JOURNAL OF MAGNETIC RESONANCE, Series B 107, 189–191 (1995)

A New 3D HCACO Pulse Sequence with Optimized Resolution and Sensitivity. Application to the 21 kDa Protein Human Interleukin-6

RENZO BAZZO,* DANIEL O. CICERO, AND GAETANO BARBATO

Istituto di Ricerca di Biologia Molecolare P. Angeletti spa, Via Pontina km 30.600, Pomezia 00040, Rome, Italy

Received December 13, 1994; revised January 31, 1995

A number of heteronuclear 3D techniques have been developed in recent years in order to obtain the complete assignment of $^{15}N/^{13}C$-labeled proteins (*1*). An experiment that often proves useful is one which correlates backbone H_α and C_α resonances with the intraresidue carbonyl resonance (CO).

In the constant-time version of the original experiment (CT-HCACO) (*2*), the pulse scheme illustrated in Fig. 1a is used. Let us briefly outline the rationale of this experiment. H_α magnetization is transferred to C_α via an INEPT scheme. At the end of the t_1 period, C_α magnetization is transferred to the CO nucleus, which evolves during the second evolution period t_2, prior to being transferred back to C_α and then to H_α for detection. During the constant-time $2T$ period, C_α frequencies are monitored, while at the same time the coupling with CO builds up (J = 55 Hz) in order to allow for the successive transfer. Unfortunately, another undesired coupling interaction, between C_α and C_β (J = 35 Hz), introduces a passive splitting of C_α resonances, which affects sensitivity negatively. Therefore, a compromise is made on the actual duration of the constant-time $2T$ period, which cannot exceed about 7 ms, thereby limiting C_α resolution. However, the sensitivity loss due to the C_α, C_β coupling is not negligible if we consider that an equivalent time for refocusing is used in the second half of the pulse sequence. The total loss is given by the expression $[\cos(2\pi J(C_\alpha, C_\beta)T)]^2$, which, for $J(C_\alpha, C_\beta)$ = 35 Hz and $2T$ = 7 ms, amounts to a factor of 2.

The new pulse sequence is illustrated in Fig. 1b, and we have named it HACACO. It follows a different rationale. The t_1 evolution of the C_α nucleus takes place within a constant-time period, which allows optimum refocusing of the C_α, C_β passive coupling: $2T(C_\alpha)$ = 28 ms = $1/J(C_\alpha, C_\beta)$. Therefore, C_α resolution can be entirely adequate, equivalent to the resolution of a constant-time 2D HSQC experiment (*3*). However, the novelty of this scheme is that the t_2 evolution period for the attached CO resonances is also obtained in a constant-time fashion [$2T$(CO)] and within the same constant time [$2T(C_\alpha)$] used for C_α resonances. Clearly, in the central part of the sequence, both nuclei, C_α and CO, are precessing in the transverse plane and are evolving as C_α, CO multiple-quantum coherences. Nevertheless, their frequencies are separately monitored by regular independent shifting of the corresponding 180° pulses.

During such a period, $J(C_\alpha, CO)$ is clearly inactive, whereas $J(C_\alpha, C_\beta)$ is active as in the rest of the $2T(C_\alpha)$ period. The intervening long-range coupling $J(CO, C_\beta)$ is very small and can be neglected. The constant-time $2T$(CO) for CO evolution amounts to 12 ms and it is sandwiched by the two periods (8 ms each) dedicated to build up and refocusing of C_α, CO coupling. The entire $2T(C_\alpha)$ period (28 ms) can be exploited for C_α frequency evolution, regardless of the different physical entities evolving in the different intervals: either single-quantum C_α coherences or multiple-quantum C_α, CO coherences. The implementation of frequency evolutions of both nuclei in a constant-time fashion renders all mutual and external coupling interactions transparent to detection.

A first caveat of this experiment is the execution of the pulse sequence if the t_1 duration exceeds the 12 ms corresponding to the central interval. Then the 180° pulse on C_α will be beyond the multiple-quantum central period, at some advanced stage of the t_1 monitoring. At this point, such a pulse must be applied in concert with a 180° pulse on CO in order to allow proper refocusing. That is the purpose of the insertion of a second 180° pulse on CO, which is conveniently kept fixed in the first part of the C_α frequency monitoring. This extra pulse (indicated with dashed lines in Fig. 1b) is clearly avoided if the C_α frequency monitoring takes place completely within the central 12 ms multiple-quantum period.

A second caveat of this experiment is the optimization of pulse phases, namely the phase of the second 90° pulse on both the carbonyls and the C_α nuclei ($\phi 6$ and $\phi 8$, respectively, in Fig. 1b). For both nuclear types, the Bloch–Siegert effect requires compensation, since the pulse sequence is not symmetrical on opposite sides of the shifting 180° pulses for either nucleus and, as is well known, pulses on CO nuclei will affect the evolution of the C_α resonances and vice versa.

* To whom correspondence should be addressed.

1064-1866/95 $6.00

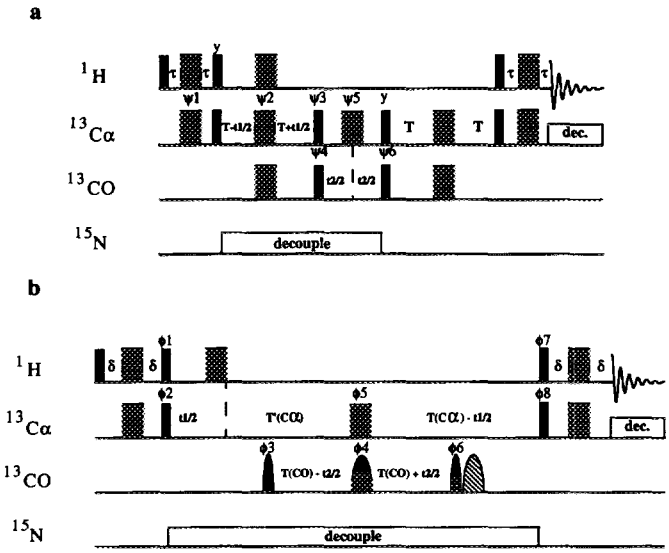

FIG. 1. (a) Pulse scheme of the CT-HCACO experiment. Phase cycling is as follows: $\psi 1 = 4x, 4(-x)$; $\psi 2 = 8x, 8(-x)$; $\psi 3 = 4y, 4(-y)$; $\psi 4 = x, -x$; $\psi 5 = 8x, 8(-x)$; $\psi 6 = 2x, 2(-x)$; Acq. $= x, 2(-x), x, -x, 2x, -x$. The durations of the fixed delays are $\tau = 1.6$ ms, $T = 3.5$ ms. (b) Pulse scheme of the HACACO experiment. Phase cycling is as follows: $\phi 1 = y, -y$; $\phi 2 = 2x, 2(-x)$; $\phi 3 = 8x, 8(-x)$; $\phi 4 = 16x, 16y$; $\phi 5 = 4x, 4y$; $\phi 7 = 32x, 32(-x)$; Acq. $= A, 2(-A), A$, where $A = [x, 2(-x), x, (-x), 2x, 2(-x), 2(x), (-x), x, 2(-x), x]$; $\phi 6 = 95°$ and $\phi 8 = 124°$ have been empirically determined to compensate for the Bloch–Siegert effect. The durations of the fixed delays are $T(\mathrm{CO}) = 6$ ms; $T(\mathrm{C}_\alpha) = 14$ ms; $T'(\mathrm{C}_\alpha) = T(\mathrm{C}_\alpha) - \tau_{180°}(^1\mathrm{H})$. The carbonyl inversion pulse indicated with dashed lines is executed in concert with the C_α inversion pulse $\phi 5$ only when and if the latter exits the $2T(\mathrm{CO})$ interval to the right while t_1 is monitored. Otherwise it is omitted.

The appropriate phases for compensating such an effect can be easily determined by an empirical search for maximum (or minimum) signal while the pulse phases are independently varied in small steps. A correct setup of the pulse phases automatically dispenses with any phase correction at the data-processing stage. With regard to the application of the 180° to the C_α nuclei, the power level must be adjusted in order to provide the shortest pulse whose excitation profile exhibits a null point in the middle of the CO resonance bandwidth. This pulse should ideally invert C_α, C_β resonances while leaving the carbonyls untouched. As to the pulses on the CO nuclei, soft pulses should be used selectively on the CO resonance bandwidth, in order to minimize interferences with the evolution of the C_α nuclei.

In this new pulse sequence (HACACO), the optimum refocusing of the passive C_α, C_β coupling allows a constant time of about 28 ms to be used for C_α evolution, thereby greatly improving the resolution with respect to the original experiment. The carbonyl evolution is conveniently inserted within the same time interval, thereby allowing for the desired 3D correlation to be performed with minimum relaxation loss. Incidentally, this experiment allows prompt assignment of all glycine C_α cross peaks, since they are not coupled to any aliphatic carbon spin and, as a result, have opposite signs to all other C_α correlations.

The sensitivity of this experiment is comparable with the corresponding constant-time 2D HSQC experiment correlating H_α with C_α (*3*). The insertion of the CO evolution in the central part of the sequence will not significantly diminish signal intensity, due to the comparatively favorable relaxation properties of carbonyls. If we compare our HACACO scheme (Fig. 1b) with the original CT-HCACO experiment (*3*) reported in Fig. 1a, we note the longer time spent by C_α magnetizations in the transverse plane (28 ms versus 14 ms). However, this is compensated by the insertion of the CO evolution in the very same delay (in a constant-time fashion) and by the refocusing of the passive C_α, C_β coupling. More-

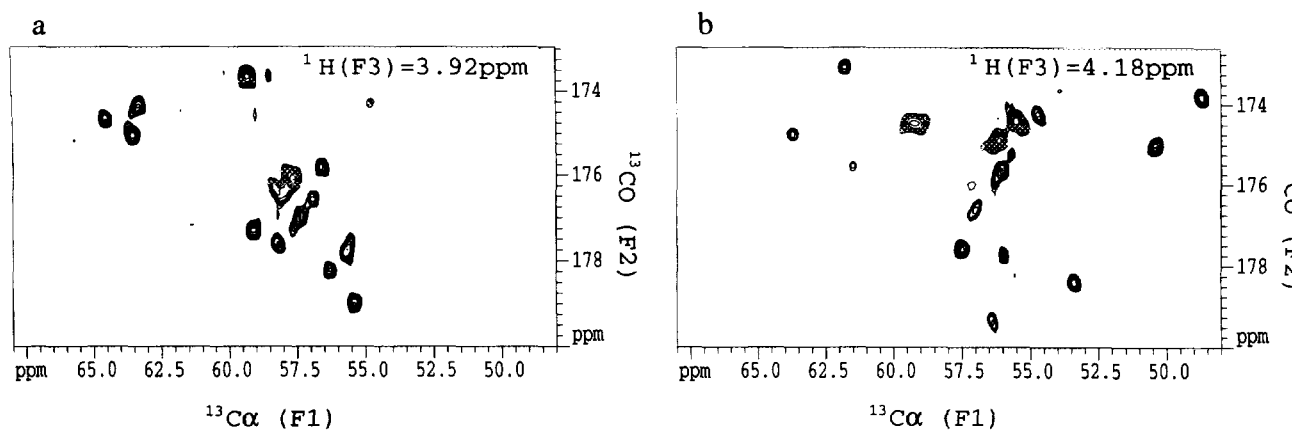

FIG. 2. Selected planes from the H_α–C_α–CO 3D correlation spectrum recorded with the HACACO scheme (Fig. 1b), for the protein human interleukin-6. C_α, CO correlations are displayed for two different chemical shifts of H_α: (a) 3.92 ppm and (b) 4.18 ppm. The spectrum is recorded using a Bruker AMX 500 MHz spectrometer and a 0.7 m*M* sample, pH 6.2, at 27°C. Acquisition times are 11.7, 11.7, and 63.5 ms in the F_1, F_2, and F_3 dimension, respectively.

over, the new pulse scheme requires fewer pulses, particularly on the C_α nuclei, which is an additional advantage in so far as pulse performance is far from ideal in systems requiring selectivity of pulses on carbonyls and aliphatic carbons, alternatively. In this respect, another advantage of the new scheme is the fact that no "simultaneous" pulsing on C_α, CO is required.

The HACACO experiment described in Fig. 1b has been applied to the backbone assignment of human interleukin-6, a protein of 184 residues with a molecular weight of 21 kDa. The sample concentration was about 0.7 m*M* at pH 6.2. Spectra were recorded at 27°C on a Bruker AMX 500 MHz spectrometer. The acquired data matrices comprised 38 complex data points in the t_1 (C_α) domain, 22 complex data points in the t_2 (CO) domain, and 512 complex data points in the t_3 (H_α) observed dimension. Spectral widths were 3269, 1886, and 8064 Hz in the C_α, CO, and H_α dimension, respectively. Effective acquisition times were 11.7, 11.7, and 63.5 ms in the C_α, CO, and H_α dimension, respectively. The total measuring time was about four days. Mirror-image linear prediction (*4*) was used in both constant times $T(C_\alpha)$ and $T(CO)$ in order to further extend the resolution. Zero filling was used in all three dimensions and the absorptive part of the final 3D spectrum consisted of 256 $\times$ 128 $\times$ 1024 data points. Shifted sine-bell filtering was used in all three dimensions. Figure 2 shows typical (F_1, F_2) planes displaying intraresidue correlations between C_α and CO for residues with a H_α chemical shift of (Fig. 2a) 3.92 ppm and (Fig. 2b) 4.18 ppm. This new HACACO experiment described here (Fig. 1b) provides a fourfold enhancement in the resolution that is attainable in the C_α dimension, compared with the original experiment CT-HCACO (Fig. 1a), without undermining sensitivity.

A related scheme has been described by Kay *et al.* (*5*) for monitoring H_α, C_α correlations in a 4D experiment. They used a shared constant-time evolution period for the H_α and C_α resonances, but limited the evolution time to the central multiple-quantum period (3 ms). In analogy with the rationale of our HACACO pulse sequence, their scheme could also be improved by extending the C_α evolution time to the entire period (about 8 ms) in which C_α resonances are in the transverse plane. Again a significant (more than twofold) resolution enhancement in the C_α dimension would result, without affecting sensitivity.

REFERENCES

1. A. Bax and S. Grzesiek, *Acc. Chem. Res.* **26**(4), 131 (1993).
2. R. Powers, A. M. Gronenborn, G. M. Clore, and A. Bax, *J. Magn. Reson.* **94**, 209 (1991).
3. G. W. Vuister and A. Bax, *J. Magn. Reson.* **98**, 428 (1992).
4. G. Zhu and A. Bax, *J. Magn. Reson.* **98**, 192 (1992).
5. L. E. Kay, M. Wittekind, M. A. McCoy, M. S. Friedrichs, and L. Muller, *J. Magn. Reson.* **98**, 443 (1992).