Correlating Backbone Amide and Side Chain Resonances in Larger Proteins by Multiple Relayed Triple Resonance NMR

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Abstract: A new three-dimensional triple resonance NMR experiment is described that correlates the amide I1 and 15N resonances of one residue simultaneously with both the 13Cα and 13Cβ resonances of its preceding residue. Sensitivity of the new experiment is comparable to that of the HN(CO)CA experiment (Bax, A.; Ikura, M. J. Biomol. NMR 1991, 1, 99-105), but the additional correlation to the Cα resonance of the preceding residue provides invaluable assignment information, previously inaccessible. The technique is demonstrated for interferon-γ, a homodimeric protein of 31.4 kDa, enriched uniformly with 13C and 15N.

The most difficult part in using the recently developed 3D triple resonance NMR for obtaining complete backbone assignments in larger proteins involves the degeneracy in the Hα-Cα region of the H-13C shift correlation spectrum. Particularly for α-helical proteins with a narrow dispersion of Hα chemical shifts, this type of degeneracy frequently can make it difficult to establish in an unambiguous manner which backbone amide is correlated with which side chain. Even for the relatively well resolved spectrum of calmodulin, 40 such cases were reported.1 Here we report a new 3D triple resonance technique that provides connectivity.

1 On leave from F. Hoffmann La Roche, Ltd., Basel, Switzerland.

References


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of the CBCA(CO)NH experiment. The scheme starts
with an INEPT transfer to enhance the polarization of I3C.
Between time points \( t \) and \( \tau \), the C\(_{\alpha}\) magnetization is transferred to the adjacent

Figure 1. Pulse scheme of the CBCA(CO)NH experiment. Narrow pulses correspond to a \( 90^\circ \) flip angle and wider pulses to \( 180^\circ \). Pulses for which no phase is indicated are applied along the x axis. The \( ^1\)H carrier is placed at 4.75 ppm until the 90\(^\circ \) CO pulse is applied. After this time the \( ^1\)H carrier is switched to 8.1 ppm. The C\(_{\alpha}\) carrier is positioned at 4.4 ppm and the power of the RF pulses is adjusted to yield zero excitation in the CO region (11.4-kHz RF for the 180\(^\circ \) and 5.1-kHz RF for the 90\(^\circ \) pulses at 151-MHz \( ^{13}\)C frequency). C\(_\alpha\) pulses are applied at 56 ppm, using an RF field of 10.5 kHz. Rounded carbonyl pulses have a 180\(^\circ \) flip angle and have the shape of the center lobe of a \( \sin^2 \) function and a duration of 202 \( \mu \)s. The second and third shaped CO pulses serve to compensate the C\(_\alpha\) phase errors caused by Bloch-Siegert effects\(^{(9)} \) related to the first and fourth shaped pulse (see text). The rectangular CO pulses are applied using an RF field strength of 4.7 kHz, yielding minimal excitation of the C\(_\alpha\) resonances. All \( ^{15}\)N pulses are applied with the carrier at 117 ppm, using a 6-kHz RF field. \( ^{13}\)C decoupling was accomplished using low power (1.5-kHz RF) WALTZ decoupling. \(^{1}H\) decoupling during the magnetization relay is accomplished with a DIPSI-2 scheme\(^{(9)} \), using a 5.5-kHz RF field. Delay durations are \( \delta = 1.5 \) ms, \( \tau = 2.3 \) ms, \( \tau_B = 3.3 \) ms, \( \tau_C = 3.7 \) ms, \( \eta = 4.5 \) ms, \( \tau_D = 11.1 \) ms, \( \tau_F = 4.4 \) ms, \( \lambda = 2.25 \) ms. The H\(_2\)O resonance was suppressed with the 1.8-ms purge pulse, SL. Phase cycling was as follows: \( \phi_1 = \phi_2 = x-x; \phi_3 = x-x; \phi_4 = 8(x, 8C, 8(x, 8C, 8(x, 8C)); \phi_5 = 4(x, 4C, 4(x, 4C)); \phi_6 = 2(x, 2C, 2(x, 2C). \phi_1 \) is 51\(^\circ \) (Bloch-Siegert phase error compensation); \( \phi_2 = 8(x, 8C); \phi_3 = x-x, 2(-x, 2(-x, 2(-x, 2(-x, 2(-x, 2(-x, 2(-x. Quadrature in F\(_1\) and F\(_2\) is obtained by altering \( \phi_1 \) and \( \phi_2 \) in the usual manner.\(^{(6)}\)

Information between the amide \( ^1\)H and \( ^{15}\)N of one residue and both the \( ^{13}\)C\(_\alpha\) and \( ^{13}\)C\(_\gamma\) resonances of its preceding residue. Consequently, this experiment removes the need for recording a HN(CO)CA type correlation\(^{(7)} \) and in addition provides the crucial \( ^1\)J connectivity between backbone and side chain resonances.

Experimental Section

A form of interon-\( ^1\)H from which the last 10 residues are missing (IFN-\( ^1\)A10) shows the highest biological activity.\(^{(8)} \) IFN-\( ^1\)A10, enriched uniformly (>98\%) with \( ^{13}\)C and \( ^{15}\)N, was obtained from E. coli. X-ray crystallographic work has shown that the protein is highly \( \alpha \)-helical and exists as a highly interwined dimer.\(^{(8)} \) The total molecular weight of the (unlabelled) dimer is 314 kDa. Details regarding the expression and purification of the protein are presented elsewhere.\(^{(9)} \) NMR experiments were carried out on a sample containing 11 mg of IFN-\( ^1\)A0.5 mL of 95\% H\(_2\)O/5\% D\(_2\)O, pH 6.2.

Experiments were carried out at 600-MHz \( ^1\)H frequency on a Bruker AMX-600 spectrometer, equipped with an external 150 W class A/B power amplifier for \( ^{13}\)C and operating with software version 91101. The 3D CBCA(CO)NH spectrum was obtained from a 512\(^*\)(t\(_x\))\times512\(^*\)(t\(_y\))\times512\(^*\)(t\(_z\)) data set, with acquisition times of 6.16 ms, 20.0 ms (t\(_x\)), and 55.3 ms (t\(_z\)). To implement the pulse program on our AMX spectrometer, \( t_z \) increments with \( 1/2 < \alpha \) (Figure 1) were executed with a different section of the total pulse program compared to values of \( t_z/2 < \alpha \). The total measuring time was 2.5 days. Mirror image linear prediction\(^{(11)} \) in both the \( t_x \) and \( t_z \) domain was used, and data were zero filled to yield a 256\(^*\)128\(^*\)1024 matrix for the absorptive part of the final 3D spectrum.

Results and Discussion

The pulse scheme used in the present work is sketched in Figure 1. A detailed description of an analogous experiment will be described elsewhere,\(^{(5)} \) and here we will outline only the general mechanism of the CBCA(CO)NH experiment. The scheme starts with an INEPT transfer to enhance the polarization of \( ^{13}\)C. Between time points a and b the aliphatic magnetization is sub-


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Figure 2. $F_1$ ($C_\alpha$, $C_\beta$) strips for residues K59-Q68, taken from the 600-MHz 3D CBCA(CO)NH spectrum of interferon-γ at the $^1$H/$^{15}$N ($F_2/F_3$) frequencies of their amides. In the leftmost strip, the amide of K59 correlates with $C_\alpha$ and $C_\beta$ of F58. Also visible in this strip is the correlation for the amide of D64 (which has a $^1$H/$^{15}$N correlation very close to that of K59) to the $C_\alpha$ and $C_\beta$ of D63. These latter correlations are observed with much higher intensity in the sixth strip.

large number of RF pulses required for the magnetization transfer.

Figure 2 demonstrates the application of the CBCA(CO)NH method to the protein interferon-γ, a homodimer of 31.4 kDa. Experiments were recorded at 27°C, and at this temperature the $^1$H and $^{13}$C line widths for residues with little internal mobility are ~40-50 Hz. Several of the recently published 3D experiments did not yield satisfactory results because of these large line widths and due to the severe overlap in the $H_\beta$-$C_\beta$ region of the $^1$H/$^{13}$C shift correlation spectrum (supplementary material). Figure 2 shows $F_1$ strips from the 3D spectrum, taken at the amide $^1$H/$^{15}$N frequencies of ten consecutive residues, located in the hydrophobic core of the protein. These results clearly illustrate the power and effectiveness of the new experiment. For each amide, both the $C_\beta$ and $C_\gamma$ chemical shifts of the preceding amino acid are identified. Note that during the initial sequential assignment stage the $C_\alpha$ chemical shift is particularly useful for determining the type of amino acid, greatly facilitating the task of making sequence specific assignments.

The present experiment provides the first example of utilizing the relatively large and uniform one-bond $J$ couplings for multiple

relayed $J$ connectivity between backbone amide and side chain resonances in proteins. For small proteins, similar kinds of connectivity can be obtained using multiple $^1$H-$^1$H relay or HOH-AHA/TROSY experiments. In larger proteins these $J_{HH}$ based experiments are usually unsuccessful, particularly if $J_{INH}$ is small. In favorable cases connectivity between the amide $^1$H/$^{15}$N and the side chain $H_\beta$ can be obtained via the $J_{NN}$ or $J_{CONH}$ coupling, but in practice these experiments fail for proteins the size of interferon-γ or if these couplings are small.

The CBCA(CO)NH experiment provides a very robust and efficient method for obtaining amide to side chain $J$ connectivity. The scheme can be readily modified to yield connectivity to the $H_\beta$ and $H_\delta$ protons by introducing an evolution period prior to the first INEPT transfer and by replacing the first constant-time evolution period by a fixed interval (i.e., by keeping $t_1 = 0$ in the scheme of Figure 1). Another potentially useful modification shifts the second constant-time evolution period from $^{15}$N to $^{13}$CO. An experiment analogous to the CBCA(CO)NH technique described here, which attempts to correlate the $C_\alpha$ and $C_\beta$ resonances directly with the intraresidue amide $^1$H and $^{15}$N resonances via $J_{NC_\beta}$, was unsuccessful for IFN-γ, due to the large $C_\beta$ line width relative to the $J_{NC_\beta}$ coupling. However, for smaller proteins this latter experiment provides a powerful complement to the CBCA(CO)NH experiment.

In principle, the amide $^1$H/$^{15}$N could also be correlated simultaneously with both $H_\delta$ and $C_\beta$ resonances by transforming the dephasing interval 26 in Figure 1 into a constant-time evolution period and recording the spectrum in a 4D manner. The pulse scheme could be executed even in a 5D or 6D manner by replacing the fixed rephasing/dephasing delays 2{ and 32 with the first constant-time evolution period from $^{15}$N to $^{13}$CO. An experiment analogous to the CBCA(CO)NH technique described here, which attempts to correlate the $C_\alpha$ and $C_\beta$ resonances directly with the intraresidue amide $^1$H and $^{15}$N resonances via $J_{NC_\beta}$, was unsuccessful for IFN-γ, due to the large $C_\beta$ line width relative to the $J_{NC_\beta}$ coupling. However, for smaller proteins this latter experiment provides a powerful complement to the CBCA(CO)NH experiment.

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Supplementary Material Available: One figure displaying the $H_\beta$-$C_\beta$ region of the $^1$H/$^{13}$C shift correlation spectrum of interferon-γ (1 page). Ordering information is given on any current masthead page.