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Improved 3D gd-HCACO and gd-(H)CACO-TOCSY experiments for isotopically enriched proteins dissolved in H_2O

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Summary

Pulsed field gradients were incorporated into the HCACO experiment for acquiring spectra on isotopically enriched protein samples dissolved in H_2O. Excellent water suppression and spectral quality were achieved using the modified pulse sequence (gd-HCACO), as demonstrated for a ^{13}C-/^{15}N-labeled sample of the SH2 domain from the hematopoietic cellular kinase dissolved in 90% H_2O/10% D_2O. Strong correlations for all residues were observed in the gd-HCACO spectrum, even for residues having α-protons resonating exactly at the H_2O frequency. The HCACO-TOCSY experiment was modified to correlate intraresidue $^{13}C^{\alpha}$ (rather than $^{1}H^{\alpha}$), carbonyl ($^{13}C'$), and aliphatic side-chain protons [(H)CACO-TOCSY]. Pulsed field gradients were also incorporated into the (H)CACO-TOCSY experiment for water suppression.

The 3D HCACO experiment correlates intraresidue $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, and carbonyl ($^{13}C'$) resonances of ^{13}C-labeled proteins (Ikura et al., 1990; Kay et al., 1990; Powers et al., 1991; Grzesiek and Bax, 1993) and is very useful for sequence-specific resonance assignments. This experiment is normally performed in D_2O since it detects $^{1}H^{\alpha}$ resonances, which can be obscured by the strong water signal for samples dissolved in H_2O. Preparation of a separate, isotopically enriched protein sample in D_2O not only increases the cost for protein production, but it also causes discrepancies in chemical shifts due to isotope effects and variation in sample conditions. In order to record all NMR spectra necessary for the determination of protein structure from a single H_2O sample, Kay and co-workers have employed pulsed field gradients to suppress the intense water signal for some experiments that detect aliphatic protons. The published pulse sequences include the gd-HCCH-TOCSY (Kay et al., 1993), ^{13}C-edited gd-NOESY-HSQC (Muhandiram et al., 1993), and an experiment that correlates intraresidue $^{13}C^{\alpha/\beta}$, $^{13}C'$ and $^{1}H^{\alpha}$ frequencies (Kay, 1993). Here we demonstrate that a modified implementation of pulsed field gradients improves water suppression for the HCACO (Grzesiek and Bax, 1993) and (H)CACO-TOCSY experiments. The (H)CACO-TOCSY experiment correlates intraresidue $^{13}C^{\alpha}$, $^{13}C'$ and aliphatic side-chain protons and is a modification from the HCACO-TOCSY pulse sequence (Kay et al., 1992), designed to improve water suppression and sensitivity. The gradient versions of the HCACO and (H)CACO-TOCSY experiments are referred to as gd-HCACO and gd-(H)CACO-TOCSY.

The gd-HCACO pulse sequence is shown in Fig. 1A and is briefly described below. During the first INEPT transfer step, $^{1}H^{\alpha}$ magnetization is transferred into heteronuclear two-spin order I_zS_z (where I_z and S_z are the z components of $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ magnetization, respectively). Magnetization from protons that are not attached to ^{13}C, such as H_2O, is in the transverse plane at this point and is dephased by the strong gradient pulse g2. This gradient pulse also removes undesired coherences. Artifacts due to imperfection of the 180° pulses in the middle of the INEPT transfer step are suppressed with the gradient pulse g1 (Kay, 1993; Kay et al., 1993). Following the evolution of the $^{13}C^{\alpha}$ chemical shift during t1 and the $^{13}C'$ frequency during t2, the magnetization of interest is in the order of S_zT_z (where T_z is the z component of $^{13}C'$ magnetization).

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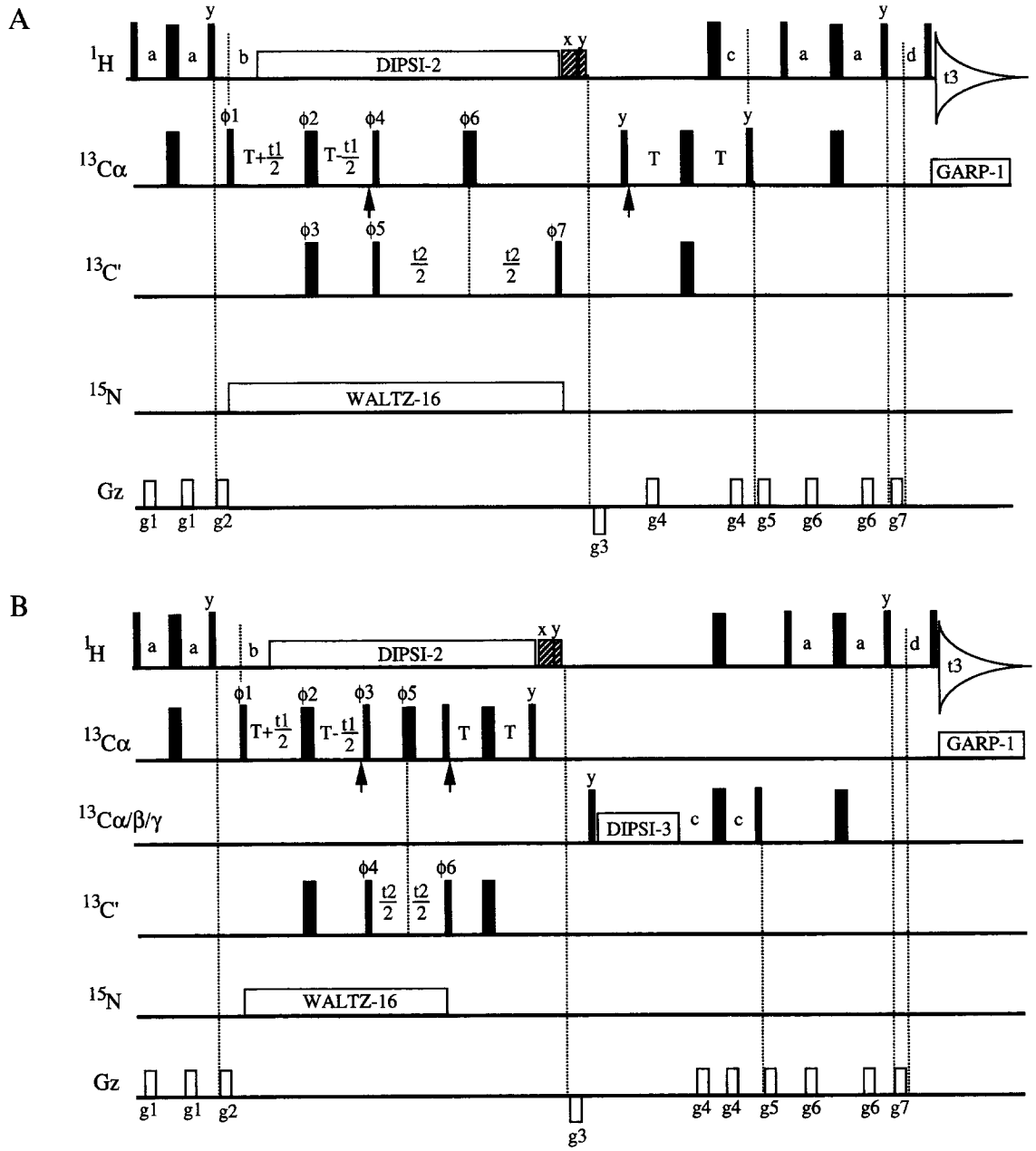

Fig. 1. (A) Pulse sequence of the gd-HCACO experiment. Narrow and wide pulses have flip angles of 90° and 180°, respectively. All pulses are applied along the x-axis, except where indicated otherwise. The carrier for $^{13}C^{\alpha}$ is placed at 56 ppm, while that for $^{13}C'$ is placed at 175 ppm. All $^{13}C^{\alpha}$ 90° and $^{13}C'$ pulses are applied at an rf field strength of 4.0 kHz, while that for the $^{13}C^{\alpha}$ 180° pulses is 9.0 kHz. The $^{13}C'$ pulses are applied as phase-modulated pulses (Patt, 1992) and the arrows indicate the positions at which the $^{13}C'$ 180° pulses are applied to compensate for the Bloch–Siegert effects (Grzesiek and Bax, 1992; Kay, 1993). A DIPSI-2 (Shaka et al., 1988) decoupling scheme was employed for proton decoupling in some parts of the sequence at an rf field strength of 6.6 kHz. ^{15}N decoupling during t1 and t2 is accomplished using WALTZ-16 modulation (Shaka et al., 1983) with an rf field strength of 1.0 kHz. ^{13}C decoupling during acquisition is achieved via GARP-1 modulation (Shaka et al., 1985) with an rf field strength of 2.5 kHz. Water purge pulses are applied at an rf field strength of 6.6 kHz and have durations of 2.3 ms (along x) and 1.3 ms (along y). The delay durations are: a = 1.6 ms, b = 2.2 ms, c = 1.1 ms, d = 0.2 ms, and T = 3.5 ms. The duration and strength of the gradient pulses are: g1 = (0.5 ms, 10 G/cm), g2 = (7.0 ms, 24 G/cm), g3 = (2 ms, −20 G/cm), g4 = (0.5 ms, 12 G/cm), g5 = (1 ms, 20 G/cm), g6 = (0.5 ms, 15 G/cm), and g7 = (0.4 ms, 15 G/cm). A delay of at least 200 μs is inserted between the end of a gradient pulse and the next rf pulse. All gradient pulses are rectangular and are applied along the z-axis. Phase cycling is ϕ1 = x, ϕ2 = 8(x),8(y), ϕ3 = 8(x),8(−x), ϕ4 = 4(x),4(−x), ϕ5 = 2x,2(−x), ϕ6 = 4x,4(−x), ϕ7 = x,−x, receiver = x,2(−x),x,−x,2x,2(−x),2x,−x,x,2(−x),x. Quadrature detection in t_1 and t_2 is achieved using the States-TPPI method (Marion et al., 1989) by incrementing the phases of ϕ1 and ϕ5, respectively. (B) Pulse scheme of the gd-(H)CACO-TOCSY experiment. Most of the details are the same as described above for the gd-HCACO experiment (A), except for the following: ^{13}C isotropic mixing is achieved using the DIPSI-3 scheme (Shaka et al., 1988) at an rf field strength of 7.6 kHz. The mixing pulse is preceded by a 1-ms trim pulse. The carrier for $^{13}C^{\alpha/\beta/\gamma}$ rf pulses is placed at 44 ppm. The $^{13}C^{\alpha/\beta/\gamma}$ 90° and 180° pulses are applied at rf field strengths of 7.6 and 15.6 kHz, respectively. The delay durations are: a = 1.6 ms, b = 3.3 ms, c = 1.1 ms, d = 0.2 ms, and T = 3.5 ms. Phase cycling is ϕ1 = x, ϕ2 = 8x,8y,8(−x),8(−y), ϕ3 = 4x,4(−x), ϕ4 = x,−x, ϕ5 = 8x,8(−x), ϕ6 = 2(x),2(−x), receiver = x,2(−x),x,−x,2(x),2(−x),2x,−x,x,2(−x),x. Quadrature detection in t_1 and t_2 is achieved using the States-TPPI method (Marion et al., 1989) by incrementing the phases of ϕ1 and ϕ4, respectively.

The H_2O signal is further suppressed by the proton x-y purge pulses (Messerle et al., 1989) and the gradient pulse g3 (Kay, 1993; Kay et al., 1993). After the S_zT_z spin order is converted back to the I_zS_z state, gradient pulse g5 is applied to remove unwanted coherences and to further dephase the H_2O magnetization. At the end of the reverse INEPT element, the ^1H magnetization of interest is brought into the I_z state by the 90° y pulse. Then, gradient pulse g7 is applied to suppress any remaining H_2O signal as well as unwanted coherences (Wider and Wüthrich, 1993). The final 90° x pulse generates transverse magnetization for detection during t3. If signals from glycine residues are not to be observed, the sensitivity of the gd-HCACO experiment can be improved by adjusting the delay durations of b and c to 3.3 and 1.8 ms, respectively. The pulse sequence for the gd-(H)CACO-TOCSY is shown in

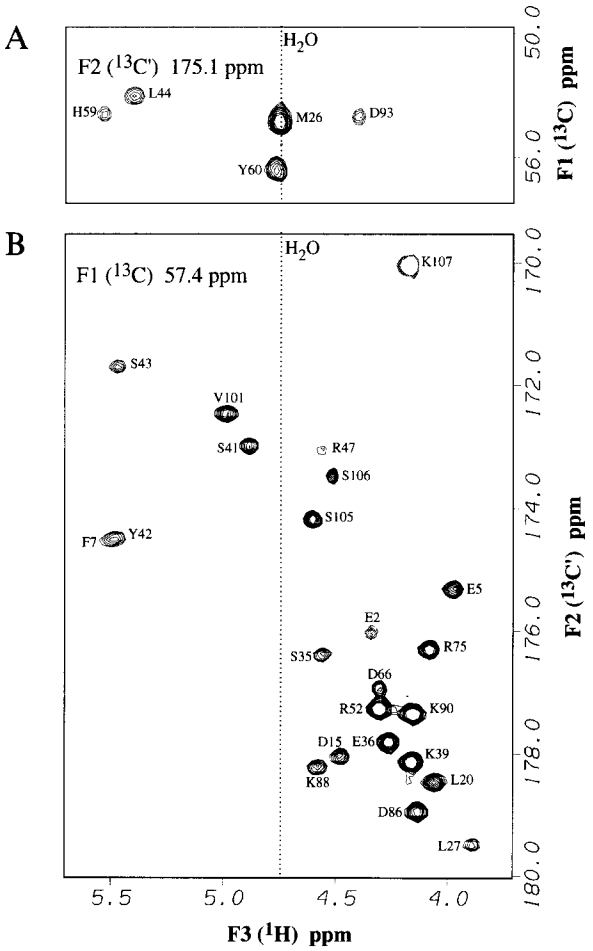

Fig. 2. Slices from the 3D gd-HCACO spectrum of Hck/SH2. (A) F1/F3 slice at the ^{13}C' chemical shift of Met^{26} (175.1 ppm). (B) F2/F3 slice at a ^{13}C chemical shift of 57.4 ppm. The data were obtained from a 24 (t_1) × 32 (t_2) × 256 (t_3) complex matrix with spectral widths of 4000, 1500 and 3000 Hz, respectively. A 16-step phase cycle and a relaxation delay of 0.8 s were used. After processing using FELIX v. 2.05 (Hare Research, Inc., Bothel, WA), including linear prediction in the t_1 and t_2 time domains, the absorptive part of the final 3D spectrum consisted of 128 × 128 × 256 real points. The position of the water resonance is indicated by dashed lines.

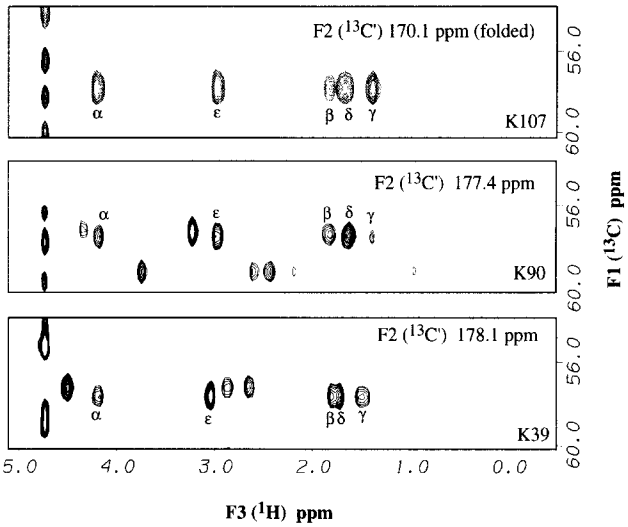

Fig. 3. F1/F3 slices taken from the gd-(H)CACO-TOCSY spectrum of Hck/SH2 at the ^{13}C' frequencies of several lysine residues with similar $^{13}C^{\alpha}$ chemical shifts. The spectrum was obtained from a 24 (t_1) × 32 (t_2) × 256 (t_3) complex matrix with spectral widths of 4000, 1400 and 6000 Hz, respectively. A 32-step phase cycle and a repetition delay of 0.7 s were employed. After processing using FELIX v. 2.05 (Hare Research, Inc.), including linear prediction in the t_1 and t_2 time domains, the absorptive part of the final 3D spectrum consisted of 128 × 128 × 256 real points.

Fig. 1B. Pulsed field gradients are incorporated in a manner analogous to that described above for the gd-HCACO experiment. The excellent water suppression achieved using the gd-HCACO and gd-(H)CACO-TOCSY experiments is demonstrated with a ^{15}N-/^{13}C-labeled sample of the SH2 domain from the hematopoietic cellular kinase, Hck (Quintrell et al., 1987). The protein (~ 3 mM) was dissolved in 90% H_2O/10% D_2O containing 100 mM NaCl and 50 mM sodium phosphate at pH 6.4. Data acquisition was performed at 30 °C using a Varian UNITY-500 NMR spectrometer equipped with a 5-mm triple resonance PFG probe.

Slices taken from the gd-HCACO and the gd-(H)CACO-TOCSY spectra are shown in Fig. 2 and in Fig. 3, respectively. Figure 2A is an F1/F3 slice taken at the carbonyl frequency of Met^{26}, showing the excellent water suppression. Although the $^1H^{\alpha}$ chemical shift of Met^{26} is the same as that of the H_2O resonance, the $^{13}C^{\alpha}$-$^1H^{\alpha}$ cross-peak is clearly observed. All 107 residues in Hck/SH2 are, in fact, observed in the gd-HCACO spectrum. Figure 2B is a slice taken at the $^{13}C^{\alpha}$ chemical shift of several lysine residues, which also have similar $^1H^{\alpha}$ frequencies. Assignment of side-chain resonances for these lysine residues is very difficult using the gd-HCCH-TOCSY experiment (Kay et al., 1993), since the chemical shifts of the side-chain protons are overlapped (data not shown). However, these lysine residues have different ^{13}C' frequencies, allowing the side-chain resonances to be separated using the gd-(H)CACO-TOCSY experiment (Fig. 3). Although water suppression for the gd-(H)CACO-TOCSY experi-

ment is not as complete as for the gd-HCACO experiment, the small residual water signal does not significantly hinder the assignment. The gd-HCACO and gd-(H)CA-CO-TOCSY pulse sequences described here are expected to find wide application in the study of isotopically enriched proteins dissolved in H_2O.

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