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# A triple-resonance pulse scheme for selectively correlating amide $^{1}H^{N}$ and $^{15}N$ nuclei with the $^{1}H^{\alpha}$ proton of the preceding residue

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## SUMMARY

A 3D $^{1}H$-$^{15}N$-$^{13}C$ triple resonance experiment is presented that contains exclusively cross peaks between the $^{1}H^{N}$ and $^{15}N$ nuclei of one residue with the $H^{\alpha}$ of the preceding residue. The pulse sequence, designed to minimize the time coherence, is transverse on nuclei with short $T_2$ values. The experiment consists of coherence transfers via one-bond couplings from the $H^{N}$ via N, CO, $C^{\alpha}$ to the $H^{\alpha}$ and back to the $H^{N}$ for detection; it is called HN(COCA)HA. The experiment was tested on uniformly $^{15}N$- and $^{13}C$-enriched T4 lysozyme.

Sequence-specific resonance assignments are a prerequisite for structural and dynamical interpretation of protein NMR spectra. Early assignment strategies have relied upon through-bond correlations in homonuclear COSY and TOCSY spectra to identify resonances associated with particular spin systems, and on NOESY spectra to sequentially connect these spin systems (Wagner and Wüthrich, 1982; Wüthrich, 1986). 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 (Ikura et al., 1990; Kay et al., 1990; Montelione and Wagner, 1990). In addition, these experiments allow measurement of coupling constants in proteins with large line widths (Montelione and Wagner, 1989; Schmieder

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et al., 1991; Wagner et al., 1991; Grzesiek et al., 1992). Recently we introduced a new triple resonance sequence called the HN(CA)HA which correlates $^1H^N$, $^{15}N$ and $^1H^\alpha$ resonances within a residue via one-bond couplings, as well as correlating $^1H^N$ and $^{15}N$ resonances of one residue with the $^1H^\alpha$ resonance of the preceding residue (Clubb et al., 1992). The latter correlation makes use of the small two-bond coupling constant between $N_i$ and $C^\alpha_{i-1}$ of ca. 7 Hz. The HN(CA)HA was shown to possess higher sensitivity to previous triple resonance sequences that provided similar correlations (Montelione and Wagner, 1990; Kay et al., 1991). In this communication we present a new triple resonance pulse scheme that correlates amide $^1H^N$ and $^{15}N$ nuclei exclusively with the $H^\alpha$ proton of the preceding residue. Only one-bond couplings are used. We call this sequence the HN(COCA)HA. It helps to distinguish intraresidue from sequential cross peaks in the HN(CA)-HA experiment and facilitates resonance assignments.

The 3D HN(COCA)HA pulse scheme is sketched in Fig. 1. The resulting spectrum contains correlations between intraresidue $^{15}N$ and $^1H^N$ resonances along $\omega_1$ and $\omega_3$ respectively, while the $^1H^\alpha$ chemical shift of the preceding residue appears along $\omega_2$. The sequence is essentially an extension of the HN(CO)CA sequence described by Bax and Ikura (1991). The coherence pathway is described below in short notation with frequency labeling indicated in parentheses.

$$H^N \rightarrow N(\omega_1) \rightarrow C' \rightarrow C^\alpha \rightarrow H^\alpha(\omega_2) \rightarrow C^\alpha \rightarrow C' \rightarrow N \rightarrow H^N(\omega_3)$$

The formalism of Sørensen et al. (1983) is used to describe the time evolution of the system; the various points on the time axis are indicated by numbers placed in boxes in Fig. 1. $H^N$, N, $C^\alpha$, C' and $H^\alpha$ are used to denote the spin operators for the respective nuclei. Relaxation effects and terms that do not result in observable magnetization during the acquisition time ($t_3$) are omitted

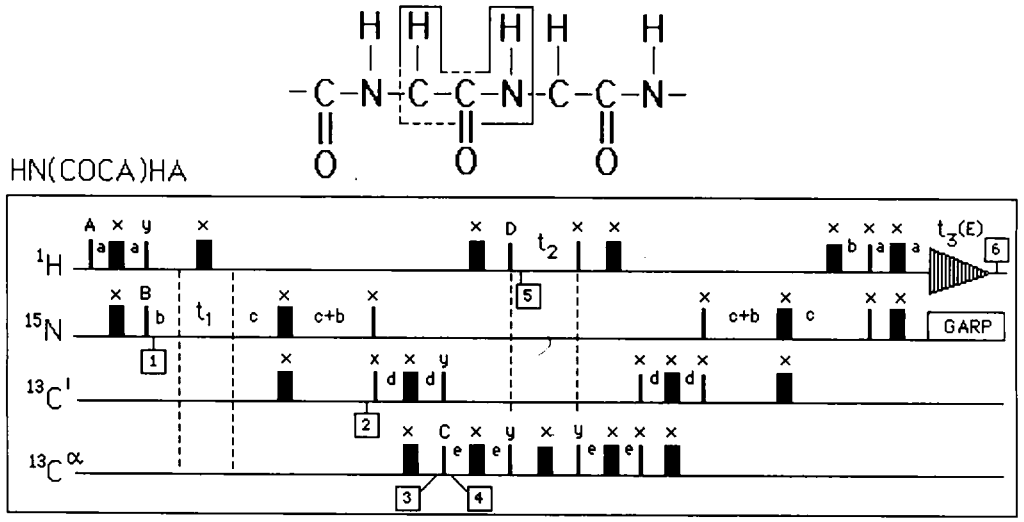


Fig. 1. The HN(COCA)HA pulse sequence. 90° and 180° pulses are represented by thin and thick vertical bars, respectively. GARP broadband decoupling (Shaka et. al., 1985) of the $^{15}N$ nuclei is applied during detection. The phase cycling scheme employed is as follows: A = 8(x), 8(−x); B = 2(x), 2(−x); C = 4(x),4(−x); D = x, −x and the receiver (E) = 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 $\omega_1$ and $\omega_2$, respectively, using the TPPI method (Marion and Wüthrich, 1983).

for clarity. The sequence begins with an INEPT transfer (Morris and Freeman, 1979) yielding $^{15}N$ coherence, in antiphase to the amide proton. The delay, a, is tuned to approximately $1/(4J_{HN\text{-}N})$. At time 1 we have:

$$\sigma_1 = 2H^N{}_z N_y \tag{1}$$

The following period achieves frequency labeling of the $^{15}N$ nucleus, refocusing of the N-H coupling and evolution of the $^{15}N$ coherence in antiphase to the $^{13}C'$ spins of the preceding residue. Ideally, $^{15}N$ should be decoupled from $^{13}C$ during the $t_1$ period. Considering the small values of the $^{15}N^{13}C$ coupling constants and the low resolution required in $t_1$, this was not considered important and was therefore omitted. At point 2 in Fig. 1 the respective terms are:

$$\sigma_2 = -2N_{iy}C'_{i-1z}\cos(\omega_N t_1)\sin(2\pi J_{Ni-C'i-1}(b+c))\sin(2\pi J_{N\text{-}HN}(b)) \tag{2}$$

The one bond coupling constants $J_{N\text{-}HN}$ and $J_{Ni\text{-}C'i\text{-}1}$ are approximately 90 and 15 Hz, respectively (Bystrov, 1976). Values of 2.75 and 8 ms were therefore used for the delays b and c, respectively, to optimize the transfer.

The following 90° pulses applied on $^{15}N$ and $^{13}C'$ create $^{13}C'$ coherence in antiphase to $^{15}N$. The delay, d, is tuned to approximately $1/4J_{C'\text{-}C\alpha}$, and an INEPT-type transfer from $^{13}C'$ to $^{13}C^\alpha$ is performed. In the experiment we used a value of 4 ms for the delay d. At point 3 the resulting coherence is described by:

$$\sigma_3 = -4N_{iz}C'_{i-1x}C^\alpha{}_{i-1z}\cos(\omega_N t_1)\sin(2\pi J_{Ni-C'i-1}(b+c))\sin(2\pi J_{N\text{-}HN}(b))\sin(2\pi J_{C\alpha i-1-C'i-1}(d)) \tag{3}$$

Following point 3 (Fig. 1), simultaneous pulses on $^{13}C'$ and $^{13}C^\alpha$ create $^{13}C^\alpha$ coherence that is in antiphase to the $^{13}C'$ nucleus. Disregarding the size of the transfer function we have at point 4:

$$\sigma_4 = -4N_{iz}C'_{i-1z}C^\alpha{}_{i-1y}(\cos\omega_N t_1) \tag{4}$$

The following delay, e, is tuned to approximately $1/4JH^\alpha C^\alpha$, and a INEPT-type transfer from $C^\alpha$ to $H^\alpha$ is executed, leading to:

$$\sigma_5 = -8N_{iz}C'_{i-1z}C^\alpha{}_{i-1z}H^\alpha{}_y(\cos\omega_N t_1) \tag{5}$$

The coherence is now labeled with the frequency of the α-proton of the preceding $(i-1)$ residue. It is important to note that, if sequential correlations to glycines are desired, a value of approximately $1/8^1J_{HC}$ should be used for the delay e. The remainder of the sequence is a return to the $H^N$ coherence for detection. The final signal detected at point 6 is described by:

$$\sigma_6 = H^N(\cos\omega_N t_1)(\cos\omega_{H\alpha i-1} t_2)(\cos\omega_{HN} t_3) \tag{6}$$

Fig. 2 demonstrates how the HN(COCA)HA experiment can be used in conjunction with the HN(CA)HA experiment to sequentially link adjacent residues. The figure shows sections of $\omega_2$–$\omega_3$ cross planes from both HN(CA)HA (left) and HN(COCA)HA (right) spectra of T4 lysozyme.

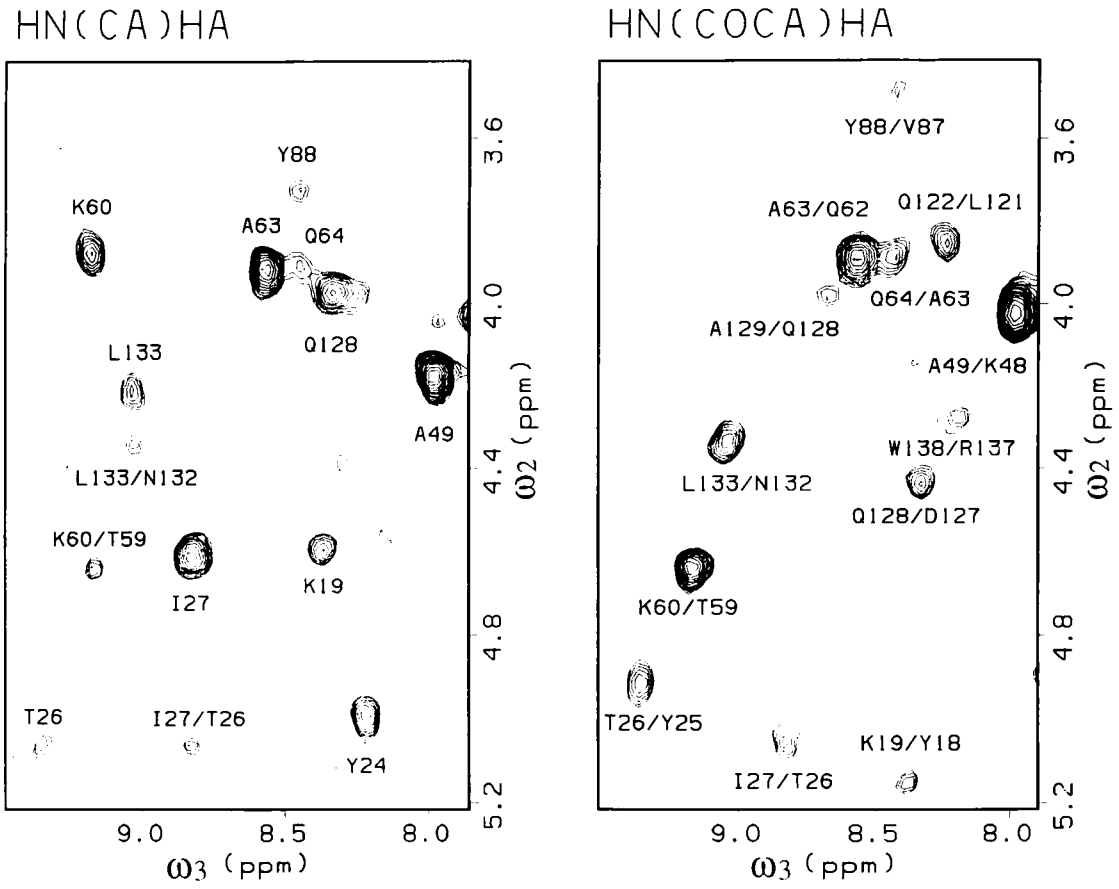


Fig. 2. Shown on the left is a ($\omega_2$, $\omega_3$) cross plane from the HN(CA)HA spectrum of a uniformly $^{15}N$ and $^{13}C$ labeled T4 lysozyme sample. A ($\omega_2$, $\omega_3$) cross plane from the HN(COCA)HA spectrum recorded on the same sample is shown on the right. The T4 lysozyme sample was 4.5 mM in a 95% $H_2O$, 5% $^2H_2O$. All spectra were recorded at 20 °C on a Bruker AMX-600 spectrometer. Cross peaks are identified by sequence number (McIntosh et. al., 1990). The HN(CA)HA spectrum was acquired as described previously (Clubb et al. 1992). The HN(COCA)HA spectrum, was acquired with 16, 56 and 512 complex data points in $t_1$, $t_2$ and $t_3$, respectively. The delays used were: a = 2.25 ms, b = 2.7 ms, c = 8 ms, d = 4 ms and e = 3.5 ms. Water suppression was achieved with continuous wave presaturation for a duration of 710 ms. The carrier was positioned on the water resonance. Linear prediction was used to extend the $t_1$ time domain to 30 complex points in both spectra (Olejniczak and Eaton, 1990). Both data sets were zerofilled to a final size of 128 × 256 × 256 real points in $\omega_1$, $\omega_2$ and $\omega_3$, respectively. A digital filter was used on the HN(COCA)HA data to reduce the residual $H_2O$ signal in the acquisition dimension ($t_3$) (Marion et al., 1989). The total acquisition time for the HN(COCA)HA spectrum was ca. 60 h. All data were processed on a Silicon Graphics 4D/35 workstation using the program FELIX (Hare Inc.).

Both cross planes are taken at $\omega_1$ frequencies corresponding to 122.5 ppm. The HN(CA)HA spectrum (left) contains predominantly intraresidue $H^N$ to $H^\alpha$ correlations for amide nitrogens that resonate at 122.5 ppm. For ca. 30% of the residues in the protein, the HN(CA)HA spectrum also contains sequential correlations ($H^N(i)$ to the $H^\alpha(i-1)$ of the preceding residue, (see K60, L133, A49 and I27 for examples). The HN(COCA)HA cross plane (right) taken at the same $^{15}N$ frequency (122.5 ppm) contains only sequential $H^N(i)$ to $H^\alpha(i-1)$ correlations. These sequential correlations

are more numerous and intense than the identical correlations in the HN(CA)HA spectrum (illustrated by K60/T59 correlation in both spectra). The HN(COCA)HA spectrum therefore serves several roles. (1) It provides complementary information to the HN(CA)HA spectrum, and the cross peaks are more intense than the sequential cross peaks in the HN(CA)HA experiment. (2) It distinguishes inter vs. intraresidue correlations in the HN(CA)HA spectrum. Normally the relative intensity of the inter- and intraresidue correlations in the HN(CA)HA spectrum allows the two to be distinguished. (3) It resolves $H^{\alpha}$ proton degeneracy ambiguities. The case of A63 demonstrates this point. The $H^{\alpha}$ protons of Q62 and A63 are nearly degenerate at 3.9 and 3.91 ppm, respectively. Therefore, both sequential and intraresidue cross peaks arising from A63 in the HN(CA)HA spectrum will be overlapped. This problem is resolved in the HN(COCA)HA spectrum (right) which clearly indicates that the $H^{\alpha}$ chemical shift of Q62 is at 3.9 ppm.

The HN(COCA)HA and HN(CA)HA are extensions of the HN(CO)CA and HNCA experiments, respectively. Each pair of experiments provides sequential information in an analogous manner. The two sets of experiments differ in the nucleus employed for sequential linkage. The HNCA-HN(CO)CA pair uses the $C^{\alpha}$ nucleus while the HN(CA)HA-HN(COCA)HA pair uses the $H^{\alpha}$ nucleus. Since there will be cases of $C^{\alpha}$ overlap but not $H^{\alpha}$ overlap, the HN(COCA)HA experiment in conjunction with the HN(CA)HA experiment should prove to be useful. The sets of experiments described in this communication are best performed in $H_2O$. They therefore avoid the need for transfer of the sample to a deuterated solvent, preventing potential sample loss during solvent exchange.

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## REFERENCES

Bax, A. and Ikura, M., (1991) *J. Biomol. NMR*, **1**, 99–104.
Bystrov, W. (1976) *Progr. NMR Spectr.*, **10**, 41–81.
Clubb, R.T., Thanabal, V. and Wagner, G. (1992) *J. Biomol. NMR*, **2**, 203–210.
Grzesiek, S., Ikura, M., Clore, G.M., Gronenborn, A.M. and Bax, A. (1992) *J. Magn. Reson.*, **96**, 215–221.
Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.
Kay, L.E., Ikura M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.
Kay, L.E., Ikura, M. and Bax, A. (1991) *J. Magn. Reson.*, **91**, 84–92.
Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.
Marion, D., Ikura, M. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 425–430.
McIntosh, L.P., Wand, A.J., Lowry, D.F., Redfield, A.G. and Dahlquist, F.W. (1990)*Biochemistry*, **29**, 6341–6362.
Montelione, G. and Wagner, G. (1989) *J. Am. Chem. Soc.*, **111**, 5474–5475.
Montelione, G. and Wagner, G. (1990) *J. Magn. Reson.*, **87**, 183–188.
Morris, G.A. and Freeman, R. (1979) *J. Am. Chem. Soc.*, **101**, 760–762.
Olejniczak, E.T. and Eaton, H.L. (1990) *J. Magn. Reson.*, **87**, 628–632.
Schmieder, P., Thanabal, V., McIntosh, L.P., Dahlquist, F.W. and Wagner, G. (1991) *J. Am. Chem. Soc.*, **113**, 6323–6324.

Shaka, A.J., Barker, P.B. and Freeman, R.J. (1985) *J. Magn. Reson.*, **64**, 547–552.
Sørensen, O.W., Eick, G.W., Levitt, M.H., Bodenhausen, G. and Ernst, R.R. (1983) *Prog. NMR Spectr.*, **16**, 163–192.
Wagner, G. and Wüthrich, K. (1982) *J. Mol. Biol.*, **155**, 347–366.
Wagner, G., Schmieder, P. and Thanabal, V. (1991) *J. Magn. Reson.*, **93**, 436–440.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York, NY.