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NOTES

A New Three-Dimensional Pulse Sequence for Correlating Intraresidue NH, N, and CO Chemical Shifts in ^{13}C, ^{15}N-Labeled Proteins

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A number of heteronuclear 3D techniques have been developed in recent years to obtain the complete assignment of ^{15}N, ^{13}C-labeled proteins (*1*). An experiment that often proves useful is one that correlates backbone NH and N resonances with the intraresidue carbonyl resonance CO (*2*). In the original experiment, named HN(CA)CO, the pulse scheme illustrated in Fig. 1a is used.

Before discussing the improved performances of the new pulse sequence, we shall briefly outline the rationale of the original experiment. NH proton magnetization is transferred to N via an INEPT scheme (*3*). After a short delay for refocussing the NH, N coupling, a constant-time monitoring of N resonance frequencies follows (t_1). During this period, the coupling N–Cα builds up, thereby allowing the coherence transfer to Cα nuclei. After an additional constant delay for the evolution of the Cα, CO coupling, the magnetization is finally transferred to the carbonyl nuclei for monitoring (t_2). Then, by reversing the pathway just described, the magnetization is brought back to the protons whence it originally started for detection (t_3). In this pulse sequence, two relatively long delays (each about 25 ms) are needed in order to transfer the magnetization from N nuclei to Cα nuclei and vice versa ($J_{N-C\alpha}$ = 11 Hz). During these delays, the magnetization resides on the N nuclei. Moreover, two additional delays (each about 7 ms) are used for the coherence to be transferred to the carbonyl nuclei from the Cα nuclei and vice versa ($J_{C\alpha-CO}$ = 55 Hz). During these periods, the magnetization resides on the Cα nuclei. In the center of the pulse sequence, the incremented t_2 period is used to monitor the carbonyl frequencies.

In Fig. 1b, we illustrate an alternative pulse scheme, which allows better sensitivity to be obtained. The magnetization sources are the Hα nuclei, whose coherence is transferred to the directly attached Cα nuclei via an INEPT scheme. A constant-time period of 26–28 ms [$2\lambda + 2T(CO)$] follows, which allows optimum refocusing of the Cα, Cβ coupling and also allows the buildup of the Cα, N coupling. At the same time, the inclusion of a constant-time [$2T(CO)$] multiple-quantum period (Cα, CO) of 10–12 ms allows the monitoring of the directly attached carbonyl frequencies (t_1). Finally, the coherence is transferred to the N resonances for constant-time [$2T(N)$] monitoring (t_2) and then to the amide protons for detection (t_3). The delay σ of 1.78 ms, followed by the proton inversion pulse, allows the refocusing of Hα, Cα couplings at the beginning of the sequence. The elimination of this coupling term is necessary, since the coherence is finally transferred to a different nucleus (namely, the amide proton) for detection. For all residues except glycines, the transfer factor to the final observable term is $\sin(2\pi J_{H\alpha,C\alpha}\sigma)$, which is equal to one for $J_{H\alpha,C\alpha}$ = 140 Hz. For glycine residues, the coupling of Cα coherences cannot be efficiently refocused with respect to both α protons at the same time, and the transfer factor is $\sin(2\pi J_{H\alpha,C\alpha}\sigma)\cos(2\pi J_{H\alpha,C\alpha}\sigma)$, which is equal to zero. Therefore, as a direct consequence of the coherence-transfer pathway of this experiment, glycine signals are expected to be filtered out or at least severely diminished, depending on the actual value of the coupling constant $J_{H\alpha,C\alpha}$ involved (*4*). The delay ρ of 2.7 ms allows the build up of the N, NH couplings prior to the final magnetization transfer to amide protons for detection.

We named our pulse sequence (HACA)CO,NH where the nuclei whose frequencies are monitored are outside brackets. The new pulse scheme (Fig. 1b) provides better sensitivity, thanks to several factors. First, the total duration of the pulse sequence is about 30 ms shorter than the original one. However, the advantage, in terms of diminished relaxation loss, is partially reduced by the increased residence time of the magnetization on the usually fast relaxing Cα nuclei (10–12 ms as multiple-quantum coherence out of 26–28 ms in total, as opposed to 14 ms in the original

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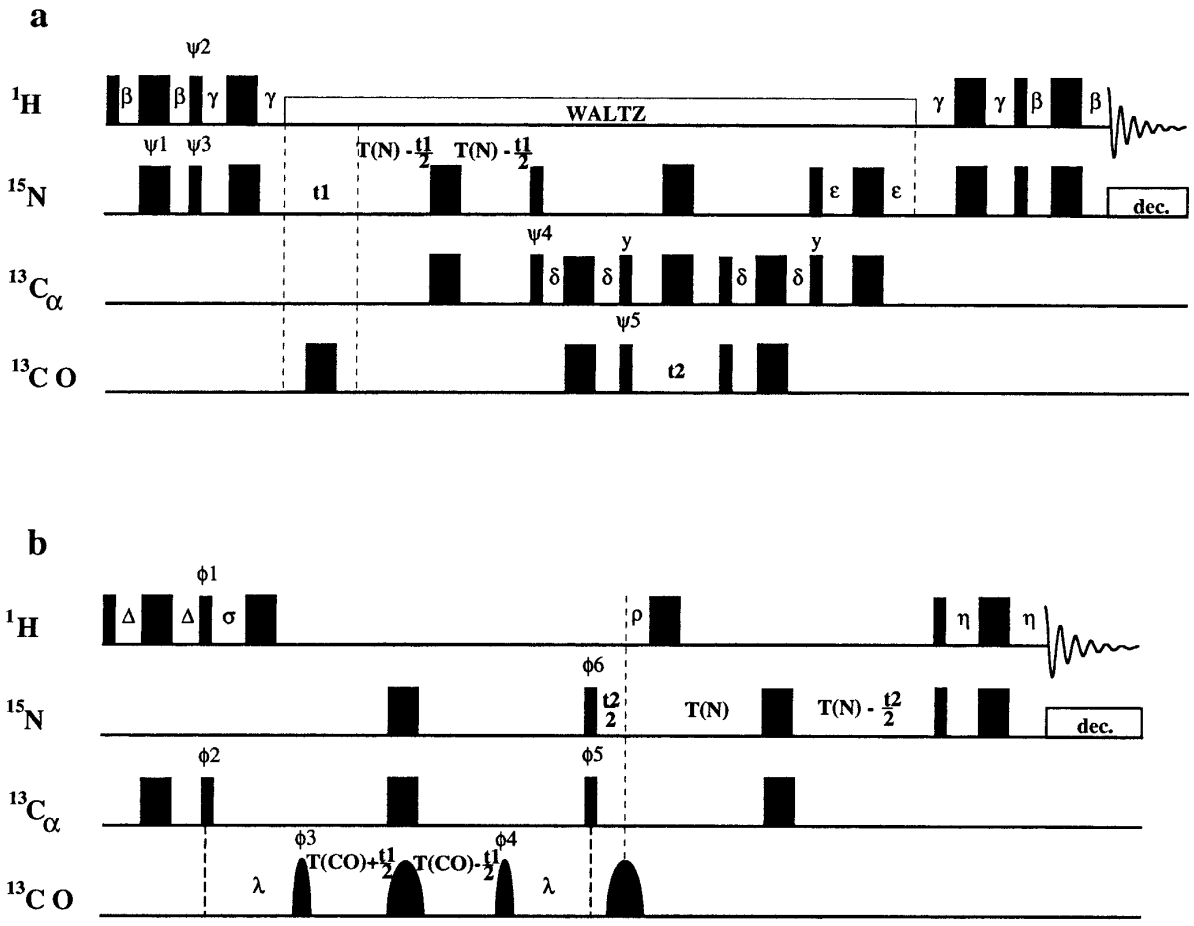

FIG. 1. (a) Pulse sequence for an HN(CA)CO experiment. Pulses (90° and 180°) are represented by thin and thick bars, respectively. Empty rectangles represent broadband decoupling. Phase cycling is as follows: $\psi_1 = x, -x$; $\psi_2 = y, -y$; $\psi_3 = 8(x), 8(-x)$; $\psi_4 = 2(x), 2(-x)$; $\psi_5 = 4(x), 4(-x)$; and receiver $= x, 2(-x), x, -x, 2(x), 2(-x), 2(x), -x, x, 2(-x), x$. ψ_3 and ψ_5 are incremented independently according to the TPPI method for quadrature detection. The delays are typically $\beta = 2.3$ ms, $\gamma = 2.7$ ms, $T(\mathrm{N}) = \epsilon = 12.5$ ms, $\delta = 3.5$ ms. (b) Pulse sequence for the (HACA)CO,NH experiment. Phase cycling is as follows: $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; $\phi_4 = 92°$; $\phi_5 = 119°$ for compensating the Bloch–Siegert effect; $\phi_5 = x$; $\phi_6 = 8(x), 8(-x)$; and receiver $= x, -x, -x, x, -x, x, x, -x, -x, x, x, -x, x, -x, -x, x$. ϕ_3 and ϕ_6 are incremented independently according to the STATES–TPPI method for quadrature detection. The delays are $\Delta = 1.4$ ms, $\sigma = 1.78$ ms, $\lambda = 8$–9 ms, $T(\mathrm{CO}) = 5$–6 ms, $\rho = 2.7$ ms, $\eta = 2.3$ ms.

sequence). On the other hand, the optimum refocusing of the passive Cα, Cβ coupling translates into a sensitivity enhancement. This gain is only slightly reduced by the occurrence of an unavoidable passive coupling of the carbonyl nuclei to their directly attached nitrogens ($J = 15$ Hz).

A further asset of the new pulse scheme is the constant-time monitoring of the carbonyl nuclei within the residence time on the Cα nuclei, so to speak, as opposed to an incremented conventional t_2 time. Both Cα and CO magnetizations are in the transverse plane while CO resonances are monitored via an HMQC (*5*) type of scheme. Moreover, fewer pulses are required in the new scheme, as is evident from a direct comparison, which also translates into a sensitivity advantage if we consider the effect of pulse imperfections. Finally, depending on the technique used for water suppression, a sensitivity gain can also derive from using Hα nuclei as the source of the magnetization. If water suppression is achieved through hard spin-lock water-dephasing pulses, as in our scheme, no sensitivity loss through cross saturation can ensue.

A possible caveat of the new experiment is the optimization of pulse phases, namely, the phase of the second 90° pulse on the carbonyl nuclei (ϕ_4) and the Cα nuclei (ϕ_5). In both cases, the Bloch–Siegert effect requires compensation, since the pulse sequence is not symmetrical on opposite sides of the central inversion pulses for either nuclei, and pulses on CO nuclei are bound to affect to some extent the evolution of Cα resonances and vice versa. The same problem, however, is also present in the original scheme (Fig. 1a), since the simultaneous inversion pulses applied on both CO and Cα nuclei are, in practice, likely to be applied consecutively. However, a practical method for compensating

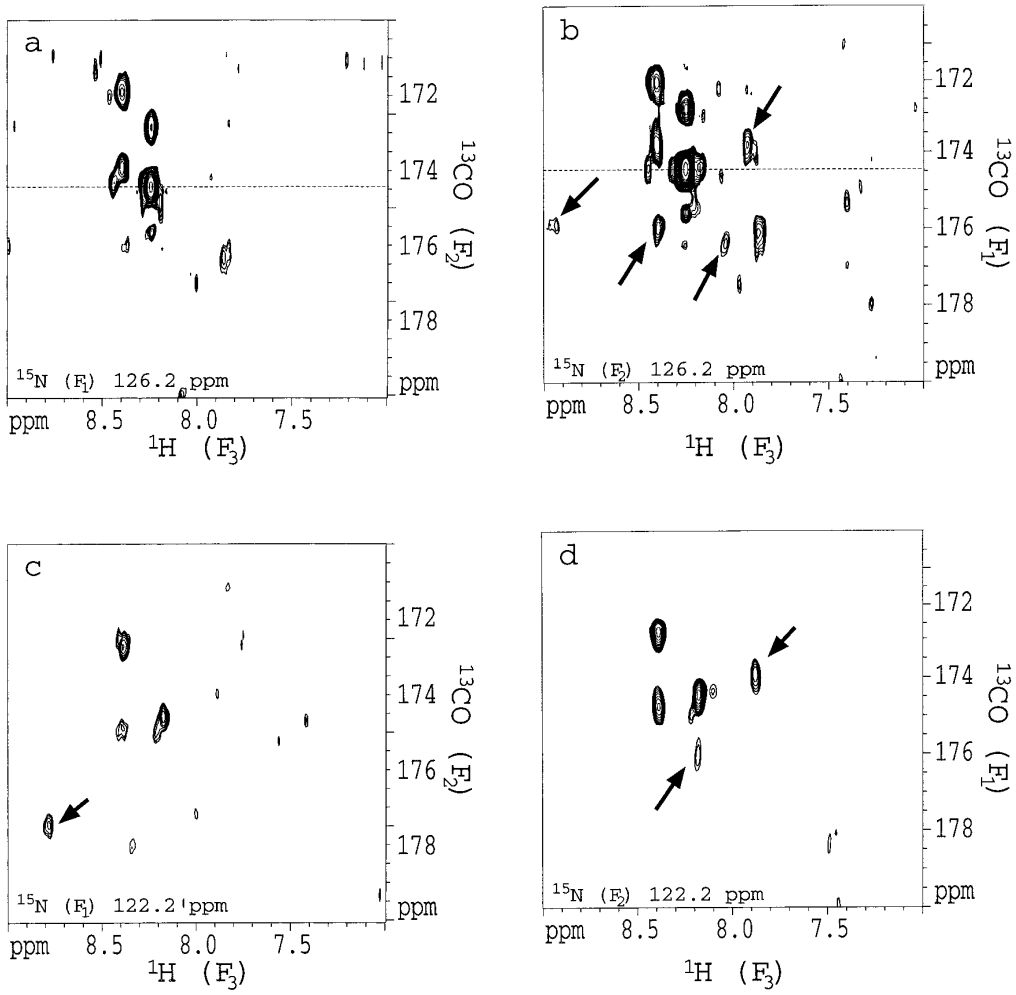

FIG. 2. Selected (f_1, f_3) planes extracted from the 3D data sets. Intraresidue correlations between NH protons and carbonyl nuclei are displayed for residues with nitrogen chemical shift of 126.2 ppm (a, b) and of 122.2 ppm (c, d). (a, c) Plots obtained from the application of the original pulse scheme HN(CA)CO. (b, d) Plots obtained from the application of the new pulse scheme (HACA)CO,NH. Correlations observed in only one of the two experiments are indicated by arrows. The spectra were recorded on a Bruker AMX 500 spectrometer, on a 0.7 m*M* sample of Il-6, at pH 6.1, at 27°C. Acquisition times used are 9.54, 24, and 63.5 ms in f_1, f_2, f_3 dimensions, respectively.

such an effect is an empirical search for maximum (or minimum) signal while the phase of the pulses is independently varied in small steps.

A correct setup of the pulse phases will automatically dispense with the need for any phase correction in the resulting signal. With regard to the execution of the inversion pulses on the Cα nuclei, the power level must be adjusted to provide the shortest pulse whose excitation profile exhibits a null in the middle of the CO resonance bandwidth. This pulse should ideally invert all the Cα, Cβ resonances while leaving the carbonyl nuclei untouched. As to the pulses on the CO nuclei, soft pulses should be used selectively on the CO resonance bandwidth only, to avoid interferences with the evolution of the Cα resonances.

The (HACA)CO,NH experiment has been applied to the backbone assignment of the human interleukin-6 (Il-6), a protein of 184 residues with a molecular weight of 21 kDa. The sample concentration was about 0.7 m*M* at pH 6.1. Spectra were recorded at 27°C on a Bruker AMX-500 MHz spectrometer. The acquired data matrices comprised 18 complex data points in the t_1 (CO) domain, 32 complex data points in the t_2 (N) domain, and 1024 complex data points in the t_3 (NH) observed dimension. Spectral widths were 15, 26, and 16 ppm in the CO, N, and NH dimensions, respectively. Effective acquisition times were 9.54, 24, and 63.5 ms in the CO, N, and NH dimension, respectively. The total measuring time was about four days. Zero filling was used in all three dimensions and the absorptive part of the final 3D spectrum consisted of $1024 \times 64 \times 128$ data points. Gaussian-to-Lorentzian apodization was used in f_3 and shifted sine-bell filtering was used in other dimensions.

The same 3D correlation experiment has been carried out

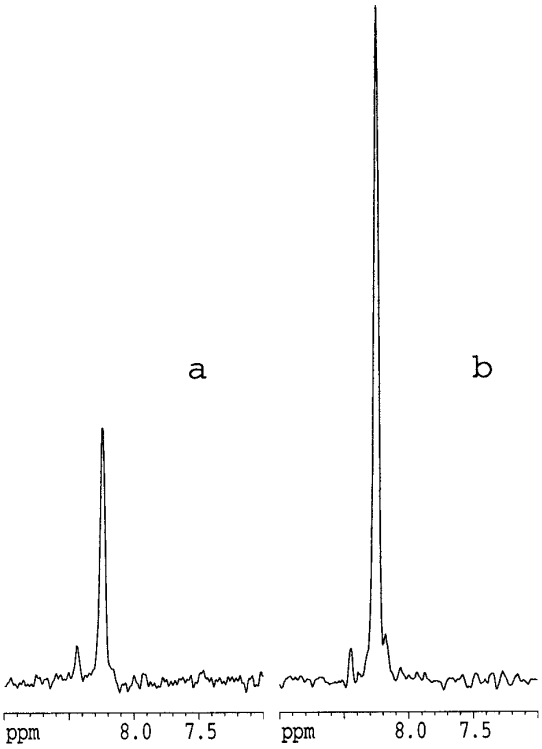

FIG. 3. Selected cross sections corresponding to the dashed lines in Figs. 2a, 2b. Plots obtained by the application of the pulse schemes HN(CA)CO and (HACA)CO,NH in (a) and (b), respectively.

using the original HN(CA)CO pulse sequence. In order to show the relative sensitivity of the two experiments, equivalent measuring time, acquisition, and processing parameters were used. In both cases, our goal of observing the complete set of intraresidue H−N−CO correlations was only partially achieved. This was not unexpected, since the sensitivity of this type of correlation is known to be critical due to a less favorable heteronuclear coupling network and relaxation behavior, compared to other heteronuclear correlation experiments. However, the percentage of observed correlations increased from 33% using the original HN(CA)CO sequence to 56% using our new (HACA)CO,NH sequence. Nevertheless, a few correlations (5%) present in the original experiment failed to show up in the new experiment.

The relative performances of the two pulse sequences are compared in Fig. 2 where representative planes corresponding to the same nitrogen frequencies are extracted from the two 3D data sets. Intraresidue correlations between NH protons and carbonyl nuclei are displayed for residues with a nitrogen chemical shift of 126.2 and 122.2 ppm in Figs. 2a, 2b and Figs. 2c, 2d, respectively. Plots in Fig. 2a, 2c are derived from the application of the original HN(CA)CO scheme, whereas plots in Figs. 2b, 2d are obtained with our (HACA)CO,NH scheme. The average sensitivity gain obtained by the new experiment is evident from the direct comparison. Additional correlations that are observed only with the new experiment are indicated by corresponding arrows (Fig. 2b, 2d). On the other hand, one particular correlation is shown in Fig. 2c which is missing in the new experiment. In summary, using our scheme, 95 correlations are observed out of the 170 theoretically expected from the number of residues excluding glycines, prolines, and the initial residue. We observed 49 additional peaks, whereas 10 are lost, compared with the original experiment. In Fig. 3, the two cross sections corresponding to the dashed line in Figs. 2a, 2b are reported. The sensitivity gain obtained for this particular correlation corresponds to a factor 2.5. However, the gain turns out to be rather variable throughout the spectrum and indeed some correlations even exhibit a loss, as already stated. If we consider the 46 peaks that are present in both experiments, we can roughly estimate an average gain of about 35%.

We can conclude that the new mechanism of coherence transfer introduced by our pulse scheme allowed significantly greater sensitivity to be obtained for most intraresidue NH−N−CO correlations. Nevertheless, the relation between sensitivity and pulse sequence turned out to be rather complex. Although a systematic analysis of all aspects of this problem is beyond the scope of this Note, the crucial element is the fast relaxation of Cα magnetizations. Indeed, the inherent better efficiency of the new method can be undermined by the very fast relaxation of particular Cα resonances. However, even in a very critical case, like that illustrated here where almost half of the expected signals turned out to be missing in the end, the overall performance of the new sequence is significantly superior compared to the original scheme.

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