NOE Measurements in the Absence of Spin Diffusion: Application to Methylene Groups in Proteins and Effects on Local Structural Parameters

Charles G. Hoogstraten,[†] William M. Westler,[†] Slobodan Macura,[‡] and John L. Markley*,[†]

Department of Biochemistry, University of Wisconsin Madison, Wisconsin 53706
Department of Biochemistry and Molecular Biology Mayo Foundation, Rochester, Minnesota 55905

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The NOESY experiment, which commonly is used in NMR structural studies of proteins, can yield results that are perturbed by multispin effects, termed spin diffusion; the major effect is a systematic distance underestimation that can lead to inaccuracies in derived structures.[1,2] In this communication, we introduce a novel pulse sequence, CBD-NOESY (complementary block-decoupled NOESY), which allows analysis of NOEs between protons in different spectral regions free of interference from magnetization transfer steps within each single region.[3] CBD-NOESY is particularly suited for analyzing NOEs to methylene groups, which are often distorted by efficient cross relaxation between the two geminal protons. CBD-NOESY allows analysis of all NOEs connecting two selected spectral blocks in a single experiment, in contrast to other recently-introduced pulse sequences that allow careful analysis of a single or very limited number of NOE interactions in the absence of all spin diffusion.[4−6] We demonstrate the use of accurate CBD-NOESY data to remove a distortion in calculated structures resulting from spin-diffusion contributions to NOESY data.

The CBD-NOESY pulse sequence is shown in Figure 1. During the mixing period, magnetization is alternated between the longitudinal and transverse (spin-lock) frames. Cross relaxation is eliminated among spins that follow this trajectory, since for rigid macromolecules the algebraic signs of longitudinal and transverse cross relaxation are opposite.[7] In CBD-NOESY, a band selective 180° pulse is used to invert a region of the spectrum. During the subsequent transverse period (SL_x), cross relaxation between the inverted and noninverted regions of the spectrum has the same sense as during the longitudinal cross relaxation period τ_N. Cross peaks between regions will therefore build up over the course of the mixing time. Within each region, there is no compensation for the destructive interference of the longitudinal and transverse periods, and cross relaxation is eliminated. To avoid signal losses due to projection of magnetization from the *xy*-plane to the spin-lock axis and back, the spin-lock period is flanked with high-power 90° pulses.[8] Since all resonances are aligned along the x-axis following the $90°_y$ pulse, a precession delay τ_p is inserted with length adjusted such that the spins at the largest offset precess by an angle equal to the inclination of their spin-lock axis from the *xy*-plane. The first $90°_x$ pulse then rotates all resonances to within a few degrees of their spin-lock axes, minimizing losses due to projection. The individual NOESY and ROESY cross-relaxation periods are set sufficiently short so that spin diffusion within each period is negligible, and the sequence is repeated to obtain the desired mixing time.

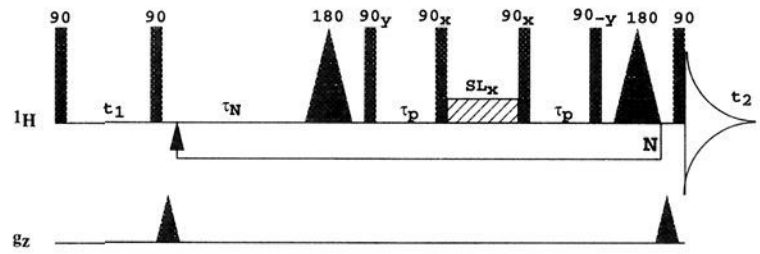

Figure 1. Pulse sequence for the CBD-NOESY experiment. Shaded rectangles represent high-power pulses; shaded triangles represent shaped, band-selective pulses adjusted to invert a single spectral block; diagonal shading indicates low-power spin lock; g_z indicates *z*-axis pulsed field gradients. NOESY and ROESY cross-relaxation periods are indicated by τ_N and SL_x, respectively, and τ_p indicates a precession delay to allow spins to fan out or refocus in the *xy*-plane. The two initial pulses and one final nonselective 90° pulse and the receiver are phase cycled in the standard manner.

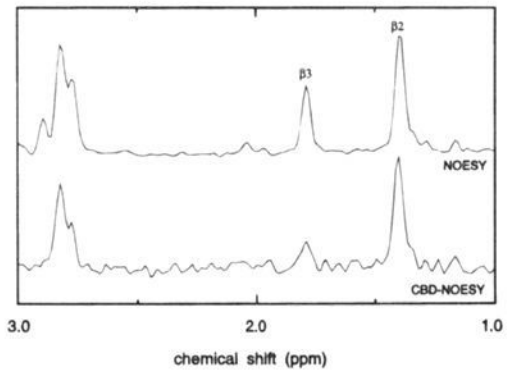

Figure 2. Slices through NOESY (top) and CBD-NOESY (bottom) spectra of OMTKY3 at the ω_2 frequency of C^{38} H^N (9.17 ppm). Spectra were acquired on a 3 mM sample of OMTKY3, 298 K, pH 4.1, on a Bruker DMX-750 spectrometer. The NOESY mixing time was 180 ms; for CBD-NOESY, the NOESY delay was set to 19.3 ms, the ROESY delay was set to 12.5 ms, the precession delay τ_p was set to 35 μs, and the mixing sequence was repeated for $N = 4$ times. The band-selective pulse was a 1.05 ms IBURP2[20] pulse applied to the aliphatic region of the spectrum. 768 t_1 points of 80 transients each were acquired using States-TPPI[21] detection in the indirect dimension. Spectra were extended by 33% in t_1 using complex linear prediction; 3 Hz exponential broadening in t_2 and a cosine-squared window in t_1 were applied, and the spectra were zero-filled and transformed to a final matrix of 4096 by 1024 points.

Because the transverse cross-relaxation rate is a function of offset, the overall cross-relaxation rate cannot be set precisely to zero at all offsets simultaneously; nevertheless, alternation of frames gives useful cancellation of cross-relaxation across the spectral width.[9] Experimentally, the ratio of longitudinal to transverse cross-relaxation periods is adjusted such that the suppression of cross relaxation is most efficient at the offset of the resonances expected to be the most serious source of spin diffusion (e.g., β-methylenes); within-block cross relaxation is sufficiently suppressed at other offsets to allow the analysis of all interblock cross peaks in a single two-dimensional experiment. CBD-NOESY is closely related to the S.NOESY experiment,[4] which selects for all processes involving a single resonance.

Figure 2 demonstrates the application of CBD-NOESY to the study of cross relaxation in a small protein, turkey ovomucoid third domain (OMTKY3). Slices through NOESY

* Author to whom correspondence should be addressed.
[†] University of Wisconsin.
[‡] Mayo Foundation.

(1) Thomas, P. D.; Basus, V. J.; James, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1237−1241.
(2) Krezel, A. M.; Darba, P.; Robertson, A. D.; Fejzo, J.; Macura, S.; Markley, J. L. *J. Mol. Biol.* **1994**, *242*, 203−214.
(3) Macura, S.; Westler, W. M.; Markley, J. L. *Methods Enzymol.* **1994**, *239*, 106−144.
(4) Fejzo, J.; Westler, W. M.; Markley, J. L.; Macura, S. *J. Am. Chem. Soc.* **1992**, *114*, 1523−1524.
(5) Boulat, B.; Burghardt, I.; Bodenhausen, G. *J. Am. Chem. Soc.* **1992**, *114*, 10679.
(6) Zwahlen, C.; Vincent, S. J. F.; Di Bari, L.; Levitt, M. H.; Bodenhausen, G. *J. Am. Chem. Soc.* **1994**, *116*, 362−368.
(7) Fejzo, J.; Westler, W. M.; Macura, S.; Markley, J. L. *J. Am. Chem. Soc.* **1990**, *112*, 2574−2577.
(8) Griesinger, C.; Ernst, R. R. *J. Magn. Reson.* **1987**, *75*, 261−271.
(9) Fejzo, J.; Westler, W. M.; Macura, S.; Markley, J. L. *J. Magn. Reson.* **1991**, *92*, 20−29.

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Table 1. Calibration of Interproton Distances in OMTKY3 from NOESY and CBD-NOESY Data

	Cys-38	$H^N-H^{\beta 2}$	$H^N-H^{\beta 3}$
NOESY	$a_{ij}{}^{1\,a}$	0.120	0.065
	$\Gamma_{ij}{}^{obs}$ $(s^{-1})^b$	2.00	1.08
	r_{calc} (nm)^c	0.23	0.25
CBD-NOESY	$a_{ij}{}^1$	0.0388	0.00678
	$\Gamma_{ij}{}^{obs}$ (s^{-1})	0.854	0.150
	r_{calc} (nm)	0.26	0.35
	$r_{X\text{-}ray}$ (nm)[10]	0.23	0.36

[a] Normalized peak intensities, calculated as ratios of integrated volumes of cross and diagonal peaks.[19] [b] Observed cross-relaxation rates, corrected as appropriate for off-resonance effects in the case of CBD-NOESY. [c] Interproton distances calculated from $\Gamma_{ij}{}^{obs}$ by assuming an isotropic correlation time of 4.8 ns.

and CBD-NOESY spectra of OMTKY3 at the ω_2 frequency of the backbone amide proton of Cys-38 (C^{38} H^N) are shown, and intraresidue NOEs to the β_2 and β_3 protons of Cys-38 are indicated. In the X-ray crystallographic structure of OMTKY3 in complex with α-chymotrypsin,[10] the $H^N-H^{\beta 2}$ distance is much shorter than the $H^N-H^{\beta 3}$ distance (see Table 1).[11] In this geometry, the indirect pathway $H^N \rightarrow H^{\beta 2} \rightarrow H^{\beta 3}$ contributes significantly to the $H^N-H^{\beta 3}$ cross peak in NOESY because of the short distance between the geminal protons, whereas this contribution is removed in CBD-NOESY. In Table 1, it is seen that the CBD-NOESY data predict a large (0.1 nm) difference between the two distances, in agreement with the X-ray structure, whereas the NOESY data predict that the two distances are essentially equal. For quantitative analysis of distances involving methylene protons, CBD-NOESY clearly is superior to NOESY. Even for semiquantitative interpretation using a "small, medium, weak" protocol,[12] the $H^N-H^{\beta 3}$ cross peak in NOESY would be misclassified as "medium" or even "strong" rather than "weak", and an inappropriately short upper distance bound would be applied.

To investigate the effects of such a single inaccurate distance on the derived protein structure, we have calculated two sets of OMTKY3 structures using the distance geometry/simulated annealing protocol implemented in the program X-PLOR/dg.[11,13] The calculations employed the 655 NOE, 29 dihedral angle, and 17 hydrogen bond constraints used in a previous solution structure determination of this protein,[2] except that the two intraresidue amide−β-methylene NOEs for Cys-38 were replaced by constraints derived from either NOESY or CBD-NOESY data as shown in Table 1. Upper and lower bounds were derived as in the original work.[2] Ten structures with no NOE violations greater than 3.5 Å or dihedral angle violations greater than 3° and good covalent geometry were calculated for each data set. The resulting sets of structures were globally very similar. The mean pairwise backbone internal rmsds (root mean squared positional deviations) for the structures derived from NOESY and CBD-NOESY data were 0.83 and 0.81 Å, respectively, whereas the mean pairwise rmsd between the two sets was 0.88 Å. Examination of local structure, however, reveals a significant perturbation. The χ_{38}^1 dihedral angle, which is spanned by these intraresidue NOEs, takes on values from −167.2° to +170.0° (mean −178.3° ± 6.7°) in structures calculated from NOESY data and from −85.1° to +168.5° (−117.9° ± 34.0°) in structures calculated from CBD-NOESY. The much smaller distribution of χ_{38}^1 values indicates that the inaccurate NOESY $H^N-H^{\beta 3}$ constraint restricts the resulting structures to much less than the full conformational space available to the molecule. Moreover, the χ_{38}^1 dihedral angle observed in the crystallographic state (−82.7°)[10] is consistent with the range observed for the CBD-NOESY structures but not for the NOESY structures. This result implies that the underestimation of the $H^N-H^{\beta 3}$ distance in the NOESY data causes the observed χ_{38}^1 rotamer population to be inaccurate, as well as unjustifiably precise.

In the calculation of solution structures from NMR data, it is commonly (if often tacitly) assumed that the dense network of NOEs normally obtained for the interiors of globular proteins yields sufficient redundancy in distance determination to compensate for the deleterious effects of individual inaccurate distances.[14] The data set used for the OMTKY3 structures calculated here contains nine NOE constraints and a covalent disulfide linkage involving the Cys-38 β-methylene group. Nonetheless, a single inaccurate distance constraint caused a significant distortion of the local structure. This result emphasizes the importance of ensuring that applied distance bounds are accurate in the sense of containing the correct average distance.[14-16]

CBD-NOESY allows the analysis of NOESY cross peaks connecting spectral blocks free of multistep magnetization transfer pathways involving one or more steps contained within a single block. All two-step magnetization transfer contributions to peaks of interest are thus eliminated, and only a single class of three-step transfers (the rare case in which each of the three steps is interblock, e.g., $H^N \rightarrow H^{\beta'} \rightarrow H^{N'} \rightarrow H^{\beta}$) is allowed.[17] The combination of CBD-NOESY with the BD-NOESY experiment for measuring cross relaxation within a spectral block[18] allows the analysis of all cross peaks observed in a NOESY spectrum under conditions of greatly reduced spin diffusion. The results reported here indicate that the analysis of large numbers of NOEs from CBD-NOESY spectra, particularly in three-dimensional heteronuclear-edited form, is likely to substantially improve the local accuracy of derived solution structures.

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(10) Fujinaga, M.; Sielecki, A. R.; Read, R. J.; Ardelt, W.; Laskowski, M., Jr.; James, M. N. G. *J. Mol. Biol.* **1987**, *195*, 397−418. Hydrogens were added using a facility in the X-PLOR package.[11]
(11) Brünger, A. T. *X-PLOR Version 3.1: A system for x-ray crystallography and NMR*; Yale University Press: New Haven, 1992.
(12) Williamson, M. P.; Havel, T. F.; Wüthrich, K. *J. Mol. Biol.* **1985**, *182*, 295−315.
(13) Nilges, M.; Clore, G. M.; Gronenborn, A. M. *FEBS Lett.* **1988**, *229*, 317−324.
(14) James, T. L. *Methods Enzymol.* **1994**, *239*, 416−439.
(15) Jardetzky, O. In *Computational aspects of the study of biological macromolecules by nuclear magnetic resonance spectroscopy*; Hoch, J. C., Poulsen, F. M., Redfield, C., Eds.; Plenum Press: New York, 1991; pp 375−389.
(16) Zhao, D.; Jardetzky, O. *J. Mol. Biol.* **1994**, *239*, 601−607.
(17) Under appropriate conditions, scalar coupling can cause magnetization transfer between the two methylene protons during the spin-lock periods and, thus, give rise to indirect magnetization via an RH (ROESY cross-relaxation in series with Hartmann−Hahn transfer) mechanism (Farmer, B. T., II; Macura, S.; Brown, L. R. *J. Magn. Reson.* **1987**, *72*, 347−352).
(18) Hoogstraten, C. G.; Westler, W. M.; Macura, S.; Markley, J. L. *J. Magn. Reson. B* **1993**, *102*, 232−235.
(19) Macura, S.; Farmer, B. T., II; Brown, L. R. *J. Magn. Reson.* **1986**, *70*, 493−499.
(20) Geen, H.; Freeman, R. *J. Magn. Reson.* **1991**, *93*, 93−141.
(21) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.