JOURNAL OF MAGNETIC RESONANCE **126,** 270–273 (1997)
ARTICLE NO. MN971153

An Easy Way for Resolution Enhancement in Out-and-Back Triple-Resonance Experiments Applied to the HCACO Sequence

Matthias Baur and Horst Kessler*

Institut für Organische Chemie und Biochemie, TU München, Lichtenbergstrasse 4, D-85747 Garching, Germany

Received October 11, 1995; revised December 18, 1996

In recent years, a set of triple-resonance experiments has been developed for the complete assignment of backbone resonances of $^{15}N/^{13}C$-labeled proteins (*1–7*). These experiments are called out-and-back experiments, because the pathway of magnetization leads from initial excitation of a proton to heteronuclei and back to the originating proton. A key element in most assignment strategies is the highly sensitive HCACO experiment, correlating H^{α}, C^{α} and intraresidual CO. With increasing number of residues the assignment task faces the problems of signal loss due to fast transverse relaxation and increasing peak overlap. To overcome these problems in the HCACO experiment, we implemented minor changes to the existing out-and-back sequence (*3*), yielding a genuine twofold increase in resolution in the critical C^{α} dimension with 14 ms constant-time evolution instead of 7 ms. The theoretical sensitivity is 64% of the original HCACO (*3*). When these modifications are applied to a HCACO with sensitivity-enhanced ^{1}H detection (*8*), the sensitivity is 64% of the sensitivity-enhanced HCACO.

For proteins of 100 and more amino acids, severe peak overlap is observed in the $H^{\alpha}-C^{\alpha}$ projection of the standard out-and-back HCACO experiment (*3*), which has 7 ms of constant-time C^{α} evolution. This often leads to misassignments of the intraresidual CO. Basically, the constant-time evolution periods of out-and-back experiments can be easily prolonged, even to exceed the optimal coupling delay length (*9*). However, constant-time evolution periods of C^{α} in uniformly ^{13}C-labeled proteins, when simultaneously used for build up or refocusing for $C^{\alpha}-CO$ antiphase magnetization, must have a period length of 7 or 28 or 50 ms, etc. These periods are a compromise between losing magnetization from the $^{1}J(C^{\alpha}, C^{\beta})$ coupling and ensuring build up or refocusing of $C^{\alpha}-CO$ antiphase magnetization. A C^{α} constant-time evolution period of 28 ms or more is prohibitive in *large* proteins or when partial aggregation or chemical exchange is present, because in these cases the single-quantum transverse relaxation time T_2 may be much shorter than 28 ms. For example, when a protein with an overall correlation time τ_c of 10 ns is observed at 600 MHz, the transverse dipolar relaxation time T_2 is only about 14 ms (*10*). Various approaches have been developed to address this problem. A postacquisitional mirror-image linear-prediction technique has been developed to double the resolution in the C^{α} domain (*11*). However, due to imperfections in the experimental data, the assumption of constant signal amplitude is often not met, and mirror-image linear prediction fails.

A different approach has been to replace the $C^{\alpha}-CO$ HSQC correlation with a $C^{\alpha}-CO$ HMQC period of 28 ms fixed length for joint evolution of C^{α} and CO in a constant-time fashion (*12, 13*). As the entire HMQC period can be used for C^{α} evolution, a genuine fourfold resolution is possible. However, the decay of transverse mixed zero- and double-quantum magnetization during the $C^{\alpha}-CO$ HMQC period is even faster than the decay of transverse C^{α} magnetization (*14*). This results in a critical loss of signal intensity in cases where the C^{α} transverse relaxation time is very short. As we faced very short C^{α} transverse relaxation times but major overlap in our sample of the 31 kDa homodimeric IIA^{Man} subunit of the mannose transporter of *Escherichia coli* (*15, 16*), we developed a modification of the standard HCACO experiment that allows simultaneous use of the $C^{\alpha}-CO$ INEPT and $CO-C^{\alpha}$ REVINEPT periods for C^{α} evolution.

The modified HCACO with sensitivity-enhanced ^{1}H detection is shown in Fig. 1. As usual, the constant-time period is set to $2T = 7$ ms. During the first 7 ms of evolution in the C^{α} domain, only the pulses and gradients shown in black are executed. This amounts to a standard HCACO (*3*) experiment with the C^{α} chemical shift evolving during the $CO-C^{\alpha}$ REVINEPT delay. A step-by-step description of the magnetization pathway is given in (*3*). During the subsequent C^{α} evolution from 7 to 14 ms the lightly marked gradients are additionally executed and the C^{α} 180° pulse in the $C^{\alpha}-CO$ INEPT period (marked *P* in Fig. 1) is shifted with each time increment. The C^{α} 180° pulse in the $CO-C^{\alpha}$ REVINEPT period (marked *Q* in Fig. 1) is left in the excentered position reached for recording the last point of the first evolution set. The additional gradients generate a

* To whom correspondence should be addressed.

1090-7807/97 $25.00

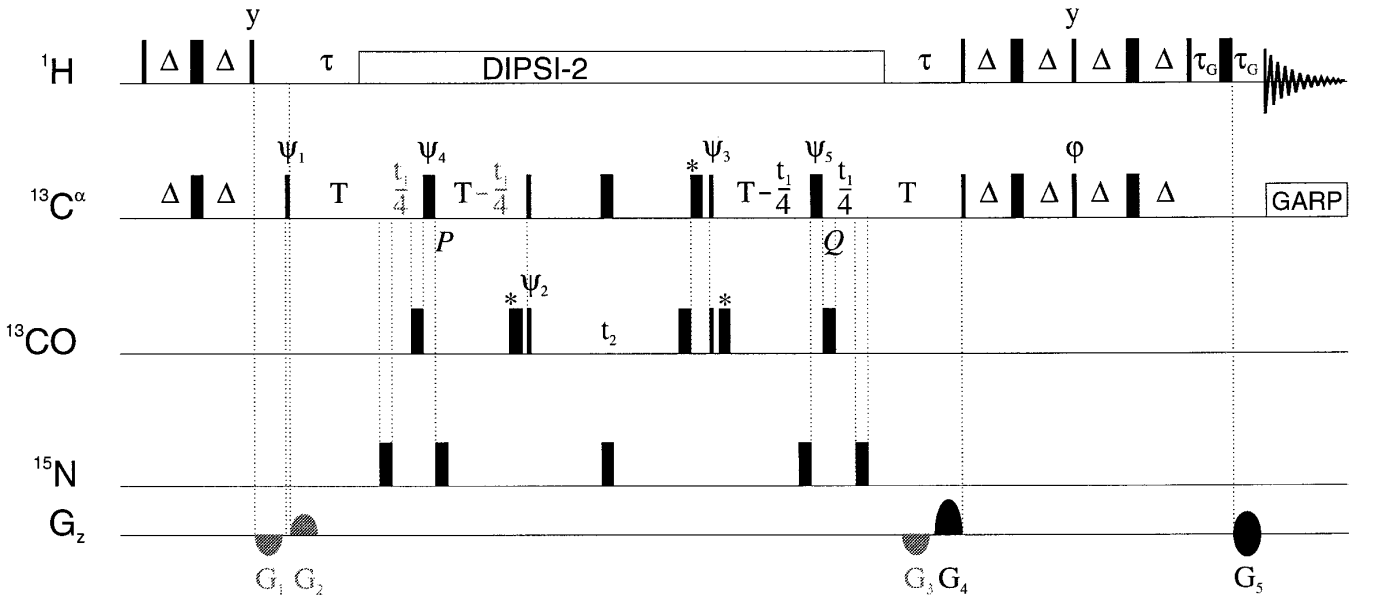

FIG. 1. Modified HCACO experiment, with genuine twofold increase in resolution in the C^α domain. Each C^α dimension was recorded in two sets of 64 points each. During the first 64 C^α points, only the pulses and gradients marked in black were applied. During the second 64 C^α points, the lightly marked gradients G_1, G_2, and G_3 were additionally applied. In a pulse program for a Bruker spectrometer (Bruker, Rheinstetten), this is achieved by repeating the pulse-sequence code twice, grouping gradients G_1, G_2, and G_3 immediately before the first ^{1}H 90° pulse in the code for the first set and placing them as shown above in the code for the second set. Quadrature detection in C^α during the first set was performed in hypercomplex mode (*8*), inverting G_5 and φ on alternate scans and storing echo and antiecho magnetization separately. Time increments were executed by shifting pulse Q in the CO–C^α REVINEPT delay. Prior to each time increment, the sign of ψ_3 and Acq. was inverted. Quadrature detection in C^α during the second set followed the same procedure, with the sole exception that time increments were executed by shifting pulse P in the C^α–CO INEPT delay, while leaving pulse Q in its excentered position. Following the acquisition of all 128 points, both pulses P and Q were reset prior to acquisition of the next CO increment. Quadrature detection in CO was performed according to the States (*17*) protocol. The phase cycling was $\psi_1 = 4(x), 4(-x)$; $\psi_2 = 2(x), 2(-x)$; $\psi_3 = x, -x$; $\psi_4 = 32(x), 32(y), 32(-x), 32(-y)$; $\psi_5 = 8(x), 8(y), 8(-x), 8(-y)$; Acq. $= 2(R, -R), 2(-R, R), 2(R, -R), 2(-R, R)$, where $R = x, -x, -x, x, -x, x, x, -x$. Pulses where no phase is given were applied along the x axis. An asterisk marks C^α and CO pulses that were included to compensate for Bloch–Siegert effects. Pulsed-field-gradient (PFG) strengths were $G_1 = -6\%$, $G_2 = 6\%$, $G_3 = -6\%$, $G_4 = 79.5\%$, and $G_5 = 20\%$, where 100% equals approximately 70 G/cm. PFGs had sine-bell shape with a duration of 0.8 ms followed by a 0.7 ms recovery delay. Delays were $\Delta = 1.7$ ms, $\tau = 3.5$ ms, $2T = 7$ ms, and $\tau_G = 1.5$ ms. The recycle delay was 1 s. RF power was 25 kHz for the ^{1}H hard pulse, 5 kHz for the ^{1}H DIPSI-2 (*18*) decoupling, 4.5 kHz for the C^α and CO pulses, 2.8 kHz for the C^α GARP (*19*) decoupling, and 5 kHz for the ^{15}N pulse.

phase shift of the magnetization in the CO–C^α REVINEPT period with exactly the phase accumulated by evolution in the C^α–CO INEPT period. A detailed description of this process is given below. Due to the activity of the additional gradients, the signal-to-noise ratio (S/N) is lowered to 1/2 of its previous value during the first 7 ms of evolution. To compensate for this loss of sensitivity, four times as many scans are accumulated. Overall, the averaged S/N of the modified HCACO is $\sqrt{2}/\sqrt{5} = 64\%$ of that observed in the standard HCACO.

When C^α is observed from 7 to 14 ms, gradients G_1, G_2, and G_3 are applied. Gradient G_2 is of equal strength and sign opposite to gradient G_3. The phase of the transverse C^α magnetization, accumulated in the C^α–CO INEPT delay, is $\zeta = \Omega(C^\alpha)t_{1IN}$, where $\Omega(C^\alpha)$ denotes the resonance frequency of the C^α nucleus and t_{1IN} denotes the length of chemical-shift evolution in the C^α–CO INEPT delay. Gradient G_2 turns the magnetization into a z coil, encoding this phase ζ. Gradient G_3 untwists the z coil upon execution, performing a phase shift of exactly $+\zeta$ on the transverse magnetization. Therefore, at the end of the CO–C^α REVINEPT period, the transverse magnetization is rotated to a phase $\zeta' = \Omega(C^\alpha)\{t_{1IN} + t_{1REV}\}$, where t_{1REV} denotes the length of chemical-shift evolution in the CO–C^α REVINEPT delay. This is exactly the same phase as if one continuous free chemical-shift evolution had taken place, and it is detected via the sensitivity-enhancement scheme of Kay *et al.* (*8*). To avoid signal loss due to diffusion, the strength of the gradients G_2 and G_3 were set to be only 4 G/cm for a duration of 0.8 ms. Gradient G_1 was necessary for obtaining good water suppression, compensating for the action of G_2 on the water signal. The additional scans during the second set of C^α evolution were used for phase cycling the C^α 180° pulse P in the C^α–CO INEPT delay in steps of 90°. This suppresses artifacts typical for constant-time schemes.

The HCACO experiment of Fig. 1 has been applied to the homodimeric IIA^{Man} subunit of the mannose transporter of *E. coli* (*15, 16*). A [U-^{15}N/^{13}C] sample was used, which

has a molecular weight of 31 kDa. The monomeric concentration was about 1 mM in 90% H_2O/10% D_2O at pH 7.5. The protein IIA^{Man} exhibits a set of NMR signals corresponding to the 135 amino acids of the monomer. The measurement was performed at 310 K on a Bruker AMX600 spectrometer. The acquisition order was CO–C^α–H^α. Figure 2 compares H^α–C^α projections processed from the first CO increment, using only the first 64 points in the C^α domain (a) or all 128 points (b). Spectra a and b are plotted at the same signal level. Resolution in Fig. 2a is identical to the result obtained with the standard HCACO sequence. Measurement time was 42 min for these 64 points. Figure 2b shows the same H^α–C^α region as that processed from the full 128 points in the C^α domain. As expected, the C^α linewidths are clearly reduced, leading to a separation of some previously unresolved peaks. Measurement time was 210 min for the 128 C^α points.

To check the S/N ratio, 1D slices were extracted from the spectra of Fig. 2 at the C^α shifts of Val134 and Glu135. Figure 3 shows the 1D slices and gives the signal-to-noise ratios. To compensate for the different measurement times (42 min for Fig. 2a and 210 min for Fig. 2b), the S/N value given for rows from Fig. 2b was divided by $\sqrt{210 \text{ min}/42 \text{ min}}$. The signal of Glu135 shows a S/N ratio of 67%, fulfilling the theoretical expectations (see above). However, the Val134 resonance has a S/N of only 55%. We determined that this difference is due to the partial excitation of C^α magnetization by the rectangular CO pulses (4.5 kHz RF power) and vice versa. The use of shaped selective pulses for C^α and CO is therefore highly recommended.

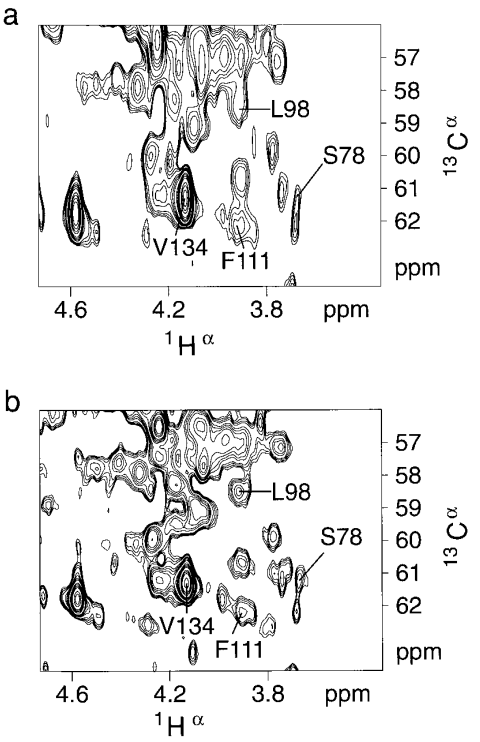

FIG. 2. Details from the H^α–C^α projection (first CO increment), recorded with the resolution-enhanced HCACO experiment of Fig. 1. (a) Only the first 64 points in C^α were processed. (b) All 128 C^α points were used. The intensities in the second 64 points of the C^α time-domain data were divided by 2 before processing to obtain a homogenous scaling of signals and noise. Spectra a and b are plotted at the same level. In the H^α domain, 1024 points were collected. Spectral widths were 4166 and 5000 Hz in the H^α and C^α domains, respectively. Both data sets were processed in the States hypercomplex manner (*20*) to a matrix of 2048 × 256 real points after application of a 90°-shifted sine-bell window function and subsequent zero filling. Measurement time for the first 64 points in C^α was 42 min (a) and for the full 128 points, 210 min (b).

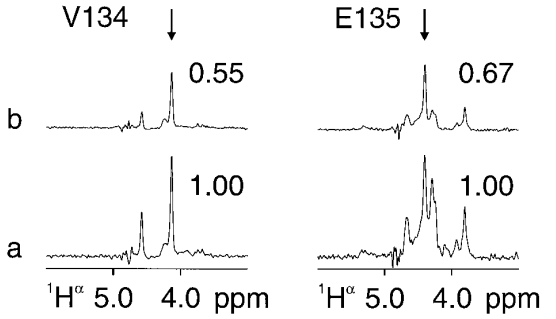

FIG. 3. One-dimensional slices from the spectra of Fig. 2a (a) and Fig. 2b (b). The numbers indicate the relative S/N compared to the slices shown in (a). To compensate for the different measuring times (42 min for Fig. 2a and 210 min for Fig. 2b), the S/N values and plots given for slices (b) were divided by $\sqrt{210 \text{ min}/42 \text{ min}}$.

In conclusion, we describe a method for obtaining a genuine twofold resolution increase in the C^α domain of the HCACO sequence. Only minor changes to the HCACO sequence are necessary. The theoretical S/N of the modified experiment is 64% of the original HCACO. The successful application of the modified HCACO experiment to the 31 kDa IIA^{Man} homodimeric protein domain was demonstrated, realizing the theoretical S/N to a good degree. The method presented is universally applicable to all out-and-back experiments using constant-time evolution, yielding a genuine doubling of resolution in the heteronuclear domains with only minor changes to the pulse sequences.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We thank G. Gemmecker (Technische Universität München) for helpful discussions.

REFERENCES

1. L. E. Kay, M. Ikura, R. Tschudin, and A. Bax, *J. Magn. Reson.* **89,** 496 (1990).
2. S. Grzesiek and A. Bax, *J. Magn. Reson.* **96,** 432 (1992).
3. R. Powers, A. M. Gronenborn, G. M. Clore, and A. Bax, *J. Magn. Reson.* **94,** 209 (1991).

4. D. R. Muhandiram and L. E. Kay, *J. Magn. Reson. B* **103,** 203 (1994).
5. J. Schleucher, M. Sattler, and C. Griesinger, *Angew. Chem. Int. Ed. Engl.* **32,** 1489 (1993).
6. L. E. Kay, M. Ikura, and A. Bax, *J. Magn. Reson.* **91,** 84 (1991).
7. S. Seip, J. Balbach, and H. Kessler, *J. Magn. Reson.* **100,** 406 (1992).
8. L. E. Kay, P. Keifer, and T. Saarinen, *J. Am. Chem. Soc.* **114,** 10663 (1992).
9. S. Grzesiek and A. Bax, *J. Biomol. NMR* **3,** 185 (1993).
10. T. Yamazaki, R. Muhandiram, and L. E. Kay, *J. Am. Chem. Soc.* **116,** 8266 (1994).
11. G. Zhu and A. Bax, *J. Magn. Reson.* **90,** 405 (1990).
12. R. Bazzo, D. O. Cicero, and G. Barbato, *J. Magn. Reson. B* **107,** 189 (1995).
13. J. Engelke and H. Rüterjans, *J. Magn. Reson. B* **109,** 318 (1995).
14. T. J. Norwood and J. Pereira, *J. Magn. Reson. A* **112,** 160 (1995).
15. B. Stolz, M. Huber, Z. Markovic-Housley, and B. Erni, *J. Biol. Chem.* **268,** 27094 (1993).
16. S. Seip, J. Balbach, S. Behrens, H. Kessler, K. Flükiger, R. de Meyer, and B. Erni, *Biochemistry* **33,** 7174 (1994).
17. D. J. States, R. A. Haberkorn, and D. J. Ruben, *J. Magn. Reson.* **48,** 256 (1982).
18. A. J. Shaka, C. J. Lee, and A. J. Pines, *J. Magn. Reson.* **77,** 274 (1988).
19. A. J. Shaka, P. B. Barker, and R. Freeman, *J. Magn. Reson.* **64,** 547 (1985).
20. A. L. Davis, J. Keeler, E. D. Laue, and D. Moskau, *J. Magn. Reson.* **98,** 207 (1992).