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A new 3D HN(CA)HA experiment for obtaining fingerprint H^N-H^α cross peaks in ^{15}N- and ^{13}C-labeled proteins

Robert T. Clubb[a,b], V. Thanabal[a] and Gerhard Wagner[a,*]

[a]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, U.S.A.*

[b]*Department of Biological Chemistry, University of Michigan, 1301 Catherine Street, Ann Arbor, MI 48109, U.S.A.*

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SUMMARY

A new 3D ^1H-^{15}N-^{13}C triple resonance experiment is presented that provides in-phase absorptive cross peaks between amide protons and α-protons of the same and the sequentially preceding residue. The experiment yields similar connectivities as those described previously by Montelione and Wagner (1990a) [*J. Magn. Reson.*, **87**, 183–188] and Kay et al. (1991) [*J. Magn. Reson.*, **91**, 84–92]. However, the pulse sequence was designed to minimize the time that transverse coherence of the $^{13}C^\alpha$ nucleus is present, since this nucleus has the shortest transverse relaxation time of all the nuclei involved in these experiments. This is achieved by using a coherence transfer pathway from $^1H^N$ to ^{15}N, $^{13}C^\alpha$, $^1H^\alpha$ and back to the $^1H^N$. In the sequence described, transverse $^{13}C^\alpha$ coherence is present only for a length of ca. $1/^1J(C^\alpha\text{-}H^\alpha)$. This reduces loss of signal due to transverse relaxation. We tested the technique on uniformly ^{15}N- and ^{13}C-enriched T4 lysozyme.

Assignment of the proton NMR spectrum is the basis for a solution structure determination of a protein. Crucial steps of the assignment process are (i) elucidation of the spin systems of the individual residues via scalar connectivities and (ii) sequential connectivities via NOESY experiments. An important part of the spin system analysis is to establish intraresidue H^N-H^α connectivities. Initially, this was achieved with the homonuclear COSY experiments recorded with a protein in H_2O (Kumar et al., 1980; Wagner and Wüthrich, 1982). The region of the H^N-H^α cross peaks was called fingerprint region. NOESY spectra were used to identify sequential connectivities (Wagner and Wüthrich, 1982). In the meantime, assignment techniques have rapidly improved, in particular by the introduction of the TOCSY (HOHAHA) experiment (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) and 3D ^{15}N dispersed NOESY and TOCSY techniques (Fesik and Zuiderweg, 1988; Marion et al., 1989a,b). For larger proteins with efficient dipole-dipole relax-

* To whom correspondence should be addressed.

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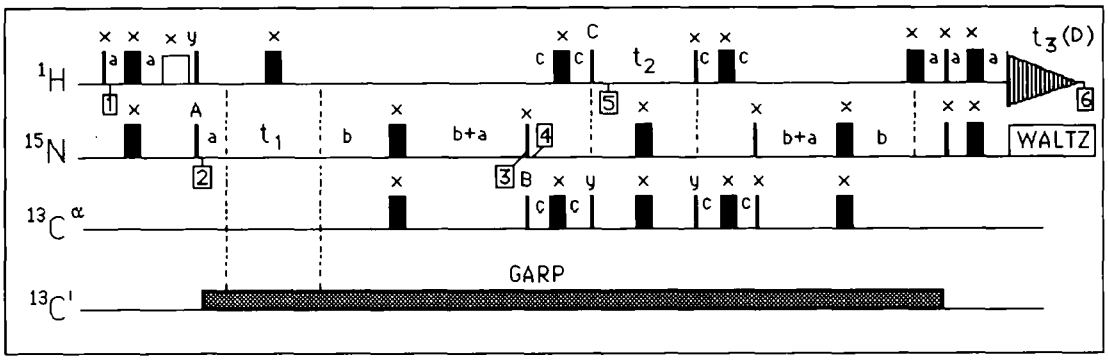

Fig. 1. Pulse sequence for the HN(CA)HA experiment. Narrow and wide bars indicate 90° and 180° pulses, respectively. The open box prior to the second ^{1}H (90°) pulse indicates a hard spin lock pulse for water elimination similar to that described by Messerle et al. (1989). Low-power GARP decoupling is applied at the C′ channel as indicated, and ^{15}N WALTZ16 decoupling is applied during detection. The phase cycles are: A: x, −x; B: 2(x), 2(−x); C: 4(x), 4(−x); D: x, −x, −x, x, −x, x, x, −x. The delays a, b and c are discussed in the text.

ation and concomitantly increased line widths, the TOCSY and COSY experiments fail to give complete connectivities, even for the direct H^N-H^α cross peaks. This is because these experiments rely on the relatively small H^N-H^α coupling constants. If these 3-bond couplings are significantly smaller than the proton line widths, these cross peaks become very weak or may be completely absent. Sequential assignments via NOESY experiments depend on the conformation of the protein, since the $d_{\alpha N}(i,i-1)$, $d_{\beta N}(i,i-1)$ and $d_{NN}(i,i-1)$ distances depend on the values of the dihedral angles Φ, Ψ and χ^1. Previously, we had developed ^{1}H-^{13}C-^{15}N triple resonance experiments that can provide intraresidue connectivities between H^N, N, C^α and H^α nuclei, exclusively via heteronuclear one-bond couplings, and conformation-independent sequential connectivities, exclusively via heteronuclear one-bond and two-bond couplings (Montelione and Wagner, 1989, 1990; Wagner et al., 1989). A number of improved and novel triple resonance experiments for conformation-independent sequential assignments via heteronuclear and homonuclear one- and two-bond couplings have been described since and made these strategies applicable to proteins (Ikura et al., 1990; Kay et al., 1991; Schmieder et al., 1991; Wagner et al., 1991). These include an optimized version of the initial triple resonance experiment by Montelione and Wagner (1990) using concatination of pulses, a careful tuning of delays and spin lock purge pulses (Kay et al., 1991). The experiment described below is yet another version of this experiment with advantageous properties.

Following the nomenclature introduced by Kay et al. (1991) we call this an HN(CA)HA experiment. The pulse sequence is shown in Fig. 1. The coherence transfer pathway can be described in a short notation as:

$$H^N \rightarrow N(\omega_1) \rightarrow C^\alpha \rightarrow H^\alpha(\omega_2) \rightarrow C^\alpha \rightarrow N \rightarrow H^N(\omega_3) \qquad (1)$$

Frequency labeling during evolution and detection periods is indicated in parentheses. We use the product operator formalism of Sørensen et al. (1983) to describe the time evolution of the system, the different instances on the time axis being indicated by small numbers placed in boxes (Fig. 1).

H^N, N, C^α and H^α are used to describe spin operators for the respective nuclei. We consider magnetization starting from the amide protons. At time 1 we have:

$$\sigma_1 = -H^N y \tag{2}$$

The INEPT transfer (Morris and Freeman, 1979; Burum and Ernst, 1980; Morris, 1980a,b) yields ^{15}N coherence, in antiphase to the amide proton. The delay a is tuned to approximately $1/(4\,J_{HN,N})$. At time 2 we have:

$$\sigma_2 = 2H^N{}_z N_y \tag{3}$$

The following period achieves frequency labeling of the N nucleus, refocusing of the N-H coupling and evolution of the ^{15}N coherence in antiphase to the C^α spins of the same and the preceding residue. The intraresidue one-bond coupling and the sequential two-bond couplings are ca. 11 and 7 Hz, respectively (Bystrov, 1976). At point 3 the relevant terms are:

$$\begin{aligned}\sigma_3 = \{&2N_{iy}C^x{}_{iz}\cos(\omega_N t_1)\sin(\pi J_{Ni\text{-}C^x{}_i}\,2(a+b))\cos(\pi J_{Ni\text{-}C^x{}_{i-1}}\,2(a+b))\\ &+ 2N_{iy}C^x{}_{i-1,z}\cos(\omega_N t_1)\sin(\pi J_{Ni\text{-}C^x{}_{i-1}}\,2(a+b))\cos(\pi J_{Ni\text{-}C^x{}_i}\,2(a+b))\}^*\\ &\exp(-2(a+b+t_1)/T_2{}^N)\end{aligned} \tag{4}$$

The first term represents a coherence transfer to the α-carbon of the same residue, the second to the α-carbon of the preceding residue. $T_2{}^N$ is the effective transverse relaxation time of the N coherence. The N-C^α coupling is active during t_1, however, it is small and can be neglected, considering the limited resolution in the N dimension. The following two 90° pulses on N and C^α create C^α coherence in antiphase to N. Disregarding the size of the transfer function and treating intraresidue and sequential transfers collectively, we have:

$$\sigma_4 = 2N_z C^x{}_y \cos(\omega_N t_1) \tag{5}$$

The following delays c are tuned to approximately $1/4J_H\alpha_C\alpha$, and an INEPT-type transfer from C^α to H^α is performed, leading to:

$$\sigma_5 = -4N_z C^x{}_z H^x{}_y \cos(\omega_N t_1) \tag{6}$$

We convert the transverse C^α coherence into z-magnetization to avoid the unfavorably fast transverse relaxation of the C^α nucleus. The coherence is now labeled with the frequency of the α-proton. The remainder of the sequence is just a return to H^N coherence for detection. Finally, at point 6 we detect:

$$\sigma_6 = H^N{}_y \cos(\omega_N t_1)\cos(\omega_H{}^x t_2)\cos(\omega_H{}^N t_3) \tag{7}$$

The backtransfer depends again on the lengths of the delays a and b. Tuning of the delay a is trivial. A proper choice of b is, however, crucial for the performance of the experiment. It should be tuned to optimize the functions

$$\sin^2(\pi J_{Ni\text{-}C^\alpha_i} 2(a + b)) \cos^2(\pi J_{Ni\text{-}C^\alpha_{i-1}} 2(a + b)) \exp(-4(a + b)/T_2^N) \quad (8a)$$

and

$$\sin^2(\pi J_{Ni\text{-}C^\alpha_{i-1}} 2(a + b)) \cos^2(\pi J_{Ni\text{-}C^\alpha_i} 2(a + b)) \exp(-4(a + b)/T_2^N) \quad (8b)$$

to maximize intraresidue and sequential cross peaks, respectively. Figure 2 shows simulated transfer functions vs. the length of the delays a + b, assuming values of 11 Hz and 7 Hz for intraresidue one-bond and sequential two-bond N-C^α coupling constants, respectively. Effective T_2^N values of 150 ms, 100 ms and 50 ms were assumed in the 3 simulations, respectively. The solid and broken lines are the transfer functions for the intraresidue and the sequential connectivities. It can be seen, that a value of a + b = 11 ms is a good compromise.

The phase cycles are given in the caption to Fig. 1. Time-proportional phase incrementation (TPPI) (Marion and Wüthrich, 1983) is applied to both A and B phases to discriminate the signs of the frequencies along ω_1 and ω_2. Water elimination could be achieved with presaturation. Here we used the method of Messerle et al. (1989) to eliminate the water signal, applying a long spin lock x-pulse prior to the second 90° proton pulse. This dephases the water signal yet does not affect the antiphase amide proton coherence $2H^N_xN_z$.

Figure 3 shows two ω_2-ω_3 cross planes of an experiment recorded with a 4.5 mM sample of T4 lysozyme. The two cross planes are at the ^{15}N frequencies of Leu^{15} (124.5 ppm) and Arg^{14} (129.3 ppm), respectively. The cross planes contain intraresidue H^α_i-H^N_i and sequential H^α_{i-1}-H^N_i cross peaks. In a preliminary inspection of the spectra, 78% of the intraresidue and more than 25% of the sequential connectivities were seen. In particular, a few H^α resonances that were not detected in

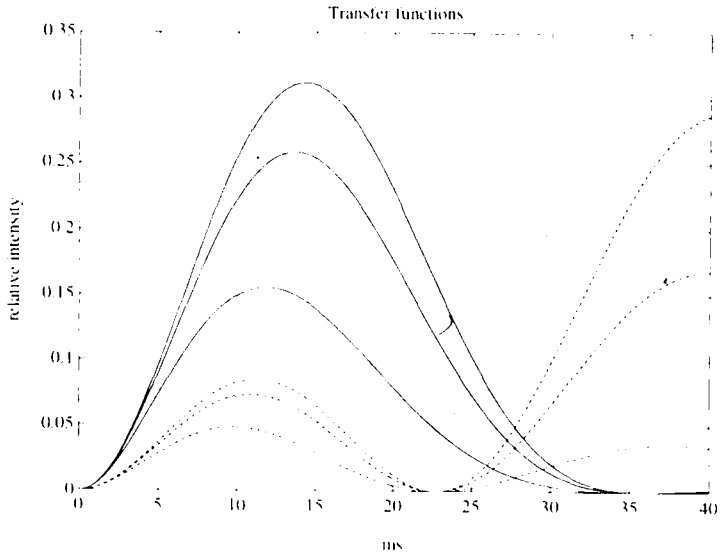

Fig. 2. Simulated transfer functions for intraresidue ( ) and sequential (-----) cross peaks. The values of the expressions in Eq. 8 were plotted vs. (a + b). Effective T_2^N values of 150 ms, 100 ms and 50 ms were assumed, with the larger values for the longer relaxation times. Values of 11 Hz and 7 Hz were used for intraresidue one-bond and sequential two-bond ^{15}N-$^{13}C^\alpha$ coupling constants, respectively (Bystrov, 1976).

conventional homonuclear COSY spectra of T4 lysozyme, were identified (McIntosh et al., 1990). These include Ile[17] (4.13 ppm), Met[1] (3.75 ppm) and Pro[86] (4.47 ppm). The latter two were identified from sequential cross peaks. Several additional assignments may be obtained after a more elaborate analysis of the data.

The experiment presented here has advantages over previously described pulse sequences (Montelione and Wagner, 1990; Kay et al., 1991) that provide similar connectivities. In our earlier work, we have discussed different versions of 3-step coherence transfers through single bonds, including transfers from H^{α} to H^{N} and vice versa. The experiment most similar to that described here uses coherence transfers from the H^{α} via C^{α} and N to the H^{N} (Montelione and Wagner, 1990). This is also the principle coherence transfer pathway in the experiment optimized by Kay et al. (1991) who introduced spin lock purge pulses, carbonyl decoupling and concatenation of pulses. A 3D version of this earlier sequence can be described in a short notation as:

$$H^{z}(\omega_1) \rightarrow C^{z} \rightarrow N(\omega_2) \rightarrow H^{N}(\omega_3) \qquad (9)$$

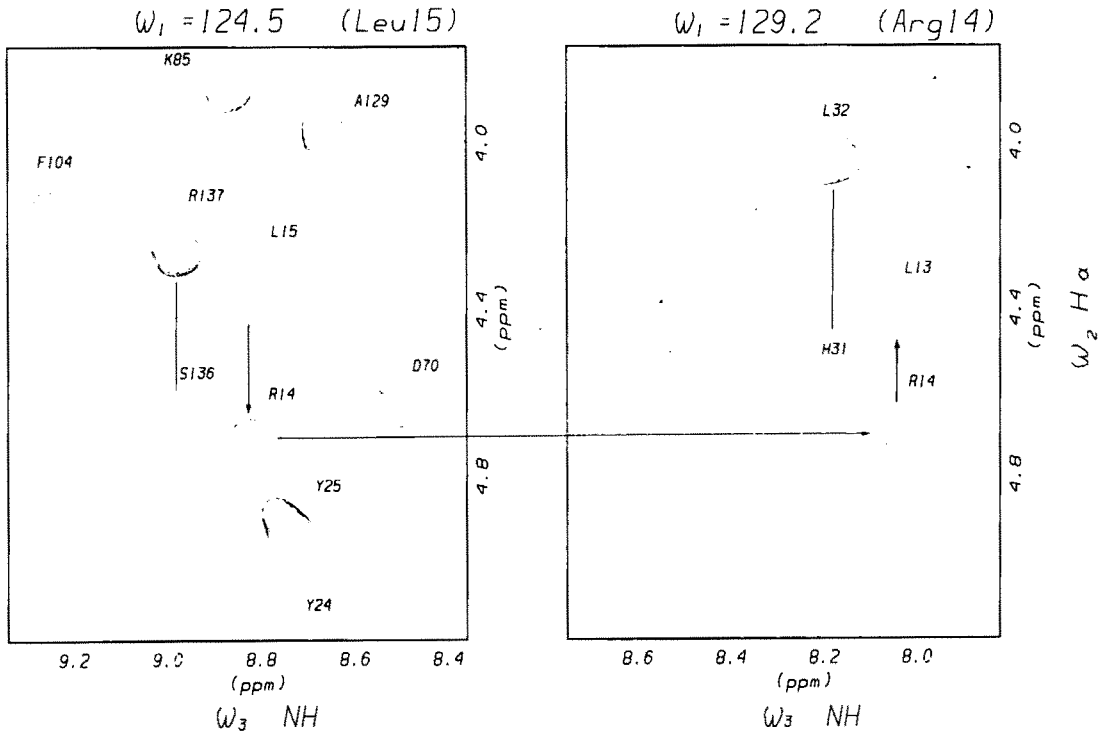

Fig. 3. Cross planes from a 3D spectrum recorded with the sequence of Fig. 1 on a 4.5 mM solution of uniformly ^{15}N and ^{13}C labeled T4 lysozyme, pH 5.5, 100 mM KCl, in 95% H_2O, 5% D_2O, 20°C. Two cross planes are shown at the ^{15}N frequencies of the sequentially adjacent residues Leu[15] and Arg[14]. Arrows indicate the sequential connectivities from Leu[15] to Leu[13]. Other cross peaks are identified with residue type and sequence number. The spectrum was recorded on a Bruker AMX-600 NMR spectrometer. GARP decoupling was applied at the C' frequency using an external GARP box (Tschudin Associates), with a 90° pulse of 400 μs. The length of the hard spin lock pulse for water elimination was 3 ms. The delays were set to a = 2.25 ms, b = 8.75 ms, c = 1.5 ms. The time domain data consisted of 15, 64 and 512 complex data points in t_1, t_2 and t_3, respectively. The total acquisition time was ca. 60 h. The data were processed with the software package FELIX.

In this experiment, the coherence is transferred from the $^{1}H^{\alpha}$ to the $^{13}C^{\alpha}$ via an INEPT sequence. The transverse $^{13}C^{\alpha}$ coherence has to evolve to antiphase coherence with respect to the peptide nitrogen, followed by a coherence transfer from C^{α} to N. Finally, the N coherence has to refocus with respect to the coupling to the C^{α}. It is important to note, that in this sequence the coherence decays with the relatively large transverse relaxation rate of the C^{α} nucleus during the time between the INEPT transfer from the H^{α} to the C^{α} and the coherence transfer to the nitrogen. After that, the coherence decays with the slower relaxation rate of the nitrogen, until it is finally converted to the amide proton coherence for detection. During the time the coherence is transverse on the α-carbon, coupling to the β-carbons is active and may lead to loss of signal. Neglecting minor details of the pulse sequences and assuming efficient decoupling of the carbonyls, the transfer functions for intraresidue and sequential cross peaks are approximately:

$$\sin^2(\pi J_{Ni\text{-}C^{\alpha}i}\,2(\tau))\cos^2(\pi J_{Ni\text{-}C^{\alpha}i\pm1}\,2(\tau))\cos(\pi J_{C\beta\text{-}C\alpha}2(\tau))\exp(-2(\tau)/T_2^{N})\exp(-2(\tau)/T_2^{C\alpha}) \qquad (10a)$$

and

$$\sin^2(\pi J_{Ni+1\text{-}C^{\alpha}i}\,2(\tau))\cos^2(\pi J_{Ni\text{-}C^{\alpha}i}\,2(\tau))\cos(\pi J_{C\beta\text{-}C\alpha}\,2(\tau))\exp(-2(\tau)/T_2N)$$
$$\exp(-2(\tau)/T_2C^{\alpha}) \qquad (10b)$$

Inspection of Eq. 10 shows that transverse relaxation of both the $^{13}C^{\alpha}$ and the ^{15}N nuclei are key factors for the sensitivity of the experiment. The delay τ is analogous to (a+b) of our new sequence (Fig. 1). Kay et al. (1991) have optimized the delays in the sequence, generally described in Eq. 9 so that the term $\cos(\pi J_{C\beta\text{-}C\alpha}\,2(\tau))$ is approximately -1. The length for which the coherence is transverse on C^{α} and N was set to ca. 25 ms and 23 ms, respectively. In the sequence described here (Fig. 1), the corresponding periods for which transverse C^{α} and N coherences are pre-

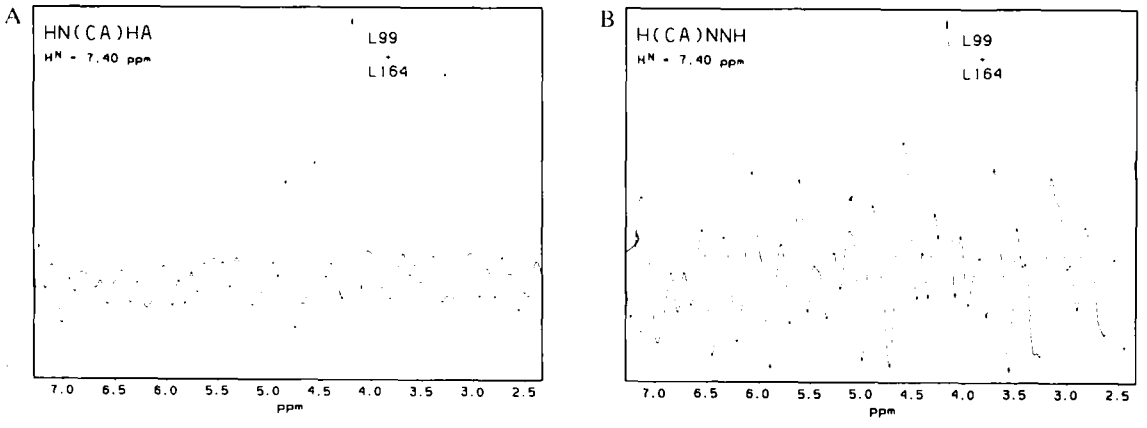

Fig. 4. Cross sections along ω_1 through the cross peaks of Leu^{99} and Leu^{164} from 2D spectra recorded with (A) the pulse sequences of Fig. 1 and (B) the sequence described by Kay et al. (1991). Equal numbers of t_1 and t_2 increments were used for both experiments. The data processing was exactly the same. The parameters for the experiment in (A) were the same as those for the experiment described in Fig. 3. The parameters for the experiment in (B) were the same as those quoted in Kay et al. (1991). However, the phase of the second proton 90 pulse was changed from x to y to correct an obvious typographical error in the list of phases given by Kay et al. (1991).

sent are 6 ms (4c) and 44 ms (2(a + b)), respectively. To our experience, the transverse relaxation rates of C^{α} nuclei are significantly faster than those of amide nitrogens (probably as much as a factor 4). Preliminary studies of the effective transverse relaxation times of C^{α} nuclei in T4 lysozyme, measured under conditions that resemble the coherence in the experiment of Kay et al. (1991) may be as short as 10 ms. Therefore, it is advisable to minimize the time spent on transverse carbon coherence, at the expense of time spent on transverse nitrogen coherence. In addition, the coupling between C^{α} and C^{β} is an insignificant problem in the sequence of Fig. 1. For comparison, we have recorded consecutively 2D versions of the experiment of Kay et al. (1991) and of the experiment shown in Fig. 1, with the same instrument setup. The nitrogen evolution was omitted so that we have 2D ^{1}H-^{1}H correlated spectra. Both experiments were performed with analogous parameters. The phase cycles as given in the figure caption of Kay et al. (1991) were checked, and the phases of the second 90° (^{1}H) pulse were rotated by 90 to make the experiment work. 192 scans were recorded per t_1 value. Figure 4 shows cross sections along ω_1 through the degenerate cross peaks of Leu^{99} and Leu^{164}. Figure 4A is the experiment of Fig. 1, and Fig. 4B is the experiment recorded with the sequence of Kay et al. (1991). The signal-to-noise ratio is significantly better with the new sequence.

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