

# An Efficient Triple-Resonance Experiment for Proton-Directed Sequential Backbone Assignment of Medium-Sized Proteins

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The three-dimensional CBCANH (1) and CBCA(CO)NH (2) triple-resonance experiments enable rapid and convenient sequential assignments of backbone  $^{13}\text{C}^\alpha$ ,  $^{13}\text{C}^\beta$ ,  $^{15}\text{N}$ , and  $^1\text{H}^N$  resonances to be obtained for medium-sized proteins by providing through-bond  $^{13}\text{C}^\alpha/^{13}\text{C}^\beta(i-1, i)-^{15}\text{N}(i)-^1\text{H}^N(i)$  and  $^{13}\text{C}^\alpha/^{13}\text{C}^\beta(i-1)-^{15}\text{N}(i)-^1\text{H}^N(i)$  connectivities, respectively. The 3D HBHA(CBCACO)NH experiment (3) is similar in spirit to CBCA(CO)NH and provides  $^1\text{H}^\alpha/^1\text{H}^\beta(i-1)-^{15}\text{N}(i)-\text{H}^N(i)$  connectivities. This experiment, however, cannot be employed alone for sequential assignment since it provides only the interresidue correlations without the corresponding intraresidue ones. Consequently, the  $^1\text{H}^\alpha/^1\text{H}^\beta$  assignment process based on the HBHA(CBCACO)NH experiment relies upon the prior assignment of the  $^{15}\text{N}$  and  $^1\text{H}^N$  resonances obtained via the sequential carbon-directed pathway (i.e., using the CBCANH and CBCA(CO)NH spectra). Thus, any backbone degeneracies in the carbon assignment pathway must be resolved through a number of previously published experiments before the HBHA(CBCACO)NH spectrum can be used to assign the backbone proton resonances. Furthermore, just as in the case of the CBCA(CO)NH experiment, all cross peaks in the HBHA(CBCACO)NH experiment are of the same sign. As a result, without prior knowledge of the  $^1\text{H}^\alpha-^{13}\text{C}^\alpha$  and  $^1\text{H}^\beta-^{13}\text{C}^\beta$  one-bond correlations, one cannot assign the  $^1\text{H}^\alpha$  and  $^1\text{H}^\beta$  resonances for certain amino acids, such as threonine and serine, purely on the basis of differences in chemical shift, as their  $\text{H}^\alpha$  and  $\text{H}^\beta$  protons resonate in the same region of the spectrum.

Using the CBCANH experiment (which provides both inter- and intraresidue correlations), however, one can immediately distinguish  $^{13}\text{C}^\alpha$  from  $^{13}\text{C}^\beta$  resonances, as their cross peaks are of opposite sign. There has been no published experiment involving the  $^1\text{H}^\alpha/^1\text{H}^\beta$  protons that is analogous to CBCANH. Such an experiment, which would complement HBHA(CBCACO)NH, would clearly be very helpful in the

backbone assignment process and could provide an independent through-bond sequential assignment pathway. While other pulse sequences which correlate the backbone  $^1\text{H}^\alpha/^1\text{H}^\beta$  nuclei with the intraresidue  $^{15}\text{N}$  and  $^1\text{H}^N$  nuclei have been published, these experiments do not consistently show interresidue connectivities (4–6). Just as the CBCANH experiment is complementary to the CBCA(CO)NH experiment, we propose an analogous experiment to complement the HBHA(CBCACO)NH experiment that will be referred to as the HBHA(CBCA)NH experiment.

The HBHA(CBCA)NH pulse sequence is shown schematically in Fig. 1 and, except for the addition of some gradients, is similar in its first part to the HBHA(CBCACO)NH pulse sequence (3) and in its latter part to the CBCANH pulse sequence (1). Only a brief description of the HBHA(CBCA)NH pulse sequence will therefore be given. The initial  $90^\circ$   $^{13}\text{C}$  purge pulse is followed by a gradient, G1, which ensures that the upcoming polarization transfer from protons will be the only source of coherent transverse carbon magnetization (7). The first  $90^\circ$   $^1\text{H}$  pulse at time point a creates  $^1\text{H}^\alpha$  and  $^1\text{H}^\beta$  transverse magnetization which evolves during a semiconstant time period (3) and simultaneously develops antiphase magnetization with the  $^{13}\text{C}^\alpha$  and  $^{13}\text{C}^\beta$  nuclei. The gradient G2 is applied at time b, when all pertinent magnetization lies along the z axis so that the gradient dephases the transverse water signal and any spurious transverse magnetization (8). After a  $90^\circ$   $^{13}\text{C}$  pulse transfers the polarization to  $^{13}\text{C}$ , the antiphase  $^{13}\text{C}^{\alpha,\beta}-^1\text{H}^{\alpha,\beta}$  magnetization is refocused after time  $\epsilon$ , and  $^1\text{H}$  decoupling is applied. During a period of total time  $2\gamma$ ,  $^{13}\text{C}^\alpha/^{13}\text{C}^\beta$  antiphase is allowed to develop. The  $90_x^\circ$   $^{13}\text{C}$  pulse at point c converts antiphase  $^{13}\text{C}_y^\beta/^{13}\text{C}_z^\alpha$  magnetization into  $^{13}\text{C}_y^\alpha/^{13}\text{C}_z^\beta$  magnetization but does not affect the  $^{13}\text{C}_x^\alpha$  term, so that these latter two sources of  $^{13}\text{C}^\alpha$  magnetization are allowed to couple with  $^{15}\text{N}$  during the subsequent time period  $2\theta$ . The time period  $2\theta$  also serves to refocus the  $^{13}\text{C}_y^\alpha/^{13}\text{C}_z^\beta$  magnetization. At point d, the  $90^\circ$   $^{13}\text{C}$  pulse flips  $^{13}\text{C}^\alpha$  magnetization to the z axis, at which time the DIPSI decoupling sequence is turned off to allow the proton carrier frequency to be moved from the water resonance to the middle of the amide region. The DIPSI

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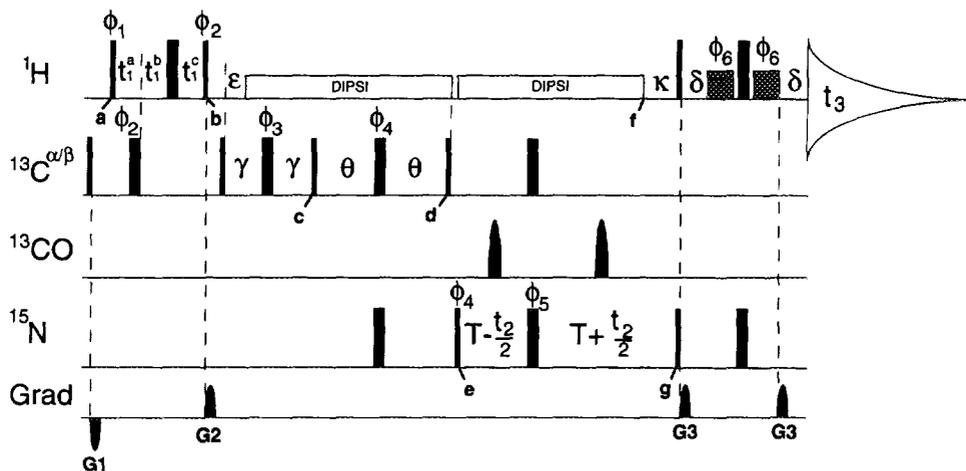


FIG. 1. Pulse scheme of the HBHA(CBCA)NH experiment. Narrow rectangular pulses correspond to a  $90^\circ$  flip angle and wider rectangular pulses to  $180^\circ$ . Rounded carbonyl pulses (of  $202 \mu\text{s}$  duration) have a  $180^\circ$  flip angle with the shape of the center lobe corresponding to a  $\sin(x)/x$  function. Pulses for which no phase is indicated are applied along the  $x$  axis. The  $^1\text{H}$  carrier is placed at 4.68 ppm except for the period between points d and f, during which time the carrier is positioned at 8.1 ppm. The  $\text{C}^{\alpha/\beta}$  carrier is positioned at 46 ppm, and the power of the RF pulses is adjusted to yield zero excitation in the carbonyl region (11.4 and 5.1 kHz RF field strength for the  $180^\circ$  and  $90^\circ$  pulses, respectively, at a  $^{13}\text{C}$  frequency of 150.9 MHz). All  $^{15}\text{N}$  pulses are applied with the carrier at 117.5 ppm, using a 6.6 kHz RF field.  $^{15}\text{N}$  decoupling was accomplished using low-power (1.6 kHz RF field strength) WALTZ decoupling.  $^1\text{H}$  decoupling during the magnetization relay is accomplished with a DIPSII-2 scheme (13), using a 4.8 kHz RF field. Delay durations are  $\epsilon = 2.1$  ms,  $\gamma = 3.1$  ms,  $\theta = 11$  ms,  $\kappa = 5.4$  ms, and  $\delta = 540 \mu\text{s}$  (such that the total delay of  $\delta +$  gradient pulse + selective water pulse is 2.25 ms). The  $\text{H}_2\text{O}$  resonance was suppressed with the WATERGATE pulse scheme (9). The WATERGATE  $\text{H}_2\text{O}$   $90^\circ$  pulses used an RF of 281 Hz. Phase cycling was as follows:  $\phi_1 = y$ ;  $\phi_2 = x, -x$ ;  $\phi_3 = 8(x), 8(y), 8(-x), 8(-y)$ ;  $\phi_4 = x, x, -x, -x$ ;  $\phi_5 = 4(x), 4(-x)$ ;  $\phi_6 = -x$ ; Rec =  $x, 2(-x), 2(x), 2(-x), x, -x, 2(x), 2(-x), 2(x), -x$ . Quadrature in  $F_1$  and  $F_2$  is obtained by altering  $\phi_1$  and  $\phi_4$  in the usual States-TPPI manner (14).

decoupling is then turned on, and the  $90^\circ$   $^{15}\text{N}$  pulse at point e creates antiphase magnetization of the form  $^{15}\text{N}_y^{13}\text{C}_z^\alpha$ . During the following constant time period (of total time  $2T$ ), the  $^{15}\text{N}$  magnetization is allowed to evolve for a total time  $t_2$ , the  $^{15}\text{N}_y^{13}\text{C}_z^\alpha$  antiphase magnetization is allowed to refocus, and the  $180^\circ$  shaped carbonyl pulses are used to decouple the carbonyl carbon from  $^{15}\text{N}$ . At point f, at a time  $\kappa$  before the end of the constant time period, the  $^1\text{H}$  DIPSII decoupling is turned off, allowing  $^{15}\text{N}$  magnetization to develop antiphase terms with respect to  $^1\text{H}^N$ . At this time the proton carrier is also returned to its position at the water resonance. At point g a  $90^\circ$   $^{15}\text{N}$  pulse flips the nitrogen magnetization to the  $z$  axis. The subsequent  $90^\circ$   $^1\text{H}$  pulse creates  $^1\text{H}_y^{15}\text{N}_z$  terms, and the two gradients G3 are used as part of the WATERGATE water-suppression scheme (9) prior to acquisition of  $^1\text{H}_x$ .

The 3D HBHA(CBCA)NH experiment is demonstrated on an  $\sim 1$  mM sample of doubly labeled  $^{13}\text{C}/^{15}\text{N}$  human (Cys<sup>35</sup>  $\rightarrow$  Ala<sup>35</sup>) thioredoxin complexed with an unlabeled peptide from the transcription factor NF $\kappa$ B. The thioredoxin mutant contains 105 residues and the peptide 13 residues, giving a total molecular weight of  $\sim 13$  kDa for the complex. The sample was buffered at pH 5.5, and the spectrum was recorded at  $35^\circ\text{C}$ . The experiment was recorded on a Bruker AMX 600 spectrometer using a Bruker triple-resonance probe equipped with a self-shielded  $z$  gradient. Sine-bell-shaped pulsed field gradients (10 G/cm at the center of the sine bell) were generated using an in-house-developed shaping unit and amplifier. Data were processed using in-house-

written NmrPipe software (F. Delaglio, unpublished), and peak positions and intensities for nonoverlapping resonances were located interactively using 3D parabolic interpolation with the program PIPP (10).

The HBHA(CBCA)NH spectrum was recorded with the pulse scheme shown in Fig. 1 as a data set comprising  $512^* (t_3) \times 60^* (t_1) \times 32^* (t_2)$  complex points with acquisition times of 63.4, 13.3, and 20.3 ms in  $t_1$ ,  $t_2$ , and  $t_3$ , respectively, using 32 scans per  $t_1/t_2$  hypercomplex increment. The total acquisition time was 69 hours. The residual  $\text{H}_2\text{O}$  resonance was removed by a solvent-suppression filter applied to the  $t_3$  domain data (11). Prior to Fourier transformation, data were doubled along the nondecaying  $t_2$  domain using mirror-image linear prediction (12), apodized by a  $90^\circ$ -shifted squared sine-bell window function in all three dimensions, and zero filled to  $128^*, 256^*,$  and  $1024^*$  complex points in the  $t_1$ ,  $t_2$ , and  $t_3$  time domains, respectively.

Figure 2 shows  $^1\text{H}(F_1)$  strips from the 3D HBHA(CBCA)NH spectrum of the human thioredoxin-NF $\kappa$ B complex in 90%  $\text{H}_2\text{O}/10\%$   $\text{D}_2\text{O}$  taken at the  $^{15}\text{N}/^1\text{H}^N$  resonance frequencies of Ala<sup>10</sup> through Val<sup>25</sup>. Connectivities can be traced for both the  $^1\text{H}^\alpha$  and the  $^1\text{H}^\beta$  pathways independently, thereby providing an alternative pathway should one of them be degenerate. Particularly helpful for assignment purposes is the difference in sign of the  $^1\text{H}^\alpha$  and  $^1\text{H}^\beta$  correlations, as illustrated in Fig. 2. Hence, there is no reliance upon proton chemical-shift differences when making assignments. Note that the correlations to the  $^1\text{H}^\alpha$  resonances of glycine residues are negative. More than 85% of the  $^1\text{H}^\beta$

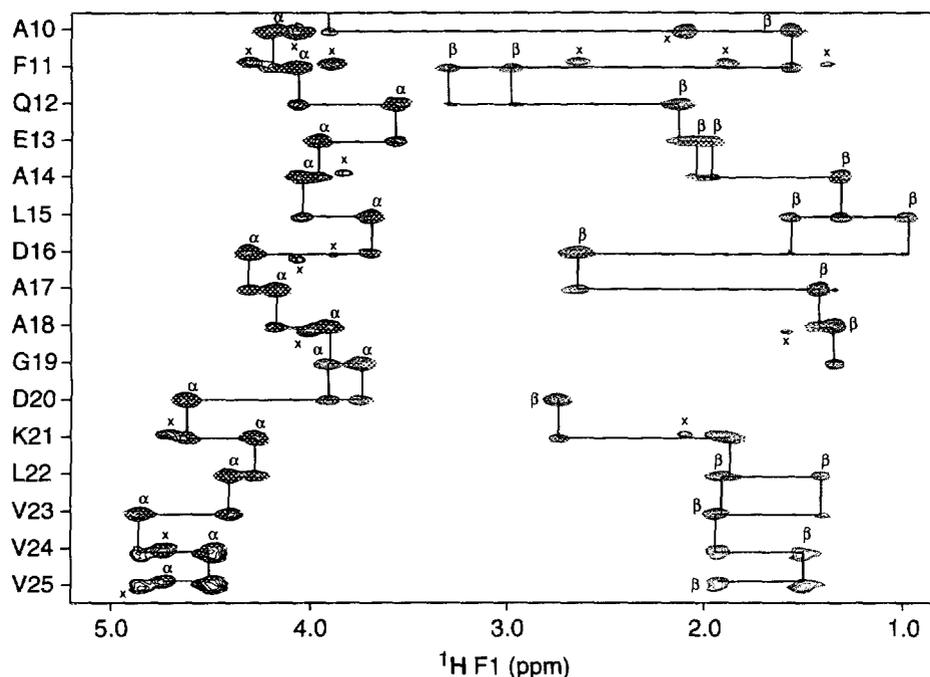


FIG. 2.  $^1\text{H}^\alpha$ ,  $^1\text{H}^\beta$  ( $F^1$ ) strips from Ala<sup>10</sup> to Val<sup>25</sup> taken from the 600 MHz 3D HBHA(CBCA)NH spectrum of an  $\sim 1$  mM sample of  $^{13}\text{C}/^{15}\text{N}$ -labeled human (C35  $\rightarrow$  A) thioredoxin complexed to a NF $\kappa$ B peptide at the  $^1\text{H}^{\text{N}}/^{15}\text{N}$  ( $F_3$ ,  $F_2$ ) frequencies of their backbone amide groups. The cross peaks are labeled at the intraresidue  $\alpha$  (solid contours) and  $\beta$  (dashed contours) correlations, and the sequential connectivity pathway is indicated by the straight horizontal and vertical lines. Peaks marked with a  $\times$  correspond to correlations to amide  $^1\text{H}$ - $^{15}\text{N}$  pairs that are close in frequency to that for which the strip has been selected.

and >95% of the  $^1\text{H}^\alpha$  thioredoxin resonances could be assigned unambiguously from the single HBHA(CBCA)NH spectrum. The missing correlations involve residues located within the mobile regions of the protein that experience severe line broadening.

The HBHA(CBCA)NH and the HBHA(CBCACO)NH spectra in combination can readily provide complete sequential assignments of backbone  $^1\text{H}^\alpha$ ,  $^1\text{H}^\beta$ ,  $^{15}\text{N}$ , and  $^1\text{H}^{\text{N}}$  resonances for medium-sized proteins in a reliable manner. Moreover, any degeneracies that are present in the carbon-directed CBCANH/CBCA(CO)NH assignment pathway can potentially be resolved by the proton-directed HBHA(CBCA)NH/HBHA(CBCACO)NH pathway and vice versa. This additional independent assignment pathway is just as efficient to use and as easy to interpret as the carbon-directed pathway. The sensitivity of the HBHA(CBCA)NH experiment is the same as that of the CBCANH experiment (1), and like the latter, is limited by transverse relaxation of  $\text{C}^\alpha$  magnetization during the relatively long dephasing period  $2\theta$ . As a result, for proteins larger than about 20 kDa, it is unlikely that a complete set of interresidue correlations will be observed.

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