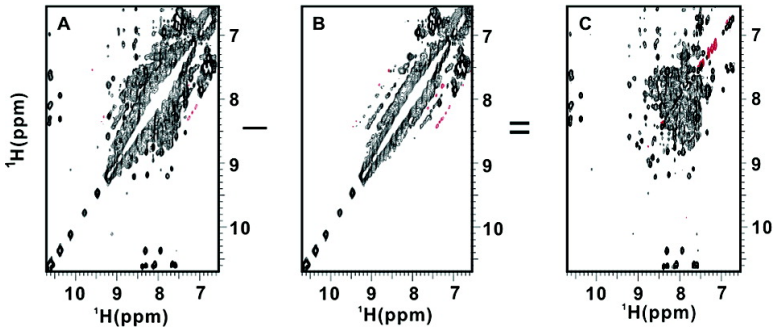

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General Method for Suppression of Diagonal Peaks in Heteronuclear-Edited NOESY Spectroscopy

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Despite recent advances in obtaining structural constraints from residual dipolar couplings, chemical shifts, *J* coupling constants, and cross-correlated relaxation, NOE remains the most important and widely used source of restraints for structure determination by NMR. The quality of NOESY spectra significantly affects the quality of solution structures of biomolecules. Diagonal peaks in NOESY spectra can be a nuisance since strong diagonals can mask many cross-peaks near the diagonals and very intense diagonals often create t_1-noise. Even in ^{15}N- or ^{13}C-edited 3D NOESY spectroscopy, interference from diagonals can be significant for systems with poor dispersion of ^{1}H chemical shifts, such as α-helical proteins, unfolded and partially unfolded proteins, and large proteins. Three distinct approaches have been designed in the past to suppress diagonal peaks. The first approach relies on difference spectroscopy to subtract data representing the diagonal peaks from the conventional NOESY spectrum.[1−4] Since the two spectra are often recorded using distinct pulse schemes with different resulting relaxation, satisfactory suppression of diagonals is difficult to achieve. The second approach eliminates magnetization that does not undergo significant chemical shift changes during the mixing period.[5] For this approach, genuine cross-peaks near diagonals are also severely suppressed. Recently, a number of experiments have been proposed to suppress diagonal peaks in ^{15}N-edited NOESY spectra on the basis of selecting the TROSY components in both proton dimensions.[6−9] While these approaches can be effective for large proteins with a large TROSY effect, they may be uneconomical for smaller proteins since the sensitivity is only 25% of the conventional NOESY experiment. In addition, suppression of diagonals is incomplete due to nonuniformity of $^1J_{NH}$ values and relaxation effects during the NOE mixing period and TROSY sequence block.

Here, we propose a novel experiment to suppress NOESY diagonal peaks more efficiently than previous experiments and provide much higher sensitivity for biomolecules with little TROSY effect. Suppression of diagonals is achieved by subtracting two data sets: one conventional NOESY spectrum and one spectrum with only diagonals. Figure 1 shows the pulse scheme for ^{15}N-edited NOESY−HSQC. The first data set (conventional NOESY) is recorded by setting τ_m to the mixing time (τ_{mix}) and τ_{m1} to zero. The second data set is acquired by setting τ_m to zero and τ_{m1} to τ_{mix}', which is slightly smaller than τ_{mix}. In the second scheme, only longitudinal two-spin magnetization, H_zX_z (H and X represent proton and heteronuclear spins, respectively) instead of H_z, exists at the beginning of the mixing period, giving rise to no cross-peaks, except for XH_2 groups in which the two protons are magnetically distinct. The diagonal peak intensities for a given proton in an XH_n group, where *n* is the number of magnetically equivalent protons in the two data sets, are governed by eqs 1 and 2.

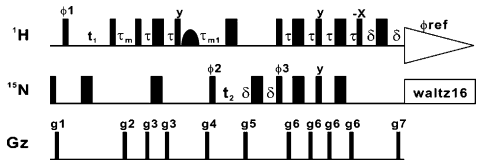

Figure 1. Pulse sequence for diagonal-suppressed ^{15}N-edited NOESY. All narrow (wide) bars represent 90° (180°) rectangular pulses. The curved shape represents the water-selective 90° pulses. The delays used are $\tau = 2.3$ ms and $\delta = 1.1$ ms. For the first data set, $\tau_m = 80$ ms and $\tau_{m1} = 0$. For the second data set, $\tau_m = 0$ and $\tau_{m1} = 74$ ms for CaM. The phase cycling employed is $\phi_1 = 2(45°), 2(225°)$; $\phi_2 = x, -x$; $\phi_3 = x$; $\phi_{ref} = x, -x, -x, x$. The durations and strengths of the sine-shaped gradients are $g1 = 1$ ms, 20 G/cm, $g2 = 1$ ms, 10 G/cm, $g3 = 1$ ms, 12.5 G/cm, $g4 = 2.4$ ms, 25 G/cm, $g5 = 1$ ms, 40 G/cm, $g6 = 1$ ms, 15 G/cm, $g7 = 1$ ms, −4 G/cm. Quadrature detection in F_1 is achieved by States-TPPI of ϕ_1, while quadrature detection in F_2 uses the enhanced sensitivity pulse gradient method. The two data sets were acquired in an interleaved manner for prevention of artifacts from subtraction.

$$-\frac{dH_z}{dt} = R(H_z)H_z + \sum\sigma(H,H^j)H_z^j \quad (1)$$

$$-\frac{dH_zX_z}{dt} = R(H_zX_z)H_zX_z + \sum\sigma(H,H^j)H_z^jX_z \quad (2)$$

where $R(H_z)$ and $R(H_zX_z)$ are longitudinal autorelaxation rates of magnetization, H_z and H_zX_z, respectively, dominated by spectral density, $J(0)$; $\sigma(H,H^j)$ is the cross-relaxation rate between proton H in the XH_n group and a nearby proton *j*. The resulting magnetization, H_z^j, from eq 1 gives rise to NOESY cross-peaks, while the resulting magnetization, $H_z^jX_z$, from eq 2 remains unobservable. The intensity ratio of the diagonal peaks in the two data sets approximates to $\exp[-R(H_z)\tau_{mix} + R(H_zX_z)\tau_{mix}']$ since $R \gg |\sigma(H,H^j)|$. To achieve optimal suppression of the diagonal peak, the two mixing times meet the following relation:

$$\tau_{mix}' = \frac{R(H_z)}{R(H_zX_z)}\tau_m \approx \left(1 - \frac{R(X_z)}{R(H_zX_z)}\right)\tau_m \quad (3)$$

where $R(X_z)$ is the longitudinal relaxation rate of spin X. Although $R(X_z)/R(H_zX_z)$ can vary widely from protein to protein, the deviation within one protein is generally small. For example, the average value $[R(N_z)/R(H_zN_z)]_{AV}$ is 0.101 with a standard deviation of 0.023 for calmodulin (CaM), while it is 0.350 ± 0.052 for ubiquitin, as measured at 25 °C on a 500 MHz NMR. The variation should be significantly smaller for larger proteins and higher fields. When τ_{mix}' is set to $[1 - [R(X_z)/R(H_zX_z)]_{AV}]\tau_{mix}$, the relative intensity of a residual diagonal peak in the difference spectrum with respect to that in the diagonal peak in the NOESY spectrum approximately equals $R(H_zX_z)\tau_{mix}[R(X_z)/R(H_zX_z) - [R(X_z)/R(H_zX_z)]_{AV}]$. The average residual diagonal peak intensity in the ^{15}N-edited NOESY

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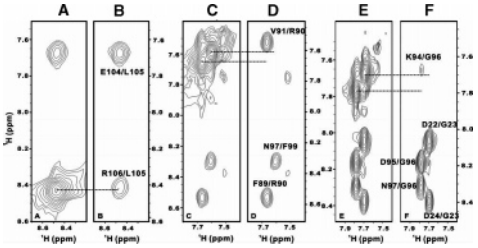

Figure 2. Representative slices from the ^{15}N-edited 3D NOESY−HSQC spectrum (A, C, and E) and the difference spectrum (B, D, and F). Each slice is labeled with NOE assignments. Positive peaks are indicated in black. No negative peaks appear at the noise level used here. The dashed lines indicate the positions of expected diagonal peaks. The experiment was recorded with uniformly ^{15}N-labeled calmodulin in a $^{1}H_2O/^{2}H_2O$ (90:10) solution (1 mM protein, 5 mM $CaCl_2$, pH 6.5, 25 °C) on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe. Complex points 90(t_1) × 36(t_2) × 512(t_3) were collected with spectral widths of 5500 (^{1}H), 1375 (^{15}N), and 8012 Hz (^{1}H). An interscan delay of 1 s, with 4 scans per increment, was used, resulting in a total experimental time of 16 h for each data set. The mixing times were $\tau_{mix} = 80$ ms and $\tau_{mix}' = 74$ ms. The post-acquisition scaling factor was 0.99 for the second data set.

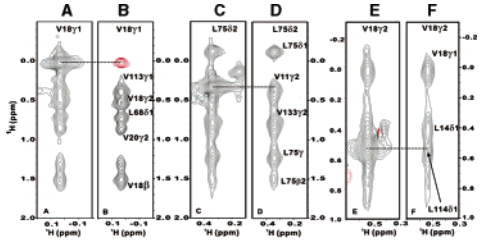

Figure 3. Representative slices from the ^{13}C-edited NOESY−HSQC (A, C, and E) and difference spectrum (B, D, and F). Positive (negative) peaks are indicated in black (red). The dashed lines indicate the positions of expected diagonal peaks. The experiment was recorded with β-chain-specific ^{13}C-labeled hemoglobin in $^{2}H_2O$ solution (~1 mM protein tetramer, 20 mM Na_3PO_4, pH 7.0, 31 °C) on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe. Complex points 40(t_1) × 67(t_2) × 512(t_3) were collected with spectral widths of 3000 (^{1}H), 3000 (^{13}C), and 8012 Hz (^{1}H). An interscan delay of 1 s, with 8 scans per increment, was used, resulting in a total experimental time of 26 h for each data set. The mixing times were $\tau_{mix} = 40$ ms and $\tau_{mix}' = 35$ ms. The post-acquisition scaling factor was 1 for the second data set.

should be smaller than 3%, even for a small protein like ubiquitin with τ_{mix} of 80 ms at a 500 MHz field.

We have applied the pulse sequence shown in Figure 1 to an ^{15}N-labeled sample of CaM, which consists mainly of α-helical structures and gives rise to very poor dispersion of amide proton shifts (Figure S1A of the Supporting Information). The optimal τ_{mix}' value was found to be 0.9 × τ_{mix} from a rough estimation of the average $R(H_zX_z)$ and $R(X_z)$ values based on 1D ^{1}H spectra recorded with pulse sequences for measuring $R(H_zX_z)$ and $R(X_z)$.[10] Experimentally, the optimal value was found to be 75 ms when $\tau_{mix} = 80$ ms. In practice, it is not necessary to optimize τ_{mix}' since one can scale the second data set to achieve best suppression of diagonal peaks in data processing. The scaling factor is adjusted so that residual diagonals are weak and opposite in sign with respect to the cross-peaks. This allows weak NOE peaks very near the diagonal, and even NOE peaks at the diagonal, to be unambiguously identified. Residues located in nonregular secondary structure regions can give rise to residual diagonal peaks with the same sign as those of the NOE peaks. Therefore, these regions should be identified prior to assigning weak NOEs at the diagonal.

Nearly complete suppression of diagonal peaks for most amides in CaM was achieved using this approach in an ^{15}N-edited ^{1}H−^{1}H NOESY (Figure S1 of the Supporting Information). Residual diagonals were typically <2% of the original and often were comparable to the noise. This enabled us to identify NOE peaks very close to diagonals (e.g., the cross-peaks, R106H_N−L105H_N, V91H_N−R90H_N, and K94H_N−G96H_N, shown in Figure 2). Using the difference spectrum, a total of 25 additional sequential H_N−H_N NOEs and 4 additional [i, i + 2] H_N−H_N NOEs were identified very close to the diagonal.

For XH_2 groups in which the two protons are magnetically distinct, cross-relaxation between the two geminal protons leads to observable NOE cross-peaks in the second spectrum (Figure S1B of the Supporting Information). In addition, eq 3 is no longer valid because the cross-relaxation rate between the two protons is comparable to the autorelaxation rate. Using the mixing time estimated from $R(H_zX_z)$ and $R(X_z)$ always results in incomplete suppression of the residual diagonal peaks for this type of XH_2 groups (Figure S1C of the Supporting Information).

Similarly, we have modified the ^{13}C-edited NOESY−HSQC and applied the sequence to uniformly ^{13}C-labeled DdCAD-1 (24 kDa). Although somewhat more-intense residual diagonal peaks were observed than were seen in the ^{15}N-edited spectrum, due to a wider range of $R(C_z)/R(H_zC_z)$ than $R(N_z)/R(H_zN_z)$, excellent suppression of diagonals was obtained (typically <6%; see Figure S2 of the Supporting Information). The NOESY−HSQC sequence with a constant-time ^{13}C evolution period has also been applied to a chain-specific ^{13}C-labeled sample of hemoglobin (65 kDa) in which the ^{1}H chemical shifts of most methyl groups distribute within a range of ~1.5 ppm. Figure 3 shows a number of slices taken from the 3D spectra of hemoglobin with ^{13}C labeling for the β-chain. With the excellent suppression of diagonal peaks, long-range CH_3−CH_3 NOEs close to diagonals (e.g., L75δ_2−V11γ_2,V18γ_2−L14δ_1, and V18γ_2−L114δ_1, Figure 3D,F) can be assigned.

One can use the conventional NOESY spectrum to assign NOEs far from the diagonal while using the difference spectrum to assign the NOEs close to diagonal peaks. Thus, the sensitivity per unit time of this method is 71% ($1/\sqrt{2}$) of the conventional one for cross-peaks far from the diagonal. For cross-peaks close to the diagonal, the sensitivity of the present method is 50% of the conventional one since the noise of the difference spectrum is $\sqrt{2}$ times more than that of the individual spectrum. However, removal of the diagonal peaks greatly enhances the effective sensitivity. Removal of intense diagonal peaks also suppresses associated artifacts, such as sinc-wiggles and t_1-noise. The experiment proposed here is far more sensitive than the TROSY-based methods for systems with little TROSY effect.

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Supporting Information Available: Two figures (S1 and S2) demonstrating excellent suppression of diagonal peaks. This material is available free of charge via the Internet at http://pubs.acs.org.

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