Constant-time NOESY: An aid in the analysis of protein NMR spectra

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SUMMARY

A constant-time version of the homonuclear NOESY experiment (CT-NOESY) is described. The experiment yields simplified protein spectra, in which cross peaks arising from protons with zero or small couplings are differentiated from other cross peaks, thus partially overcoming the problem of signal overlap. In addition, the CT-NOESY spectrum provides information on the magnitude of $^3J_{NH-\alpha}$ and $^3J_{\alpha\beta}$ coupling constants, and is thus useful to determine torsion angle constraints and to perform stereospecific assignments of $C_\beta HH'$ protons in the case of $^3J_{\alpha\beta}$ constants.

The assignment of resonances to individual protons is the crucial step in the determination of the three-dimensional structure of proteins in aqueous solution. In particular, cross peaks in NOESY spectra must be unambiguously assigned before they can be translated into upper-limit distance constraints during calculation of the structure. When using exclusively ^1H homonuclear information, signal overlap is one of the main problems found in the assignment of resonances and NOE cross-correlations. Thus, methods which could alleviate the overlap problem by introducing some kind of partitioning are always considered beneficial. Here, we concentrated on designing an experiment able to provide an edition of cross-correlations of singlets (methyl groups in Met residues) and resonances with small coupling constants ($-NH_2$ in side chains of Asn and Gln) in the NOESY spectra. The identification of NOE cross peaks arising from these protons is normally difficult because of severe overlapping in the regions where they resonate, and yet their assignment is important in defining the conformational state of their corresponding side chains. Within the framework of constant time 2D-NMR spectroscopy, we have devised a method which, in addition to providing a prompt and simple identification of NOE cross-correlations arising from protons with zero or small couplings, provides information about Φ-angle constraints through discrimina-
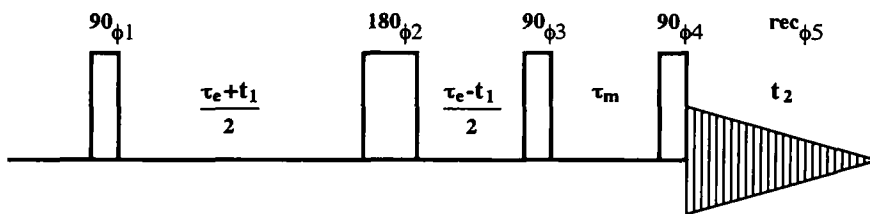
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Fig. 1. Pulse sequence for CT-NOESY experiment. Phase cycling was $\varphi_1 = x$, $-x$; $\varphi_2 = y$; $\varphi_3 = 8(x)$, $8(-x)$; $\varphi_4 = 2(x)$, $2(y)$, $2(-x)$, $2(-y)$; $\varphi_5 = \varphi_1 + \varphi_3 + \varphi_4$. After 16 scans, the phase of the 180° pulse was inverted without changing the receiver phase. Quadrature in F_1 was achieved via a TPPI of φ_1.

tion for $^3J_{\alpha NH}$ coupling values as well as for stereospecific assignment of $C_\beta HH'$ and NH_1H_2 in side chains of, respectively, AMX systems and Asn and Gln residues.

Constant-time 2D NMR spectroscopy was introduced by Bax (Bax et al., 1979; Bax and Freeman, 1981) to collapse the multiplet structure in the F_1-domain of COSY spectra, thus avoiding overlapping of cross-peak multiplets. Later Rance et al. (1984) quantitatively analysed the technique and showed that it could be extended to other types of homonuclear correlation experiments, such as relayed correlation and SECSY. The same principle of constant-time spectroscopy has also been used to reduce signal losses due to relaxation during the evolution time in the COLOC sequence and, more recently, to optimize magnetization transfer in several 3D experiments (Ikura et al., 1991; Powers et al., 1991; Grzesiek et al., 1992) and to collapse carbon–carbon couplings in Overbodenhausen experiments (Santoro and King, 1992; van de Ven and Philippens, 1992; Vuister and Bax, 1992).

In spite of the advantages of constant-time experiments, they are not free of pitfalls. In particular, the amplitude of the cross peaks depends on the number and magnitude of the coupling constants, so that for proton spectra it is difficult to obtain correlation spectra with an appreciable intensity for all cross peaks. Moreover, because of relaxation during the fixed interval, constant-time experiments are usually less sensitive than regular experiments. These undesirable aspects have limited the use of homonuclear constant-time spectroscopy to very particular cases. Notably, the constant-time NOESY experiment, although suggested in a figure caption in the work of Rance et al. (1984), has been, as far as we know, neither analysed theoretically nor achieved experimentally. In this report, we show that this experiment simplifies the NOESY spectra of proteins, thereby providing information for the assignment process.

The pulse scheme for the constant-time NOESY experiment (CT-NOESY) is displayed in Fig. 1 where the evolution time, t_1, has been replaced, as usual, by a fixed interval, τ_e. A 180° pulse is applied in this interval, in such a way that the evolution under the chemical shift is effective only in the t_1 period, while coupling constants remain effective during the whole constant period, τ_e. Hence the longitudinal magnetization at the beginning of the mixing period is

$$-I_z \cdot \cos \omega_I t_1 \cdot \prod \cos \pi J_{IS} \tau_e$$

were S extends to all ^1H nuclei coupled with I. Thus the coupling constant terms do not lead to a multiplet structure in F_1, but rather to a modulation of the cross-peak amplitude that depends both on the choice of τ_e and on the number and magnitude of the coupling constants of the spin
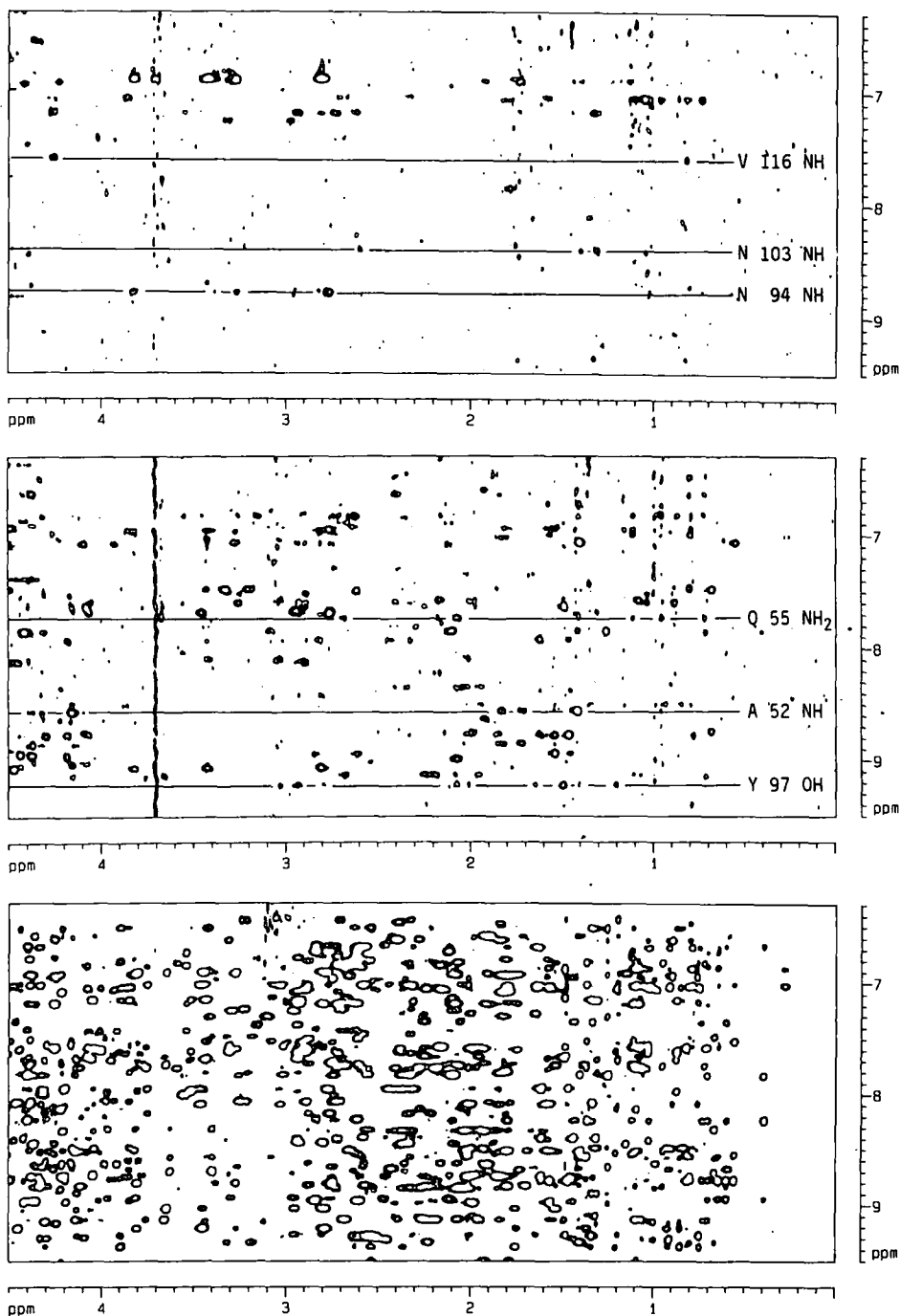
Fig. 2. Selected NH region of CT-NOESY (top and middle) of RNase A (4 mM, 35 °C, pH 4.0, τ_m = 150 ms). Negative cross peaks corresponding to protons with coupling constants greater than 7 Hz (i.e., NH of Asn[94], NH of Asn[103], NH of Val[116] are shown in the top, and positive cross peaks corresponding to protons with coupling constants less than 7 Hz (i.e., OH of Tyr[97], NH of Ala[52], NH₂ of Gln[55]) are plotted in the middle. A reference regular NOESY spectrum recorded with the same experimental conditions is shown for comparison on the bottom.

active during the evolution period. This variable amplitude response can be exploited, as indicated later (1) to simplify the final NOESY spectrum by attenuating some signals below the detection limit (those for which $\tau_e \approx 1/2J$), and (2) to obtain information about the magnitude of the coupling constants.

NOESY spectra of bovine pancreatic ribonuclease A (RNase A) have been intensively studied by our group and others, and a complete assignment of the corresponding ^{1}H NMR spectrum by sequence-specific methods has been achieved (Rico et al., 1989, 1992; Robertson et al., 1989). Because of overlap problems, some NOE interproton cross-correlations as well as stereospecific assignments still needed to be done, so we thought that this protein would be suitable for testing the CT-NOESY pulse sequence. RNase A (Type XIIa) was obtained from Sigma Biochemical Corporation and was used without purification. NMR spectra were obtained for a 4 mM solution of protein in 90% H_2O–10% D_2O, 0.2 M NaCl, pH 4.0 at 35 °C. Spectra were recorded on a Bruker AMX-600 spectrometer with 512 t_1 values, 2048 data points in t_2 and 96 scans per increment. The NOESY mixing time, τ_m, was 150 ms, and τ_e was set to 71.4 ms, corresponding to $\tau_e = 1/2J$ for J = 7 Hz. Phase-sensitive spectra were obtained by increasing the phase of the first 90° pulse in the sequence according to the TPPI method (Marion and Wüthrich, 1983). Before Fourier transformation, the data matrix was multiplied with a phase-shifted ($\pi/3$) square sine-bell function in t_1 and t_2. The spectrum obtained with the CT-NOESY scheme showed the characteristics expected from the theory: (1) it was highly asymmetrical (the intensity of the A→B transfer depends on the coupling constants of spin A, while that of the transfer B→A depends on the couplings of B), and (2) it showed positive and negative cross peaks, and a lower sensitivity than the regular experiment, because the T_2 relaxation during the time τ_e attenuates the signals by $\exp(-\tau_e/T_2)$. Although this loss of intensity is partially compensated for by the collapse of the multiplet structure in the F_1 dimension, the signal-to-noise ratio of the constant-time experiment was approximately 20% of that of a regular NOESY experiment. This result was expected, given the size of RNase A (124 residues). However, the resulting sensitivity was still adequate to provide a useful spectrum. Analysis of the CT-NOESY spectrum showed four zones of interest as a result of its simplification: the amide region, the NH side-chain region, the ββ′ region of AMX spin systems, and the methyl region.

Figure 2 shows the spectral region of the CT-NOESY corresponding to negative and positive NH correlations along the F_1 dimension. A reference regular NOESY spectrum is plotted for comparison. Forty-five amide resonances from a total of 119 were observed as positive cross-correlations. These corresponded to amino acids in which the coupling constant $^3J_{NH-H\alpha}$ was less than 7 Hz. NOESY cross-correlations from OH protons (Tyr[97] and Ser[75]) also appeared as positive signals. Negative cross peaks corresponding to 24 NH protons with $^3J_{NH-H\alpha}$ greater than 7 Hz were also observed. Thus, for a well-structured protein like RNase A, with similar amounts of α-helix and β-sheet structure, the CT-NOESY technique provides an alternative way of partitioning the spectrum, complementary to the conventionally used technique of running spectra in D_2O. Furthermore, it has the following advantages: (1) a freshly prepared sample is not required for each experiment, (2) the results are less sensitive to experimental conditions (pH and temperature), and (3) more NH cross-correlations are observed in a CT-NOESY experiment in H_2O (69) than in a conventional NOESY experiment in D_2O (43).

One of the major advantages of the CT-NOESY experiment is that it provides isolated NH side-chain resonances as a result of attenuation of the aromatic signals. RNase A has 9 Asn and
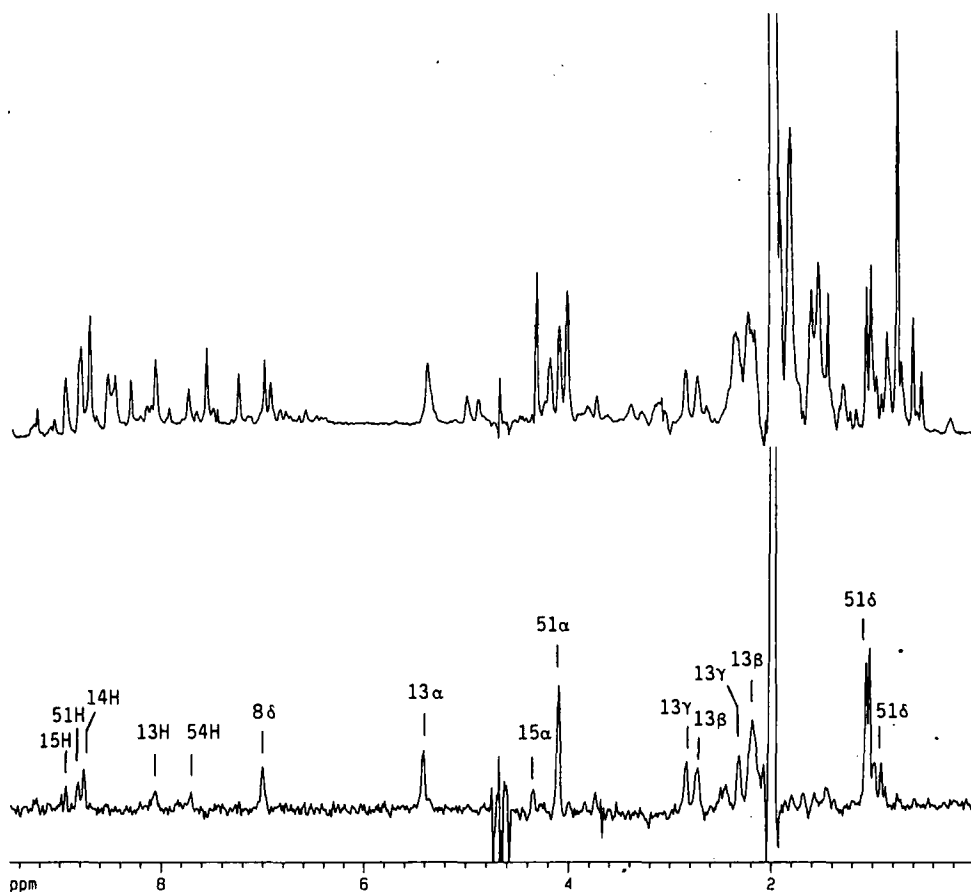
Fig. 3. Comparison of row cross-sections at 2.03 ppm of CT-NOESY (bottom) and regular NOESY (top) of RNase A (4 mM, 35 °C, pH 4.0, τ_m = 150 ms). NOEs.belonging to methyl protons of Met[13] are labelled in the CT-NOESY cross-section.

7 Gln residues in its sequence. NOESY correlations for most of them were detected in the F_1 dimension. The assignment of these NOE cross-correlations is important in deciding whether these side chains are freely rotating or locked into a distinct spatial arrangement. The characterization of the preferred side-chain conformation in the latter case can be improved by the stereospecific assignment of protons NH_1 and NH_2. This assignment can be performed on the basis of individual NH_1 or NH_2 NOE intensities to their own $C_\beta HH'$ or $C_\gamma HH'$ protons, since the ones for H_2 protons (*anti* to the CO bond) are expected to be largest with independence of the χ_2 (Asn) or χ_3 (Gln) torsion angle value. This kind of stereospecific assignment is difficult to perform from a conventional NOESY spectrum because of overlap of the weak NH_2 side chain cross peaks with strong NOE cross-correlations from aromatic protons. The CT-NOESY sequence avoids this problem, thus aiding the identification of NOEs from NH_1H_2 of Asn or Gln residues and the evaluation of their corresponding relative intensities.

Another region of interest is the one corresponding to $C_\beta HH'$ protons. With the τ_c value used in our experiment, the signal corresponding to $C_\beta HH'$ protons of long side-chain amino acids did

not appear in the spectrum because of their high number of coupling constants ($J_{\alpha\beta}$, $J_{\beta\beta}$, $J_{\beta\gamma}$), which strongly attenuated their intensity. Only signals arising from $C_{\beta}HH'$ protons of AMX spin systems showed appreciable intensity, thus greatly simplifying this spectral region and providing a cross-check of the classification of spin systems obtained from the analysis of COSY and TOCSY experiments. Furthermore, NOE correlations from these protons showed different signs depending on the magnitude of the $^3J_{\alpha\beta}$ coupling constant. So, β protons with a large $^3J_{\alpha\beta}$ ($^3J_{\alpha\beta} > 7$ Hz) appeared as positive signals, and β protons with a smaller coupling ($^3J_{\alpha\beta} < 7$ Hz) as negative signals. This discriminative information, when used together with the evaluated intensity of intra-residue and sequential NOEs from the NH and α protons to the β protons, makes it possible to assign the β protons stereospecifically and to impose χ_1 torsion angle constraints.

The region located between 0 and 2.5 ppm also appeared to be simplified. Although only NOEs from methyl groups of methionines were expected in this region, NOEs arising from methyl-group protons of Ala, Thr and Val were also detected, due to incomplete elimination of their strong signals. It should be noted that clean and complete NOE patterns from methyl groups in methionines were obtained, which are of great use in confirming the sequence-specific assignments as well as in delineating information for the entire side-chain. As an illustration, Fig. 3, shows cross-sections along the F2 dimension at the F1 shift of 2.03 ppm (Met[13], methyl group) obtained with the CT-NOESY sequence and with the conventional one. In the conventional spectrum, in addition to cross peaks corresponding to the Met[13] methyl group, cross peaks of other protons resonating approximately at the same frequency were also visible. It is to be noted that these additional peaks were absent from the CT-NOESY spectrum.

In summary, the CT-NOESY experiment can be used as an aid in the analysis of the ^1H NMR spectra of proteins. Although the sensitivity of the experiment is rather low, it functions well for proteins as large as RNase (124 residues), and probably can be applied to slightly larger proteins. Many of the spectral regions are simplified, allowing an easier assignment of several kinds of NOE cross peaks, mainly those arising from methyl groups in Met residues and NH_2 groups in Asn and Gln. In addition, the CT-NOESY experiment allows a qualitative estimation of the values of the $^3J_{NH-\alpha}$ and $^3J_{\alpha\beta}$ coupling constants, which are normally difficult to measure for large proteins in E-COSY (Griesinger et al., 1985) and P.E.-COSY (Müller, 1987) experiments. Although alternative methods exist for the measurement and evaluation of these couplings (Kay and Bax, 1990; Clore and Gronenburg, 1991), they require the use of ^{15}N-labelled proteins, which are not always available.

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