JOURNAL OF MAGNETIC RESONANCE 97, 213-217 (1992)

A Constant-Time Three-Dimensional Triple-Resonance Pulse Scheme to Correlate Intraresidue $^{1}H^{N}$, ^{15}N, and $^{13}C'$ Chemical Shifts in ^{15}N-^{13}C-Labeled Proteins

ROBERT T. CLUBB,*† V. THANABAL,* AND GERHARD WAGNER,*‡

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115; and †Department of Biological Chemistry, University of Michigan, 1301 Catherine Street, Ann Arbor, Michigan 48109

Received November 12, 1991

Sequence-specific resonance assignments are a prerequisite for structural and dynamical interpretation of protein NMR spectra. For proteins smaller than 10 kDa assignment strategies have relied upon through-bond correlations in homonuclear COSY and TOCSY spectra to identify resonances associated with particular spin systems. Conformation-dependent nuclear Overhauser effects are then employed to sequentially connect these spin systems (*1–5*). In larger proteins, however, extensive resonance overlap and decreased sensitivity of experiments utilizing ^{1}H-^{1}H scalar couplings have hindered this approach.

^{1}H-^{13}C-^{15}N triple-resonance experiments provide a conformation-independent approach for the assignment of backbone resonances in ^{13}C-^{15}N-labeled large proteins (*6–13*). In addition these experiments allow accurate measurement of coupling constants in proteins with large linewidths (*14, 15*). These experiments exploit large heteronuclear one-bond couplings to transfer magnetization with the sensitivity of indirect detection. As demonstrated in calmodulin (*16, 17*) backbone assignments utilize four triple-resonance experiments [HNCa, HNCO, HCaCO, and HCA(CO)N] and the 3D TOCSY-HMQC experiment (*18*). A fifth triple-resonance experiment [H(CA)NHN] is a useful complement to the TOCSY-HMQC experiment, correlating $^{1}H^{N}$, ^{15}N, and $^{1}H^{\alpha}$ resonances (*7, 9, 13*). Furthermore, a sixth experiment, the HN(CO)CA, has been introduced, providing additional sequential information (*11*). Of the experiments listed above, those which detect amide protons [HNCA, HNCO, H(CA)NHN, HN(CO)Ca] need to be collected in H_2O, while those with detection of α protons [HCACO and HCA(CO)N] are best performed in $^{2}H_2O$. This causes difficulties if assignments are based on alignment of C′ chemical shifts, because these resonances may show significant isotope shifts, depending on whether the carbonyl is hydrogen bonded to a proton or a deuteron. It is therefore desirable to perform the majority of experiments in the same solvent (H_2O), restricting oneself to those experiments which detect amide protons [HNCA, HNCO, HN(CO)CA, H(CA)NHN].

‡ To whom correspondence should be addressed.

0022-2364/92 $3.00

We present here a 3D triple-resonance experiment that correlates intraresidue $^1H^N$, ^{15}N, and $^{13}C'$ chemical shifts that we call the HN(CA)CO. This experiment should prove a useful supplement to triple-resonance-based assignment strategies. At present intraresidue carbonyl assignments are obtained solely from the HCACO experiment recorded in ^2H_2O. These correlations in conjunction with those from HNCA, 3D TOCSY–HMQC, and/or H(CA)NHN spectra recorded in H_2O allow the assignment of intraresidue nuclei ($^1H^N$, ^{15}N, $^1H^\alpha$, $^{13}C\alpha$, and $^{13}C'$). These assignments can then be compared with sequential information obtained from the HNCO, HCA(CO)N, and HNCA [HN(CO)CA] experiments to sequentially link groups of nuclei.

Two problems arise during this process that can be overcome by information obtained from the HN(CA)CO experiment. As stated above sequential linkages based on carbonyl chemical shifts are obtained by comparison of two spectra obtained under different solvent conditions, the HNCO in H_2O and the HCACO recorded in ^2H_2O. Carbonyl chemical shifts between these two experiments are therefore influenced by isotope shifts and any pH variations between the two samples. Carbonyl chemical shifts obtained from the HN(CA)CO spectrum are directly comparable to shifts from the HNCO, since both experiments are recorded in H_2O. A second problem overcome by use of the HN(CA)CO experiment results when pairs of $^1H^\alpha$–$^{13}C^\alpha$ resonances are degenerate. $^1H^\alpha$–$^{13}C^\alpha$ chemical-shift degeneracies result in ambiguous assignment of intraresidue ^{15}N and $^{13}C'$ nuclei from HNCA and HCACO spectra, respectively. The HN(CA)CO experiment provides a direct correlation between intraresidue ^{15}N and $^{13}C'$, thereby resolving this ambiguity.

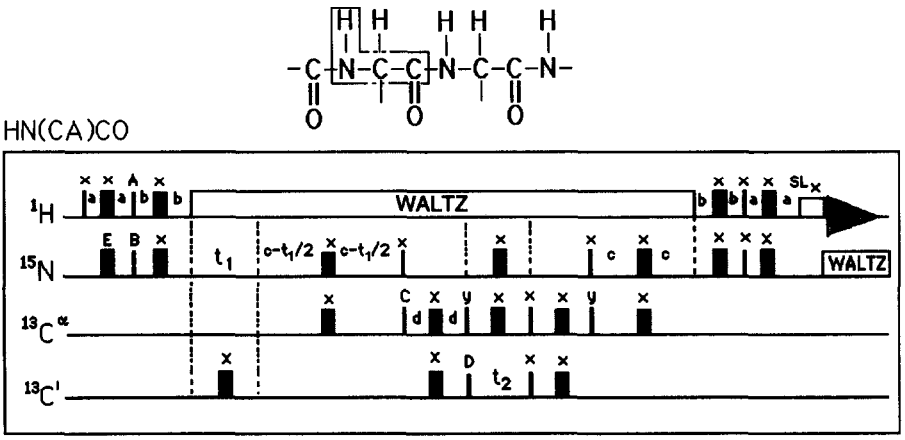

FIG. 1. Pulse sequence for a HN(CA)CO experiment; 90° and 180° pulses are represented by thin and thick vertical bars, respectively. Empty rectangles represent either broadband decoupling or purge pulses as labeled. ^1H and ^{15}N decoupling was achieved with WALTZ-16 broadband decoupling (*20*). Water suppression was achieved with continuous-wave presaturation for 650 ms. A purge pulse (SL_x) of 1.97 ms in duration was also employed to further attenuate the water resonance. The carrier was positioned on the water resonance. The phase cycling scheme employed is as follows: $A = y, -y$; $B = 8(x), 8(-x)$; $C = 2(x), 2(-x)$; $D = 4(x), 4(-x)$; $E = x, -x$ and receiver = $x, 2(-x), x, -x, 2(x), 2(-x), 2(x), -x, x, 2(-x), x$. The phases of B and D were incremented independently by 90° to obtain sign distinction in ω_1 and ω_2, respectively, using the TPPI method (*27*).

The 3D HN(CA)CO pulse scheme is sketched in Fig. 1. The resulting spectrum contains correlations between intraresidue ^{15}N, $^{13}C'$, and $^{1}H^{N}$ resonances in ω_1, ω_2, and ω_3, respectively. The sequence begins with a refocused INEPT (*19*) transfer from $^{1}H^{N}$ to ^{15}N. During the t_1 period ^{15}N magnetization evolves under the influence of ^{15}N chemical shift with $^{13}C'$ and $^{1}H^{N}$ decoupled. $^{13}C'$ decoupling is achieved by a 180° pulse and the protons are decoupled by WALTZ-16 decoupling (*20*). This has the advantage that the nitrogen coherence is kept in-phase with respect to the amide proton, since it has been shown that in-phase nitrogen coherence has a significantly longer T_2 than antiphase coherence (*21–24*). During the t_1 period and the subsequent two $[c-(t_1)/2]$ periods ^{15}N magnetization becomes antiphase with respect to $^{13}C^{\alpha}$. Simultaneous 90° pulses on ^{15}N and $^{13}C^{\alpha}$ channels result in an INEPT-type transfer with $^{13}C^{\alpha}$ magnetization now transverse and antiphase with respect to ^{15}N. The subsequent period $(2d)$ allows active coupling between $^{13}C^{\alpha}$ and $^{13}C'$ while ^{15}N is decoupled. The 90° pulses on $^{13}C^{\alpha}$ and $^{13}C'$ create transverse $^{13}C'$ magnetization that is antiphase with respect to ^{15}N and $^{13}C^{\alpha}$. During t_2 the magnetization is allowed to evolve under the effects of $^{13}C'$ chemical shift with ^{15}N and $^{13}C^{\alpha}$ decoupled by 180° pulses. At the end of the t_2 period 90° pulses on $^{13}C^{\alpha}$ and $^{13}C'$ begin the process of refocusing the magnetization in a manner symmetrical to that already described.

The sequence in Fig. 1 has been optimized for sensitivity. In particular the t_1 period is used to develop coupling between the evolving ^{15}N magnetization and $^{13}C^{\alpha}$. As the t_1 period is incremented the delay labeled c is decremented so that the total time allowed for coupling remains constant. This amounts to time savings of up to 8 ms at $t_{1\,\max}$. In addition "constant-time" evolution in t_1 should facilitate the use of "mirror image" linear prediction, thereby improving resolution (in t_1) and shortening acquisition times (*10, 25*). A refocused INEPT has been employed instead of the shorter concatenated INEPT transfers used in other sequences (*9*), allowing proton broadband decoupling. This avoids oscillation between in-phase and antiphase ^{15}N coherence and reduces the effective transverse ^{15}N relaxation (*21*).

The pulse sequence of Fig. 1 yields intraresidue $^{15}N_i(\omega_1)-^{13}C'_i(\omega_2)-^{1}H^{N}_i(\omega_3)$ cross peaks. However, some sequential $^{15}N_i(\omega_1)-^{13}C'_{i-1}(\omega_2)-^{1}H^{N}_i(\omega_3)$ cross peaks can be observed due to coherence transfer from $^{1}H^{N}_i$ to $^{15}N_i$, $^{13}C_{\alpha i-1}$, $^{13}C'_{i-1}$ and back. Tuning the delay c to maximize the functions

$$\sin[\pi J_{NiC\alpha i-1}(2c)]\cos[\pi J_{NiC\alpha i}(2c)]\exp(-2c/T_{2N}) \quad [1]$$

or

$$\cos[\pi J_{NiC\alpha i-1}(2c)]\sin[\pi J_{NiC\alpha i}(2c)]\exp(-2c/T_{2N}) \quad [2]$$

selects for sequential and intraresidue correlations, respectively. A value of $c = 12.5$ ms was used in our experiments. The delay d should be tuned to maximize the function

$$\sin[\pi J_{C'C\alpha}(2d)]\cos[\pi J_{C\alpha C\beta}(2d)]\exp(-2d/T_{2C\alpha}). \quad [3]$$

A value of 3.5 ms was used in our experiments.

Figure 2 (left) shows a (ω_2, ω_3) slice of the 3D spectrum of T4 lysozyme recorded with the HN(CA)CO pulse sequence in Fig. 1. The slice is taken at the ^{15}N frequency of the amide nitrogen of Asn132. The experiment is most useful when compared with an HNCO spectrum. Figure 2 (right) is a (ω_2, ω_3) slice of the HNCO spectrum of T4

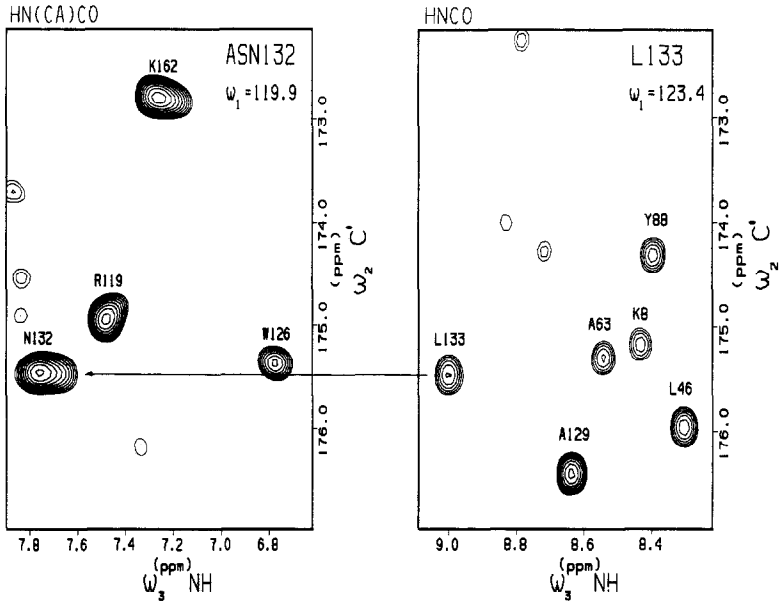

FIG. 2. Sections of (ω_2, ω_3) slices from HN(CA)CO and HNCO 3D spectra of 4.5 m*M* T4 lysozyme recorded on Bruker AMX500 and AMX600 spectrometers, respectively. (Left) A section of the HN(CA)CO spectrum recorded with the sequence in Fig. 1. The delays were tuned to a = 2.3 ms, b = 2.7 ms, c = 12.5 ms, and d = 3.5 ms. The carbon carrier was placed in the center of the C^α spectrum. The carbonyl pulses were generated as DANTE pulses. The left slice is taken at a ^{15}N frequency of 119.9 ppm containing the intraresidue correlations of Asn132. (Right) A section of the HNCO spectrum recorded with the pulse sequence described by Kay *et al.* (*8*) at a ^{15}N frequency of 123.4 ppm containing the sequential correlations of Leu133. Both HN(CA)CO and HNCO 3D spectra were acquired with 16 and 512 complex data points in t_1 and t_3, respectively; 68 and 48 complex data points were acquired in t_2 for the HNCO and HN(CA)CO experiments, respectively. The HN(CA)CO spectrum was acquired in 48 hours. Both spectra were zero-filled to a final size of 512 × 256 × 128 real points in ω_1, ω_2, and ω_3, respectively. A baseline correction was applied in t_3 to remove residual H_2O artifacts in the HN(CA)CO spectrum. Spectra were processed on a Silicon Graphics Iris 4D/35 workstation using the program FELIX (Hare, Inc.).

lysozyme taken at the ^{15}N frequency of Leu133. The sequential peak in the HNCO spectrum (Fig. 2, right) is at a $^{13}C'$ frequency (ω_2) identical to the intraresidue cross peak of Asn132 in Fig. 2 (left; an arrow indicates the linkage). Analysis of the HN(CO)CA spectrum reveals predominantly intraresidue cross peaks ($\omega_1 = {}^{15}N_i$, $\omega_2 = {}^{13}C'_i$, $\omega_3 = {}^{1}H^{N}_i$), with only a few sequential cross peaks ($\omega_1 = {}^{15}N_i$, $\omega_2 = {}^{13}C'_{i-1}$, $\omega_3 = {}^{1}H^{N}_i$) analogous to those in the HNCO experiment.

In theory, a set of HN(CA)CO and HNCO spectra can provide complete sequential assignment of the $^1H^N$, ^{15}N, and $^{13}C'$ resonances. Similarly the set of HNCA and HN(CO)CA experiments can provide complete sequential assignment of $^1H^N$, ^{15}N, and $^{13}C^\alpha$ resonances. In practice, both assignment routes appear to be useful. The HN(CA)CO experiment will also be useful when combined with the assignment strategy used for calmodulin (*16, 17*) to resolve ambiguities caused by $^1H^\alpha$–$^{13}C^\alpha$ pair chemical-shift degeneracies. This type of resonance overlap has recently been pointed

out by Bax and co-workers, who have proposed a 4D sequence that would help to resolve this problem (*26*).

ACKNOWLEDGMENTS

We express our appreciation to Drs. L. P. McIntosh and F. W. Dahlquist for supplying the T4 lysozyme sample. The sample was prepared with support by NSF (Grant DMB-8905322). We thank Dr. Christina Redfield for writing the peak-picking program used in this work. We thank Dr. D. Hare for providing the program FELIX. This research was supported by NIH Grant GM38608.

REFERENCES

1. A. DUBS, G. WAGNER, AND K. WÜTHRICH, *Biochim. Biophys. Acta* **577,** 177 (1979).
2. G. WAGNER, A. KUMAR, AND K. WÜTHRICH, *Eur. J. Biochem.* **114,** 375 (1981).
3. M. BILLETER, W. BRAUN, AND K. WÜTHRICH, *J. Mol. Biol.* **155,** 321 (1982).
4. G. WAGNER AND K. WÜTHRICH, *J. Mol. Biol.* **155,** 347 (1982).
5. K. WÜTHRICH, "NMR of Proteins and Nucleic Acids," Wiley, New York, 1986.
6. G. T. MONTELIONE AND G. WAGNER, *J. Am. Chem. Soc.* **111,** 5474 (1989).
7. G. T. MONTELIONE AND G. WAGNER, *J. Magn. Reson.* **87,** 183 (1990).
8. L. E. KAY, M. IKURA, R. TSCHUDIN, AND A. BAX, *J. Magn. Reson.* **89,** 496 (1990).
9. L. E. KAY, M. IKURA, AND A. BAX, *J. Magn. Reson.* **91,** 84 (1991).
10. R. POWERS, A. M. GRONENBORN, G. M. CLORE, AND A. BAX, *J. Magn. Reson.* **94,** 209 (1991).
11. A. BAX AND M. IKURA, *J. Biol. NMR* **1,** 99 (1991).
12. V. THANABAL, R. T. CLUBB, J. W. PENG, AND G. WAGNER, *J. Magn. Reson.*, submitted.
13. R. T. CLUBB, V. THANABAL, AND G. WAGNER, *J. Biol. NMR*, in press.
14. G. WAGNER, P. SCHMIEDER, AND V. THANABAL, *J. Magn. Reson.* **93,** 436 (1991).
15. P. SCHMIEDER, V. THANABAL, L. P. MCINTOSH, F. W. DAHLQUIST, AND G. WAGNER, *J. Am. Chem. Soc.* **113,** 6323 (1991).
16. M. IKURA, L. E. KAY, AND A. BAX, *Biochemistry* **29,** 4659 (1990).
17. M. IKURA, L. E. KAY, M. KRINKS, AND A. BAX, *Biochemistry* **30,** 5498 (1991).
18. D. MARION, P. C. DRISCOLL, L. E. KAY, P. T. WINGFIELD, A. BAX, A. GRONENBORN, AND G. M. CLORE, *Biochemistry* **28,** 6150 (1989).
19. G. A. MORRIS AND R. FREEMAN, *J. Am. Chem. Soc.* **101,** 760 (1979).
20. A. J. SHAKA, J. KEELER, AND R. FREEMAN, *J. Magn. Reson.* **53,** 313 (1983).
21. J. W. PENG, V. THANABAL, AND G. WAGNER, *J. Magn. Reson.* **95,** 421 (1991).
22. J. W. PENG AND G. WAGNER, *J. Magn. Reson.* **95,** 421 (1991).
23. S. CAMPBELL-BURK, P. DOMAILLI, AND L. MÜLLER, *J. Magn. Reson.* **93,** 171 (1991).
24. A. BAX, M. IKURA, L. E. KAY, D. A. TORCHIA, AND R. TSCHUDIN, *J. Magn. Reson.* **86,** 304 (1990).
25. G. ZHU AND A. BAX, *J. Magn. Reson.* **90,** 405 (1990).
26. L. E. KAY, M. IKURA, G. ZHU, AND A. BAX, *J. Magn. Reson.* **91,** 422 (1991).
27. D. MARION AND K. WÜTHRICH, *Biochem. Biophys. Res. Commun.* **113,** 967 (1983).