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Correlation of the $^{15}N(i+1)$, $^{13}C\alpha(i)$, and $^{1}H\alpha(i)$ Backbone Resonances in $^{13}C/^{15}N$-Labeled Proteins by the (CO)N(CO)CAH Experiment

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Recently, a novel three-dimensional, triple-resonance experiment was described which correlates intraresidue ^{13}CO, $^{13}C\alpha$, and $^{1}H\alpha$ backbone resonances in ^{13}C-labeled proteins via relatively large one-bond J couplings (*1*). This experiment differs from the HCACO experiment (*2, 3*), which shows the same type of correlations, in that not the proton magnetization, but the carbonyl magnetization is initially employed to produce the final signal. Therefore, the experiment was called the COCAH experiment. HCACO or COCAH spectra can be used to assign the $^{1}H\alpha$ backbone protons in proteins enriched in ^{13}C on the basis of assigned $^{13}C\alpha$ and ^{13}CO resonances. Assignment of these nuclei is generally accomplished by recording multidimensional heteronuclear NMR experiments in which each specific experiment correlates a number of different backbone nuclei. Usually, the $^{1}H\alpha$ protons can then be assigned from the HCACO or COCAH spectra unambiguously. In medium-sized proteins, however, there is a considerable chance that the ^{13}CO and $^{13}C\alpha$ frequencies of a number of residues coincide. In such cases the $^{1}H\alpha$ protons cannot be assigned unambiguously. To resolve this ambiguity, we propose an experiment that is complementary to the COCAH experiment and correlates the $^{1}H\alpha$ and $^{13}C\alpha$ spins of one residue with the backbone ^{15}N spin of the next residue. The HCACO equivalent of this experiment is the HCA(CO)N experiment (*2, 3*).

The pulse sequence of the (CO)N(CO)CAH is shown in Fig. 1. The sequence starts with a carbonyl $\pi/2$ pulse, creating transverse ^{13}CO magnetization. Subsequently, this magnetization is transferred partly into antiphase magnetization with respect to the backbone nitrogen via the $^{1}J_{NCO}$ coupling, which evolves during a delay α. The first $\pi/2$ nitrogen pulse then converts this magnetization into $^{13}CO/^{15}N$ multiple-quantum coherence. ^{15}N chemical-shift evolution takes place during the period t_1. The second ^{15}N $\pi/2$ pulse converts the frequency-labeled magnetization back into single-quantum carbonyl magnetization, antiphase with respect to the nitrogen, which then evolves into in-phase carbonyl magnetization again. Evolution under the $^{1}J_{C\alpha CO}$ coupling takes place effectively during a delay 2β. This is achieved by timing the two selective π pulses on the ^{13}CO and $^{13}C\alpha$ spins in a similar fashion as in the COCAH experiment (*1*). The period 2β is tuned to $(2\,{}^{1}J_{C\alpha CO})^{-1}$. The next $\pi/2$ pulse converts the carbonyl single-quantum coherence (antiphase with respect to the $C\alpha$) into $C\alpha$ single-quantum coherence (antiphase with respect to the carbonyl). From this point on, the pulse-sequence is equivalent to the original COCAH experiment, which is described in (*1*).

Pulsed field gradients were applied to suppress the strong $^{1}H_2O$ signal and other spurious signals. Gradients G_1 and G_2 were used to defocus unwanted magnetization created by imperfections of the π refocusing pulses (*8*). Gradient G_3 purges unwanted transverse components during the last INEPT step (*8*).

In our implementation of the (CO)N(CO)CAH experiment, the ^{13}C carrier was positioned in the chemical-shift range of the $^{13}C\alpha$ resonances during the whole pulse sequence. The ^{13}C $\pi/2$ pulses were all nonselective, rectangular pulses of 12.2 μs duration, short enough to excite the ^{13}CO spins also. Selective excitation of either the $^{13}C\alpha$ or ^{13}CO spins was achieved by selective π pulses (labeled ϕ_2 and ϕ_3 in Fig. 1), which were phase cycled in steps of 90°, while inverting the receiver phase (*5*). The selective carbonyl π pulses were applied at an offset of 15 kHz from the carrier frequency by phase modulation (*6*). Apart from the Bloch–Siegert shifts, which were compensated by shaped π pulses at the start of delays α and $(T - t_2/2)$, we had to repair the off-resonance effects caused by the CO-excitation pulses being applied 15 kHz off-resonance. In the COCAH experiment, such off-resonance effects lead only to a phase error during the carbonyl evolution period, which can easily be corrected for. In the present experiment, carbonyl chemical-shift evolution is always refocused, and any phase deviations will lead to a loss of signal. Therefore, we had to adjust the phase of the first ^{13}C $\pi/2$ pulse to optimize

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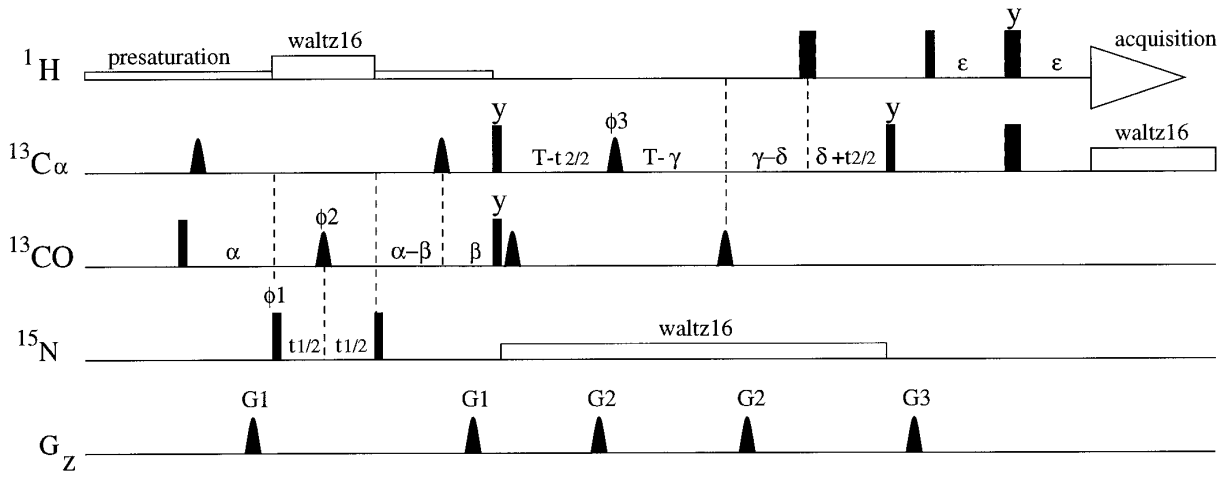

FIG. 1. Pulse scheme for the (CO)N(CO)CAH experiment. Narrow and broad rectangles symbolize nonselective $\pi/2$ and π pulses, respectively. The nonrectangular pulses represent π pulses shaped like the central lobe of a $\sin(x)/x$ function of 100 μs ($^{13}C\alpha$) or 200 μs (^{13}CO) duration. Fixed phases x were used unless indicated otherwise. The phases indicated as ϕ_{1-3} and the receiver were cycled as follows: $\phi_1 = x, -x$; $\phi_2 = 2x, 2y$; $\phi_3 = 4x, 4y$; acquisition $= x, 2(-x), x, -x, 2x, -x$. The following delays were used: $\alpha = 18.0$ ms; $\beta = 4.5$ ms; $T = 4.3$ ms; $\gamma = 4.1$ ms; $\delta = 1.5$ ms, $\epsilon = 1.7$ ms. Pulsed field gradients were applied along the z axis, and were sine bell shaped. The pulse lengths and maximum field strengths of the gradients were $G_1 = 0.5$ ms, 10 G/cm; $G_2 = 0.5$ ms, 15 G/cm; and $G_3 = 2.0$ ms, 20 G/cm. Forty-six t_1 increments and 64 t_2 increments were recorded, with spectral widths of 2000 and 5000 Hz in the ω_1 (^{15}N), and ω_2 ($^{13}C\alpha$) dimensions, respectively. Per t_1/t_2 combination, 24 transients were accumulated, leading to a total measuring time of 30 h. Quadrature detection was realized by cycling the first ^{15}N $\pi/2$, and the last ^{13}C $\pi/2$ pulse according to the TPPI protocol (*4*).

the conversion of antiphase carbonyl magnetization into antiphase Cα magnetization by the second ^{13}C $\pi/2$ y pulse.

Like in the COCAH experiment, the obvious loss by a factor of $\gamma_H/\gamma_C = 4$ in signal intensity, which arises from our choice to excite the carbonyl spins initially, is compensated by the favorable relaxation properties of the carbonyl spins, which have no proton directly attached. In the HCA(CO)N experiment, two extra periods (of 3.5 and 6 ms duration) are included for magnetization transfer from $^1H\alpha$ to the carbonyl spins. The extra losses due to relaxation, incomplete evolution of the Cα–CO J coupling, and dephasing due to Cα–Cβ J coupling during these periods amount to a factor of approximately

$$\exp(-3.5 \times 10^{-3}/T_{2H\alpha})\exp(-6 \times 10^{-3}/T_{2C\alpha}) \times \sin(\pi J_{C\alpha CO}\, 6 \times 10^{-3})\cos(\pi J_{C\alpha C\beta}\, 6 \times 10^{-3}) = 0.45$$

for a medium-sized protein. For larger proteins, this disadvantage will become more important. On the other hand, due to the relatively long carbonyl T_1's, in comparison with T_1's of Hα's, the relaxation delay for the COCAH and (CO)N(CO)CAH experiments must be chosen longer than for the experiments that start with $^1H\alpha$ excitation. This makes the (CO)N(CO)CAH experiment less sensitive by a factor of $\sim(T_{1CO}/T_{1H\alpha})^{1/2} = \sim 1.5$.

It is noteworthy that, as in the COCAH experiment, presaturation of the H_2O resonance and, unavoidably, of Hα resonances nearby is harmless. This is because the magnetization that is finally detected at these Hα frequencies was initially excited as carbonyl magnetization, and as such was not distorted by a H_2O presaturation pulse. This is not true for the HCACO and HCA(CO)N experiments.

Employing the pulse scheme shown in Fig. 1, we recorded a (CO)N(CO)CAH spectrum on $^{13}C/^{15}N$-labeled PDI-*a*, a 13.3 kDa molecular-weight protein consisting of the first 120 residues of human protein disulfide isomerase (*9, 10*). The sample consisted of 2 m*M* protein dissolved in 93% ^1H_2O/7% ^2H_2O (v/v) at 300 K, pH 5.1. The spectrum was recorded on a three-channel Varian UNITY-500 spectrometer, equipped with waveform generators on all channels to enable amplitude and phase modulation of all pulses.

Figure 2 shows two orthogonal projections of the (CO)N(CO)CAH spectrum of PDI-*a*. Most of the expected cross peaks corresponding to the backbone $^1H\alpha$ and $^{13}C\alpha$ spins of residue i and the backbone ^{15}N spin of residue $i + 1$ could be detected in this spectrum (*9, 10*). Apart from the backbone correlations, the (CO)N(CO)CAH experiment also shows correlations for side-chain resonances of Asn (Cβ, Hβ, Nγ) and Gln (Cγ, Hγ, Nδ) residues. All the expected correlations were found in the spectrum, some folded in the ^{13}C domain (Fig. 2A), confirming published assignments (*9, 10*).

All ambiguities present in the COCAH spectrum could be solved by comparison with the (CO)N(CO)CAH spectrum. An example is shown in Fig. 3 for the assignment of the $^1H\alpha$ of residue C39 of PDI-*a*. Sequential assignments of the

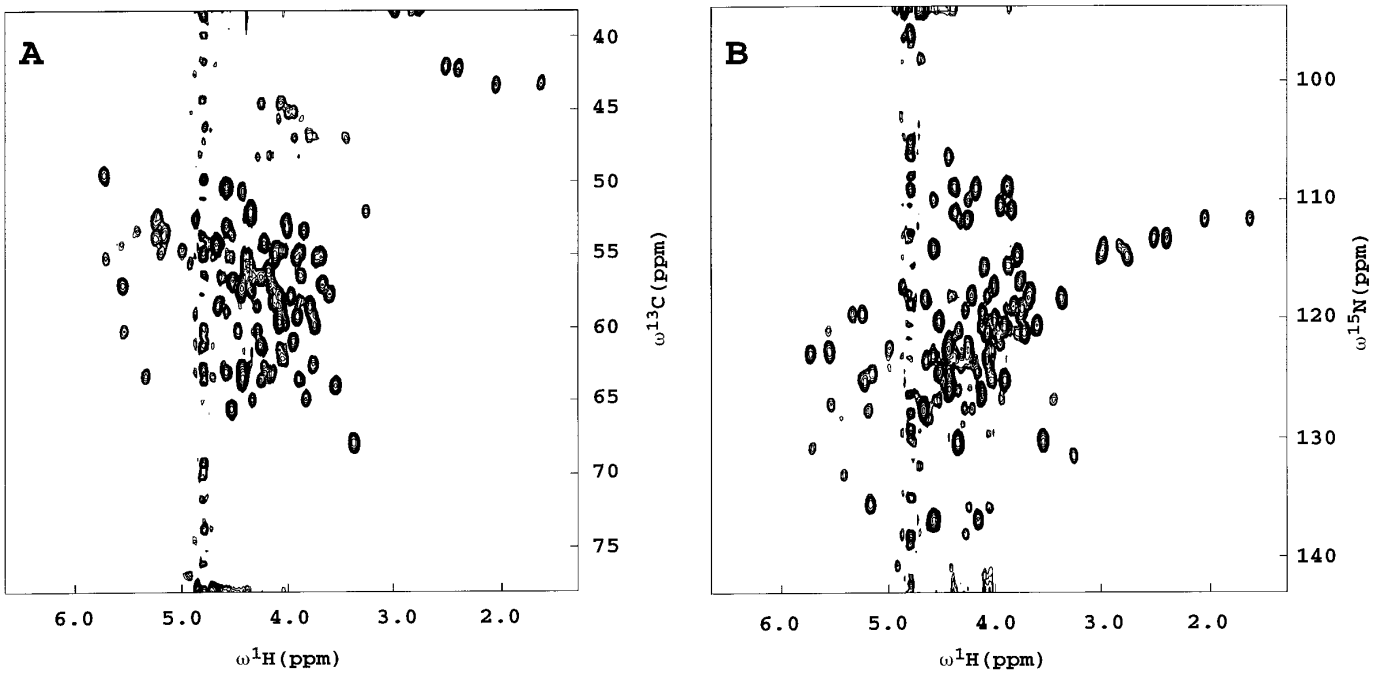

FIG. 2. Projections of the 3D (CO)N(CO)CAH spectrum of PDI-*a*: on the $\omega_3\omega_2$ (^{1}Hα ^{13}Cα) plane (A) and the $\omega_3\omega_1$ (^{1}Hα ^{15}N) plane (B). The cross peaks with a ^{1}H chemical shift smaller than ~3 ppm arise from the side-chain carbonyl groups of Asn and Gln.

backbone ^{15}N, ^{1}H^N, ^{13}Cα, and ^{13}CO spins of PDI-*a* (*9*) were accomplished by recording a set of four 3D experiments: HNCA, HN(CO)CA, HNCO, and HN(CA)CO. A plane taken from the COCAH experiment at the carbonyl frequency of C39 (δ_{CO} = 176.5 ppm; Fig. 3A) shows three peaks at the ^{13}Cα frequency of C39 ($\delta_{C\alpha}$ = 63.5 ppm). Only one of these

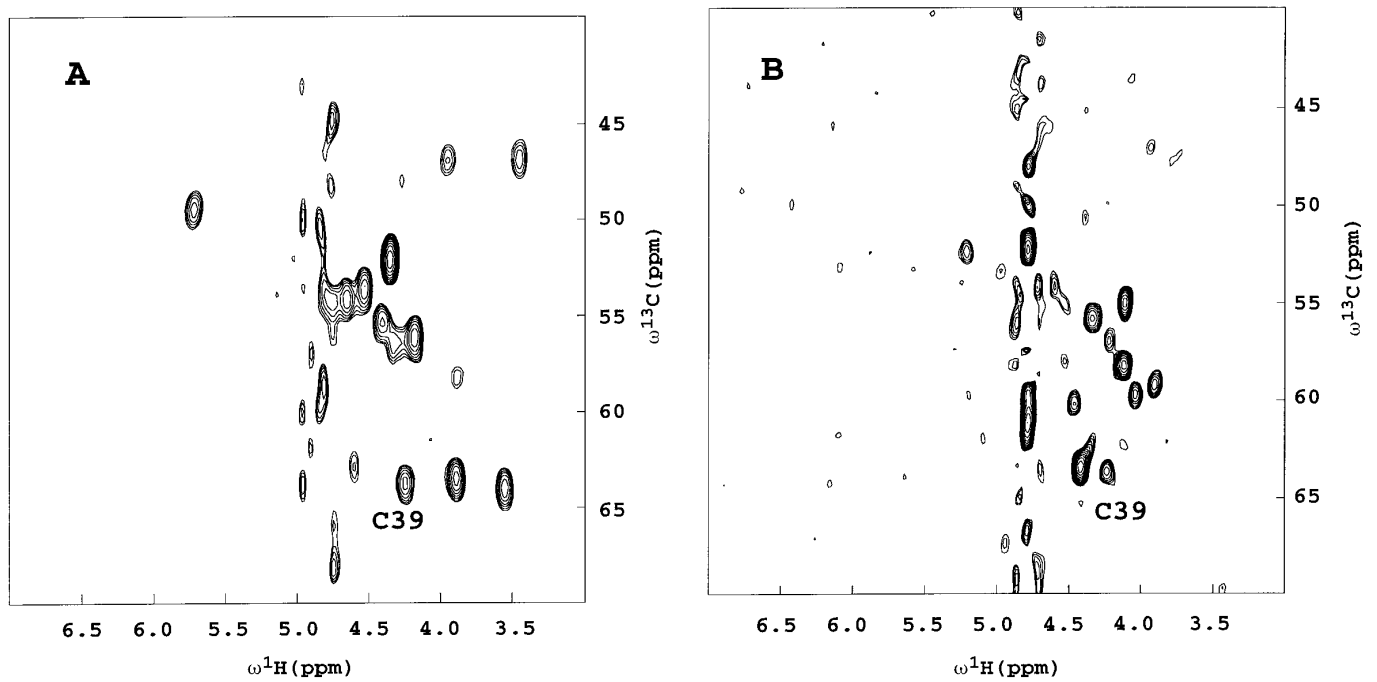

FIG. 3. Hα/Cα planes taken from a COCAH experiment at δ_{co} = 176.5 ppm (A) and from a (CO)N(CO)CAH experiment at δ_N = 125.8 ppm (B) of PDI-*a*. Indicated in (A) is a cross peak arising from an intraresidue ^{1}H$\alpha - ^{13}$C$\alpha - ^{13}$CO correlation in residue C39. In (B), a correlation between the ^{1}Hα and ^{13}Cα of residue C39 with the backbone ^{15}N of residue K40 is indicated.

peaks matches a peak present in a plane taken at the backbone ^{15}N frequency of residue K40 (δ_N = 125.8 ppm) from the (CO)N(CO)CAH spectrum of PDI-*a* (Fig. 3B). The ^{1}Hα of residue C39 could therefore be assigned unambiguously to the peak at δ_H = 4.24 ppm.

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