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Simplification of Two-Dimensional NOE Spectra of Proteins by ^{13}C Labeling

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Two-dimensional NOE spectroscopy provides a convenient method for measuring interproton distances in solution, permitting detailed conformational studies of nucleic acids and small proteins in solution. Unfortunately, many proteins of interest are not yet accessible by this type of experiment because of severe overlap in the 2D spectrum. Limited resolution in the aliphatic region, for example, can obscure "long-range" interactions which can provide information regarding tertiary structure. To overcome these limitations, heteronuclear labels may be introduced (*1–3*). We demonstrate here the feasibility of a ^{13}C-edited NOESY experiment in which only those interactions involving at least one ^{13}C-attached proton are observed.

Griffey and Redfield (*4*) have previously proposed a method for determining which NOE cross peaks involve a ^{13}C-attached proton by using ^{13}C decoupling during the evolution period but not during the detection period. Interactions involving ^{13}C-attached protons appear as asymmetric cross peaks in the resulting 2D spectrum. However, the final spectrum is of the same complexity as an ordinary NOESY spectrum. In the application to proteins it is desirable to keep the final 2D NOE spectrum as simple as possible. The pulse scheme we propose is outlined in Fig. 1. The scheme differs from the regular NOESY experiment by the insertion of an extra delay, Δ, of duration $1/J_{CH}$ at the end of the preparation period. A ^1H 180° pulse is applied at the center of this Δ interval; in odd-numbered scans a 180° ^{13}C pulse is also applied at the center of this interval, and data are subtracted from those acquired in even-numbered scans. At time $t_1 = 0$, all ^1H magnetization in even-numbered scans will be aligned along the $+y$ axis; in odd-numbered scans magnetization of ^{13}C-attached protons will be aligned along the $-y$ axis and all other ^1H magnetization will be along the $+y$ axis.

Subtraction of odd-numbered scans from even-numbered scans will thus result in the cancellation of ^1H signals that do not involve an interaction with a ^{13}C-attached proton. The basic eight-step phase cycle is presented in Table 1. In addition, phase cycling of the final 90° ^1H pulse is used to reduce the magnitude of coherent cross peaks (*5*) and CYCLOPS phase cycling (*6*) is used to suppress quadrature artifacts. A ^{13}C 180° pulse at the center of the evolution period is used to remove the effect of heteronuclear coupling in the F_1 dimension of the final spectrum, simplifying the

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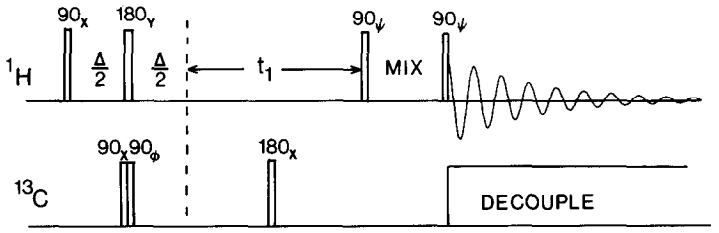

FIG. 1. Pulse scheme of the ^{13}C-edited 2D NOE experiment. The phase cycling is given in Table 1. The delay Δ is adjusted to $1/J_{CH}$ or slightly shorter to minimize losses due to transverse relaxation. The phase ϕ is cycled along the $\pm x$ axis, resulting in 0 and 180° ^{13}C pulses at the center of the Δ period in alternate scans.

spectrum and maximizing the signal-to-noise ratio of cross peaks. To allow measurement of cross peaks close to the diagonal it is also desirable to employ ^{13}C decoupling during the detection period. Without this heteronuclear decoupling, the diagonal will split into two parallel "diagonals," separated by J_{CH} in the F_2 dimension.

The method is illustrated for a 1.5 m*M* solution of the N-terminal domain of λ-repressor protein (molecular mass 11 kDa) in D_2O, pH 11.2. λ-Repressor is a specific DNA-binding protein from bacteriophage λ and provides a model system of biophysical interest (*7*). At basic pH the repressor dimer dissociates into monomers without loss of tertiary structure (*8*). The domain was purified from an overproducing strain of *Escherichia coli* constructed by R. Sauer and co-workers (*9*). The cells were grown in a minimal medium (*10*) containing alanine ^{13}C-enriched in the C_β position, resulting in a 60% ^{13}C labeling of the alanine methyl groups in the protein (*11*). The T_1 values of the ^{13}C-labeled Ala methyl protons were less than 1.0 s, permitting a relatively short delay (1.3 s) between scans in the 2D NOE experiment. The ^{13}C-edited NOE spectrum obtained with a mixing time of 350 ms is shown in Fig. 2. Only NOE interactions with the ^{13}C-labeled methyl protons are visible in this spectrum. On the diagonal some

TABLE 1

Phase Cycling in the Pulse Scheme of Fig. 1

Step	ϕ	ψ	Acquire[a]	
1	x	x	x	
2	$-x$	x	$-x$	
3	x	y		y
4	$-x$	y		$-y$
5	x	$-x$	x	
6	$-x$	$-x$	$-x$	
7	x	$-y$		y
8	$-x$	$-y$		$-y$

[a] Data acquired in steps 1, 2, 5 and 6 are stored separately from data acquired in steps 3, 4, 7 and 8 and data are processed in the standard manner (*13*) to obtain a 2D absorptive spectrum.

weak signals are also observed from protons coupled to natural abundance ^{13}C. Figure 3 compares the boxed region of Fig. 2 with the corresponding part of a regular NOESY spectrum in which ^{13}C decoupling was employed during both evolution and detection periods. Both spectra were recorded and processed under identical conditions, with the exception of the total number of scans and the delay times between scans. Spectra result from $2 \times 128 \times 1024$ data matrices, zero-filled in both dimensions to yield a 1024×1024 matrix for the absorptive part of the final spectra. The acquisition times in the t_1 and t_2 dimensions were 25.6 and 102.4 ms, respectively. In the t_1 dimension, 20 Hz exponential line narrowing followed by 40 Hz Gaussian broadening was used. In the t_2 dimension, 15 Hz exponential line narrowing followed by 20 Hz Gaussian broadening was used. In the regular NOE spectrum, 32 scans were recorded per t_1 value, with a delay time between scans of 2.6 s, resulting in a total measuring time of 3.5 h. In the ^{13}C-edited NOE spectrum, 192 scans were recorded per t_1 value, with a 1.3 s delay between scans and a total measuring time of 12 h. The ^{13}C 90° pulse width was 150 μs (5 W rf power), and the ^{13}C carrier was positioned in the center of the Ala-C_β region, 18 ppm downfield from TSP.

Figure 3 demonstrates the degree of spectral simplification obtainable with the ^{13}C

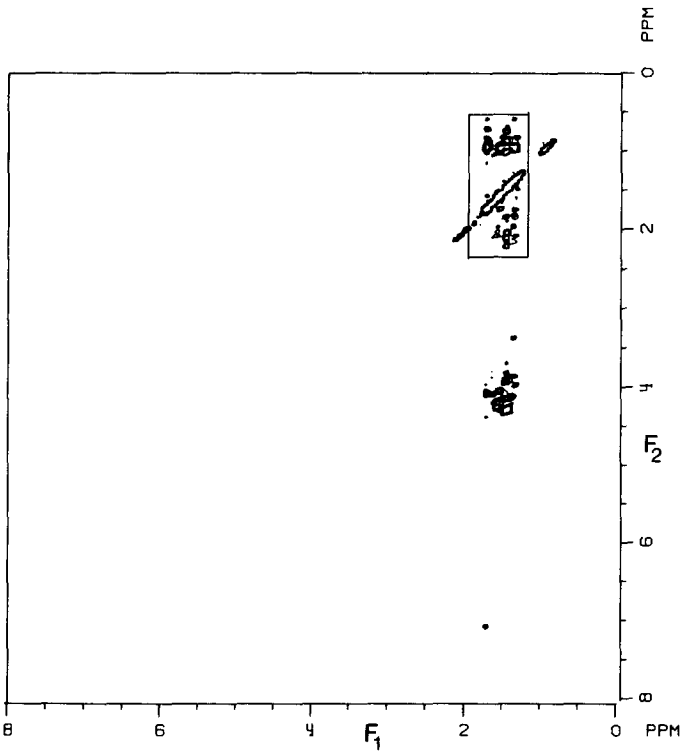

FIG. 2. ^{13}C-edited 2D NOE spectrum of the Ala-$^{13}C_\beta$-labeled λ-repressor, recorded at 25°C, using a 350 ms mixing time. Only protons that have an NOE interaction (direct or via spin diffusion) with the ^{13}C-labeled alanine methyl protons give cross peaks in this type of spectrum. The protein was dissolved in a buffer containing 100 m*M* KCl, 10 m*M* $K_2HPO_4 \cdot KOH$ (pH 11.2) and 0.1 m*M* EDTA.

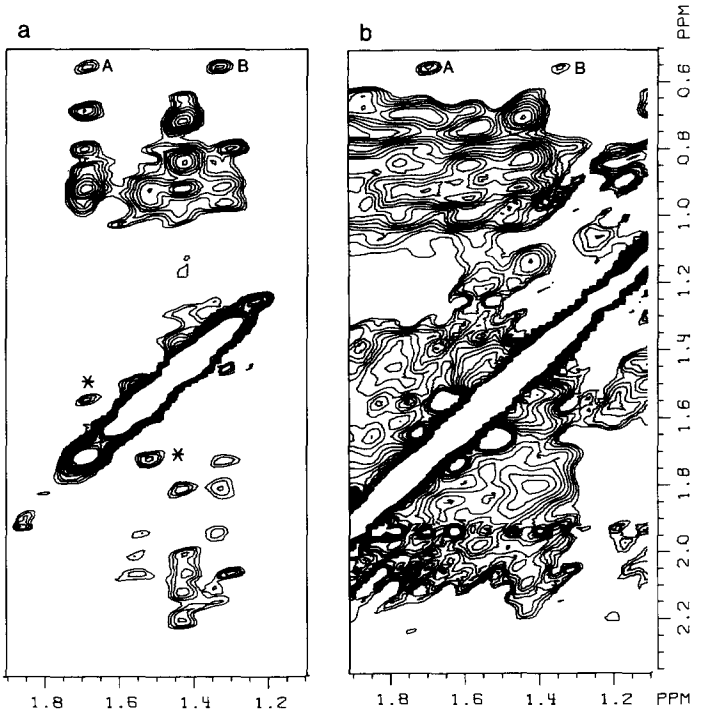

FIG. 3. Comparison of (a) an expanded region of the spectrum of Fig. 2 with (b) the corresponding region of a regular NOE spectrum recorded under similar conditions. Cross peaks due to a unique alanine–alanine interaction are labeled with asterisks. On the basis of the crystal structure the alanines involved are Ala-66 (downfield) and Ala-63 (upfield).

labeling approach. Whereas the regular NOE spectrum yields only a few resolved resonances in the displayed region, the ^{13}C-edited spectrum provides a wealth of resolved cross peaks. The ^{13}C-edited spectrum is asymmetric about the diagonal because only ^{13}C-attached protons are present in the F_1 dimension of the spectrum. Only interactions between ^{13}C-labeled sites give rise to cross peaks that are in symmetric positions with respect to the diagonal. An example is the NOE interaction indicated by asterisks in Fig. 3a between the methyl protons of a previously unassigned alanine and Ala-66 (*11*). Comparison with the X-ray crystal structure of the N-terminal domain of λ-repressor (*12*) assigns the first alanine to Ala-63. Additional assignments and implications regarding repressor structure will be presented elsewhere. The differences in the relative intensities of the two correlations marked A and B at the top of Fig. 3a and 3b are largely the result of a small baseline distortion in Fig. 3b, caused by the nearby presence of the forest of intense NOE cross peaks.

Incorporation of ^{13}C- and ^{15}N-labeled amino acids in proteins for which the gene has been cloned is relatively straightforward. Recording of an edited NOE spectrum, as described in this communication, then provides a powerful method for studying interproton distances in such molecules, expanding the applicability of the NOE experiment to proteins much larger than those studied to date.

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