JOURNAL OF MAGNETIC RESONANCE 87, 183–188 (1990)

Conformation-Independent Sequential NMR Connections in Isotope-Enriched Polypeptides by ^{1}H–^{13}C–^{15}N Triple-Resonance Experiments*

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Received November 28, 1989

Sequence-specific resonance assignments provide the basis for the structural interpretation of polypeptide and protein NMR data. A key step in making these assignments involves identifying connections between spin systems of sequential amino acid residues. So far, this has mainly been achieved using conformation-dependent nuclear Overhauser effects (*1–5*). Recently, alternative methods of establishing the sequential order of spin systems have been developed including long-range heteronuclear correlation between backbone protons and the carbon carbonyl or amide nitrogen of the peptide bond (*6–9*), and direct correlation of ^{15}N amide and ^{13}C carbonyl chemical shifts of peptide bonds (*10, 11*). The former technique relies on observation of antiphase cross peaks where the active couplings depend on the dihedral angles ϕ and ψ, respectively. The latter method requires isotope enrichment with both ^{15}N and ^{13}C. In this Communication we describe a family of new ^{1}H–^{13}C–^{15}N triple-resonance experiments which provide sequential connectivity information in polypeptides enriched with either ^{15}N or ^{13}C. These relayed coherence transfer experiments use only one- and two-bond heteronuclear couplings, all of which are greater than 7 Hz and are essentially independent of the polypeptide backbone conformation. All cross peaks are in-phase and therefore do not suffer from intrapeak cancellation. Aside from differential relaxation, all sequential cross peaks are equally intense. Since there are only intraresidue and sequential cross peaks in these spectra, they are simpler and more amenable to automated analysis than NOESY or ROESY spectra. They also provide $^{13}C^{\alpha}$ and ^{15}N backbone amide assignments in the course of determining sequence-specific ^{1}H assignments.

The pulse sequences we have developed are shown in Fig. 1. The first sequence (Fig. 1A) we call a 2D H^{α}–$C^{\alpha}(\omega_1)$–N–$H^{N}(\omega_2)$ heteronuclear relayed coherence transfer experiment (HETERO-RELAY). It begins with transfer of α-proton polarization to directly bound ^{13}C nuclei using refocused INEPT (*12–15*), tuned for a one-bond ^{1}H–^{13}C coupling constant of 140 Hz. During t_1, this ^{13}C in-phase magnetization

* An account of this work was also presented at the Experimental Nuclear Magnetic Resonance Spectroscopy Conference, Pacific Grove, California, on April 5, 1989.

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0022-2364/90 $3.00

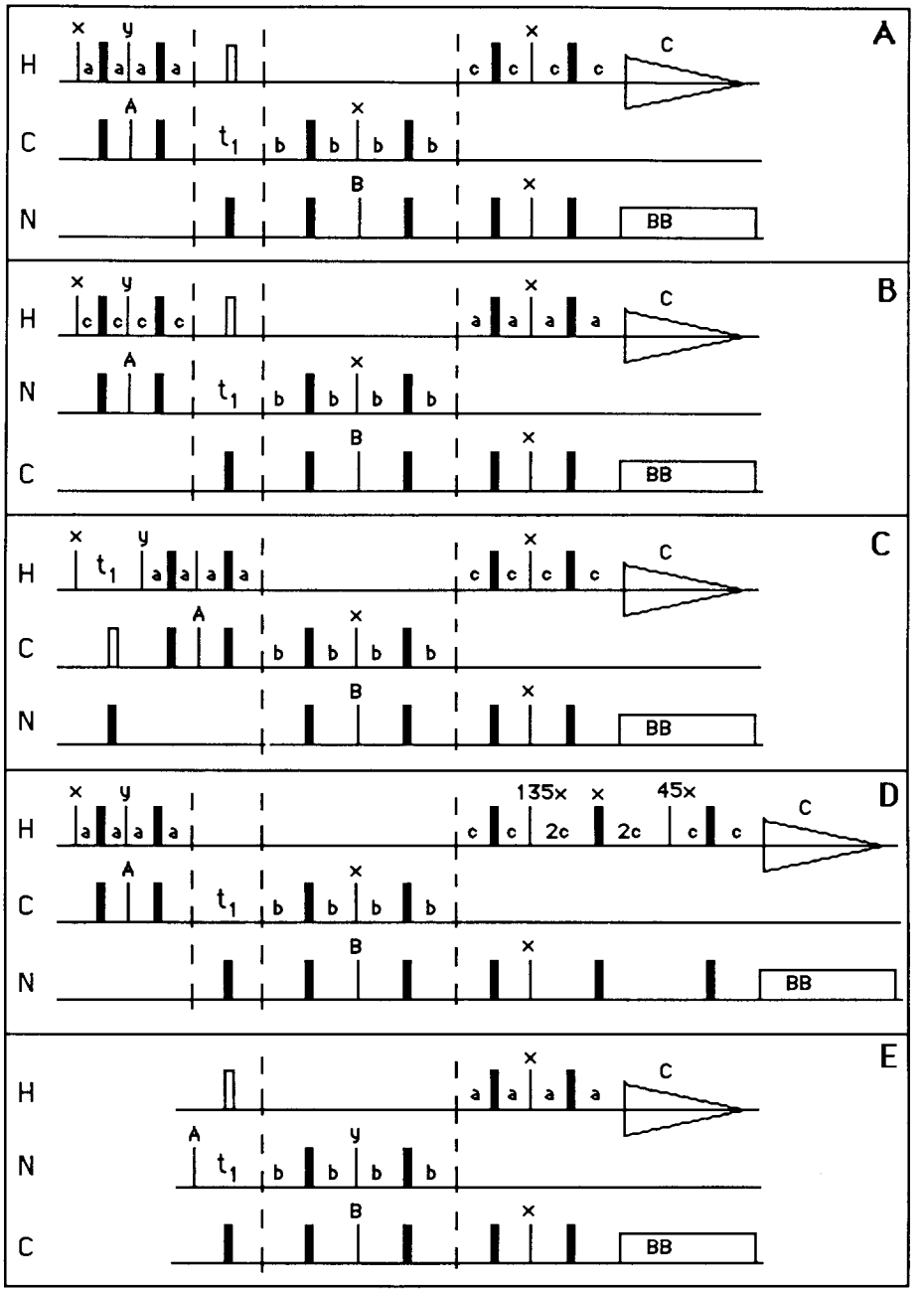

FIG. 1. Pulse sequences of triple-resonance heteronuclear ^{1}H–^{13}C–^{15}N RELAY experiments. H^{α}–$C^{\alpha}(\omega_1)$–N–$H^{N}(\omega_2)$ (A), H^{N}–$N(\omega_1)$–C^{α}–$H^{\alpha}(\omega_2)$ (B), $H^{\alpha}(\omega_1)$–C^{α}–N–$H^{N}(\omega_2)$ (C), H^{α}–$C^{\alpha}(\omega_1)$–N–selective–$H^{N}(\omega_2)$ (D), and $N(\omega_1)$–C^{α}–$H^{\alpha}(\omega_2)$ HETERO-RELAY experiments for determining sequential connections in polypeptides. The narrow bars represent 90° pulses, except for the sequence D where we

evolves, decoupled from ^1H and ^{15}N nuclei. Next, this α-carbon magnetization is transferred to ^{15}N using a second refocused INEPT-type transfer tuned for a ^{13}C–^{15}N coupling constant of 10 Hz. This step provides simultaneous polarization transfer from the C^α atoms of residue i and $i+1$ to the amide nitrogen of residue $i+1$ through the direct (approximately 11 Hz) and two-bond (approximately 7 Hz) $^{13}C^\alpha$–^{15}N coupling constants. This magnetization is then transferred to the directly bound amide proton by a reverse refocused INEPT sequence and detected with broadband decoupling of ^{15}N. For a simple dipeptide system, the resulting spectrum has sequential and intraresidue peaks which are centered at

$$[\omega_1, \omega_2] = [\omega(C_i^\alpha), \omega(H_{i+1}^N)]$$
$$[\omega_1, \omega_2] = [\omega(C_{i+1}^\alpha), \omega(H_{i+1}^N)].$$

An example of this is shown for the terminally blocked tripeptide Ac-Asn–Pro–[^{15}N]Tyr-NHMe (*16–18*) in Fig. 2A. The same experiment carried out on a uniformly ^{15}N-enriched polypeptide would result in a network of $C_i^\alpha \rightarrow H_{i+1}^N \rightarrow C_{i+1}^\alpha \rightarrow H_{i+2}^N \rightarrow C_{i+2}^\alpha \rightarrow$ etc. cross peaks which provide sequential connectivity information. These conformation-independent data, combined with an identification of the spin systems associated with each C^α and H^N resonance by standard homonuclear and heteronuclear 2D NMR experiments, can provide sequence-specific assignments in polypeptides.

Figures 1B–1E are variations of the experiment described above. The sequence of Fig. 1B has the ^{15}N and ^{13}C channels interchanged. It is an H^N–$N(\omega_1)$–C^α–$H^\alpha(\omega_2)$ RELAY experiment. Figure 2B shows the experimental spectrum obtained with the same tripeptide as discussed above. It has sequential and intraresidue cross peaks at

$$[\omega_1, \omega_2] = [\omega(N_{i+1}), \omega(H_i^\alpha)]$$
$$[\omega_1, \omega_2] = [\omega(N_{i+1}), \omega(H_{i+1}^\alpha)].$$

Figure 1C has proton frequency labeling in both the ω_1 and the ω_2 dimensions. It is an $H^\alpha(\omega_1)$–C^α–N–$H^N(\omega_2)$ RELAY experiment. The experimental spectrum is shown in Fig. 2C. The cross peaks are at

$$[\omega_1, \omega_2] = [\omega(H_i^\alpha), \omega(H_{i+1}^N)]$$
$$[\omega_1, \omega_2] = [\omega(H_{i+1}^\alpha), \omega(H_{i+1}^N)].$$

This experiment produces an asymmetric homonuclear spectrum. Exchanging the

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have a 135° pulse and a 45° pulse; the full thick bars are 180° pulses. Open thick bars represent optional 180° pulses which can be used to achieve heteronuclear decoupling during t_1. All experiments shown in Fig. 2 used this decoupling with the exception of the experiment in Fig. 2D. The vertical broken lines indicate boundaries of refocused INEPT periods. BB stands for broadband decoupling which was done with WALTZ-16 (*21*). The delays are tuned in the following way: a = $(4\ ^1J_{H\alpha\text{-}C\alpha})^{-1}$ = 1.8 ms, b = $(4\ ^1J_{N\text{-}C\alpha})^{-1}$ = 25 ms, and c = $(4\ ^1J_{N\text{-}H})^{-1}$ = 2.8 ms. The phase cycles used were A, $x, -x$; B, $x, x, -x, -x$; C, $x, -x, -x, x$. Time-proportional 90° incrementation of phase A provided quadrature detection in ω_1. Water suppression can be performed by preirradiation of the solvent signal.

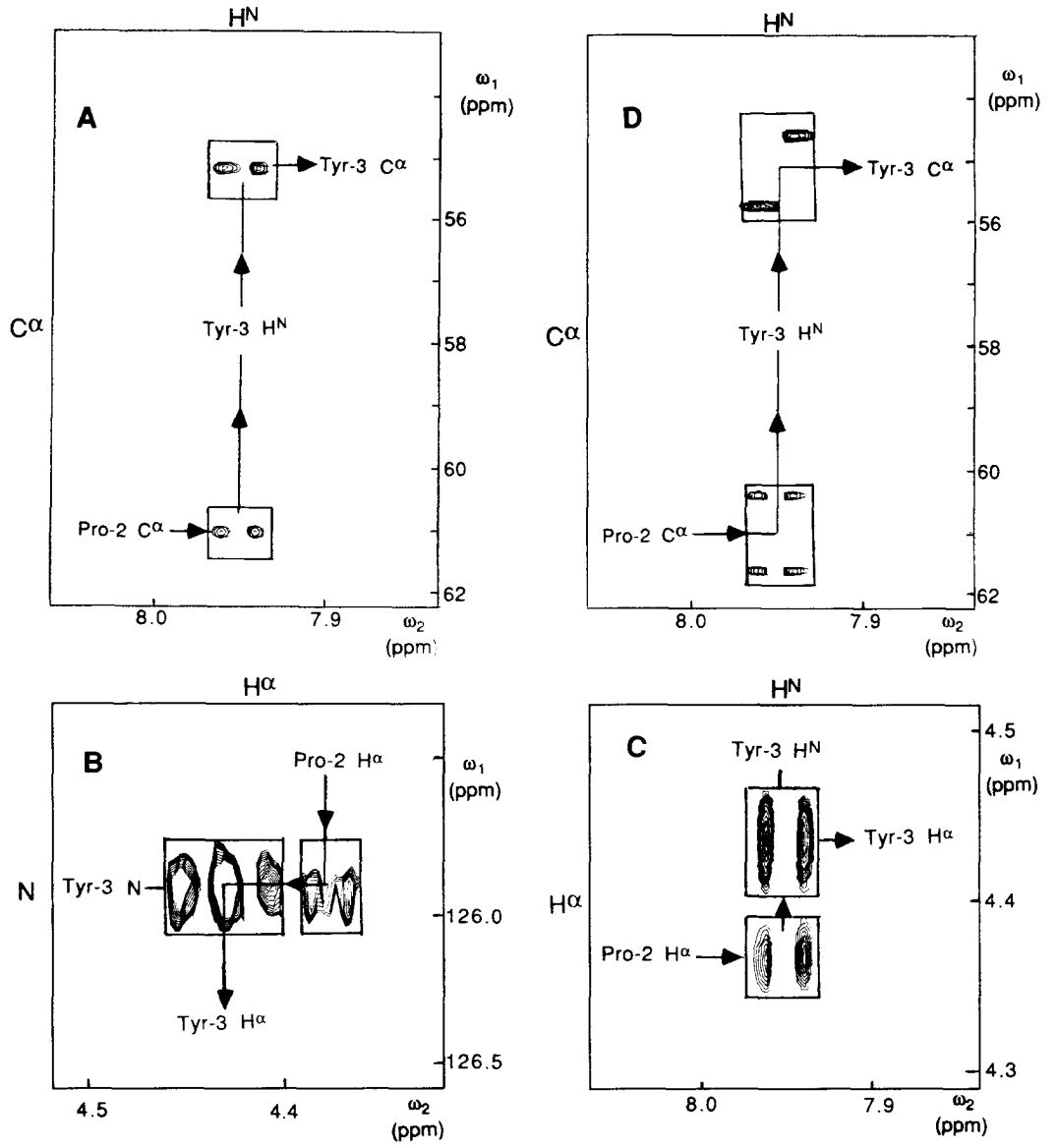

FIG. 2. (A–D) Expanded regions of spectra recorded with Ac-Asn–Pro–(^{15}N)Tyr-NHMe using the pulse sequences from Figs. 1A–1D, respectively. For clarity, the cross peaks are identified with rectangular boxes. All four experiments contain just one intraresidue and one sequential cross peak. The positions of the connected resonances are identified by three-letter abbreviations of the amino acids, the sequence number, and the type of nucleus (N, C^{α}, H^{α}, and H^{N}). All experiments used heteronuclear decoupling during t_1, except for the experiment in (D) where the splitting along ω_1 provides the coupling constant $^3J(H^N, H^{\alpha})$ of the intraresidue cross peak (*19*). The synthesis and NMR assignments for this peptide have been described previously (*16–18*). The sample was prepared at 30 m*M* concentration in d_6-dimethyl sulfoxide (Cambridge Isotopes) and the data were recorded on a General Electric GN-500 spectrometer using a custom-built triple-resonance probe and a modified transceiver board (details available on request).

^{15}N and the ^{13}C channels results in a spectrum (not shown) which is the mirror image with respect to the $\omega_1 = \omega_2$ diagonal compared to the spectrum of Fig. 2C.

Simple modification of these triple-resonance experiments provide an approach for accurate measurements of coupling constants (*19*). For example, in Fig. 1D is an H^{α}–$C^{\alpha}(\omega_1)$–N–selective–$H^{N}(\omega_2)$ 2D HETERO-RELAY pulse sequence related to that of Fig. 1A. The resulting experimental spectrum is shown in Fig. 2D. This experiment (*19*) differs from that of Fig. 1A in two respects. First, the ^{13}C–^{1}H coupling during t_1 is not decoupled, resulting in an approximately 140 Hz splitting in the ω_1 dimension with two coherence components arising from the ensembles of ^{13}C nuclei with the H^{α} spins up and down, respectively. Second, the 90° ^{1}H pulse of the reverse refocused INEPT pulse sequence is replaced with a TANGO (*20*) sequence, which is a selective 90° pulse for protons bound to ^{15}N. Because H^{α} nuclei do not experience a mixing pulse, this sequence transfers carbon magnetization to H^{N} without mixing the two coherence components which have evolved during t_1. For intraresidue cross peaks, the ensemble of molecules for which the C^{α} nucleus was coupled to an H^{α} spin up during t_1 also has the H^{α} spin up after transfer to H^{N} during detection. The same argument holds for the ensemble of molecules with the H^{α} spin down. As can be seen in Fig. 2D, this results in only two components of the intraresidue cross peak, corresponding to the sets of molecules with the H^{α} spin either up or down in both t_1 and t_2. This E.COSY-like pattern provides accurate measurements of the $^{3}J(H^{N}$–$H^{\alpha})$ vicinal coupling constant as we have discussed previously (*19*). For sequential cross peaks, all four components are observed (Fig. 2D), because there is no correlation between the spin states of H_i^{α} and H_{i+1}^{α}. In this way, intraresidue and sequential cross peaks are distinguished by their characteristic fine structure.

The H^{N}–$N(\omega_1)$–C^{α}–$H^{\alpha}(\omega_2)$ RELAY experiment of Fig. 1B has special significance because it provides sequential connectivities to proline residues, and it can also be used to determine sequence-specific assignments in systems for which the amide protons have been exchanged for deuterons, are exchange broadened, or are otherwise absent. For this case we use the modified sequence shown in Fig. 1E which does not include the initial polarization transfer from the proton to the nitrogen. This makes the experiment, in theory, less sensitive by a factor of $\gamma_N/\gamma_H = 0.1$. In practice, the initial polarization transfer of Fig. 1B is not perfect and provides a significantly smaller sensitivity enhancement. Furthermore, in macromolecules, ^{15}N T_1 values are shorter than ^{1}H T_1 values. Thus, the repetition rate can be two to three times faster so that the effective sensitivity of the experiment of Fig. 1E comes near to that of Fig. 1B. Of course, the sequences of Figs. 1A and 1D can be modified similarly by replacing the preparation period with a 90° carbon pulse.

The experiments described here can be expanded to multidimensional techniques, such as 3D $H^{\alpha}(\omega_1)$–$C^{\alpha}(\omega_2)$–N–$H^{N}(\omega_3)$ HETERO-RELAY, 3D $H^{\alpha}(\omega_1)$–C^{α}–$N(\omega_2)$–$H^{N}(\omega_3)$ HETERO-RELAY, or 4D $H^{\alpha}(\omega_1)$–$C^{\alpha}(\omega_2)$–$N(\omega_3)$–$H^{N}(\omega_4)$ HETERO-RELAY. Many of these experiments are complementary; thus, overlapping connectivities in H^{α}–$C^{\alpha}(\omega_1)$–N–$H^{N}(\omega_2)$ 2D HETERO-RELAY may be resolved in H^{N}–$N(\omega_1)$–C^{α}–$H^{\alpha}(\omega_2)$ experiments. In all of these experiments, the total time needed for the three refocused INEPT periods (approximately 125 ms) is comparable to typical mixing times for NOESY or ROESY spectra used for determining sequential connectivities in polypeptides. However, applications to macromolecules require

a proper tuning of the delays taking account of the different T_1 and T_2 values of ^1H, ^{13}C, and ^{15}N. These applications are still under development.

ACKNOWLEDGMENTS

We thank Dr. Y. C. Meinwald and Professor H. A. Scheraga for their generous gift of Ac-Asn-Pro-[^{15}N]Tyr-NHMe. This work was supported by NIH (Grant 1GM38608), by NSF (Grants DMB86-16059 and BBS-8615223), and by the Damon Runyon-Walter Winchell Cancer Research Fund, DRG-920.

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