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A Gradient-Enhanced HCCH–TOCSY Experiment for Recording Side-Chain ^1H and ^{13}C Correlations in H_2O Samples of Proteins

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The development of pulsed-field-gradient technology has led to a number of useful applications in high-resolution NMR spectroscopy (*1–4*). The use of gradients allows the selection of particular magnetization-transfer pathways (*5, 6*), the elimination of coherences producing artifacts (*7*), a decrease in the number of phase-cycling steps, leading to a concomitant reduction in experimental acquisition times for samples of sufficient concentration (*3, 7, 8*), and the elimination of the intense solvent signal from samples recorded in water (*9, 10*). The use of gradients to remove the water signal efficiently, in principle, allows the possibility of recording all spectra needed for protein assignment and structure determination from a single H_2O sample. This avoids complexities in the assignment introduced by isotope shifts and slightly different sample conditions associated with H_2O and D_2O samples as well as reduces the cost of sample production.

Recently a gradient-enhanced sequence for correlating intraresidue $^{13}C\beta$, $^{13}C\alpha$, $^{13}C'$, and $^1H\alpha$ chemical shifts in uniformly ^{13}C-labeled protein samples dissolved in H_2O was described (*11*). The approach presented allows the recording of all triple-resonance experiments for backbone assignment using a single H_2O sample. Following the assignment of the backbone resonances in $^{15}N,^{13}C$-labeled proteins, the side-chain ^1H and ^{13}C chemical shifts can be obtained from experiments such as the HCCH–COSY (*12*) and HCCH–TOCSY (*13*). Because aliphatic ^1H magnetization is detected, these experiments have been recorded on samples dissolved in D_2O. However, the ability to record such spectra on H_2O samples is clearly advantageous. In the present Communication, a gradient-pulse scheme is presented for recording the HCCH–TOCSY experiment in H_2O (gd-HCCH–TOCSY). Gradients are employed to suppress the water signal so that ^1H resonances close to or at the H_2O line can be observed and also to assist in the removal of artifacts.

Figure 1 illustrates the pulse sequence used to record the gd-HCCH–TOCSY experiment in H_2O. The scheme is very similar to the original sequence of Bax *et al.* (*13*) and therefore only the differences originating specifically from the use of gradients will be discussed in detail. The flow of magnetization in this experiment can be described briefly by

$$^1H_i(t_1) \xrightarrow{J_{HC}} {}^{13}C_i(t_2) \xrightarrow{J_{CC}} {}^{13}C_j \xrightarrow{J_{HC}} {}^1H_j(t_3),$$

where $^1H_{i(j)}$, $^{13}C_{i(j)}$ are one-bond coupled, $^{13}C_i$, $^{13}C_j$ are part of the same side-chain spin network, and the active couplings involved in each transfer step are indicated above each arrow. Bax and Pochapsky have described the use of various gradient building blocks to suppress artifacts in spectra (*7*). Many of these building blocks are incorporated into the gd-HCCH–TOCSY sequence. For example, the gradient-pulse pair g_1 and g_2 is applied to ensure that only ^1H transverse magnetization is present during the $2\tau_a + t_1$ period. Imperfections in the first ^{13}C 180° pulse can lead to artifacts as a result of the generation of multiple- and zero-quantum $^1H–^{13}C$ coherences. These potential artifacts are eliminated by the application of this gradient pair. In nongradient applications the phase of this RF pulse would normally be inverted for half of the scans (*13*). This gradient pair also eliminates artifacts created by imperfections in the ^1H 180° refocusing pulse which might transform transverse into longitudinal magnetization. In addition, ^1H magnetization which is not refocused by the action of the ^1H 180° pulse is also eliminated. Gradient-pulse pairs (g_4, g_5), (g_6, g_7), (g_{10}, g_{11}), and (g_{12}, g_{13}) suppress artifacts in a similar manner to (g_1, g_2).

The utility of the gradient g_3 is twofold. At the point of application of g_3 in the sequence, the magnetization of interest is of the form I_zC_z, where I_z and C_z are the z components of ^1H and ^{13}C magnetization, respectively. Because this term is proportional only to z spin operators, it is invariant to the effects of the z gradient pulse. However, undesired coherences, such as proton magnetization not returned to the z axis by the action of the second ^1H 90° pulse (90°_y), are eliminated by this gradient. In the nongradient HCCH–TOCSY experiment, the phase of the 90°_y ^1H pulse would normally be cycled ±, with a concomitant inversion

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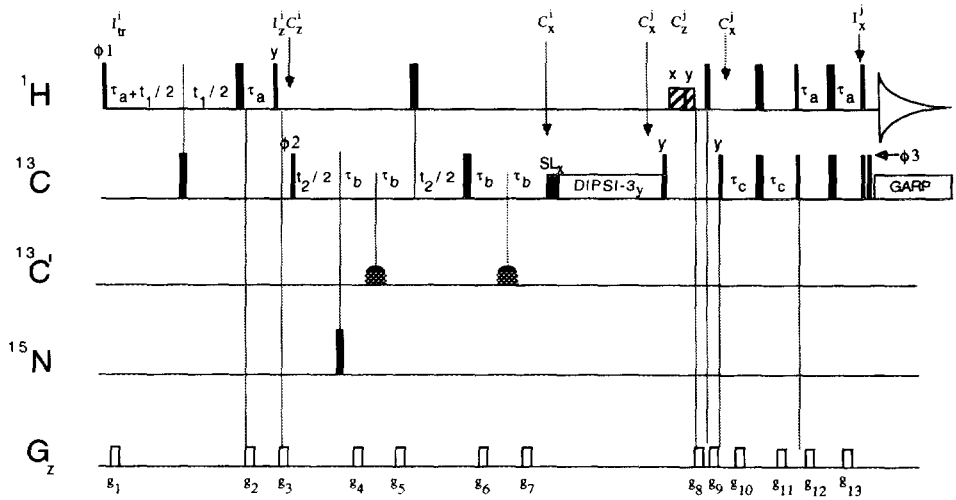

FIG. 1. Pulse scheme of the gd-HCCH-TOCSY experiment. All narrow (wide) pulses have flip angles of 90° (180°). The two C′ pulses are 180° pulses with a shape profile given by a 180° element of the SEDUCE-1 (*15*) decoupling sequence. All ^{13}C pulses are applied at a field strength of ~18 kHz, with the exception of the 2 ms SL_x pulse, the DIPSI-3 mixing scheme (*16*), and the ^{13}C 90° pulse immediately following the DIPSI scheme, where RF field strengths of 8.2 kHz were employed. All of the pulses in the DIPSI sequence are applied along $\pm y$. The ^{13}C carrier is positioned at 43 ppm. The C′ 180° pulses (250 μs) are applied as phase-modulated pulses. Carbon decoupling during acquisition is achieved using the GARP decoupling sequence (*17*) with a 3.5 kHz RF field. The application of ^{13}C pulses at a field strength of ~18 kHz minimizes off-resonance effects. Note that at the time of application of the high-power ^{13}C 180° pulse immediately after the second $t_2/2$ period the C′–C″ J coupling is refocused. Hence perturbations of the C′ spins by this pulse do not produce artifacts or loss of signal. The values of τ_a, τ_b, and τ_c are 1.6 ms, 475 μs, and 1.1 ms, respectively. The phase cycle used is $\phi_1 = x, -x$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; rec = $x, -x, -x, x$. Quadrature in t_1 and t_2 is obtained by States–TPPI (*18*) of ϕ_1 and ϕ_2. The duration and strengths of the gradients are $g_1 = g_2 = (0.5$ ms, 8 G/cm); $g_3 = (2.0$ ms, 15 G/cm); $g_4 = g_5 = g_6 = g_7 = (0.3$ ms, 8 G/cm); $g_8 = (7.0$ ms, 30 G/cm); $g_9 = (4.4$ ms, 30 G/cm); $g_{10} = g_{11} = (0.5$ ms, 8 G/cm); $g_{12} = g_{13} = (0.5$ ms, 8 G/cm). All gradients are applied along the z axis and are rectangular. The relevant magnetization terms are indicated above the pulse sequence using the product-operator notation. (I = ^{1}H, C = ^{13}C, and I_{tr} is the transverse component of I magnetization.)

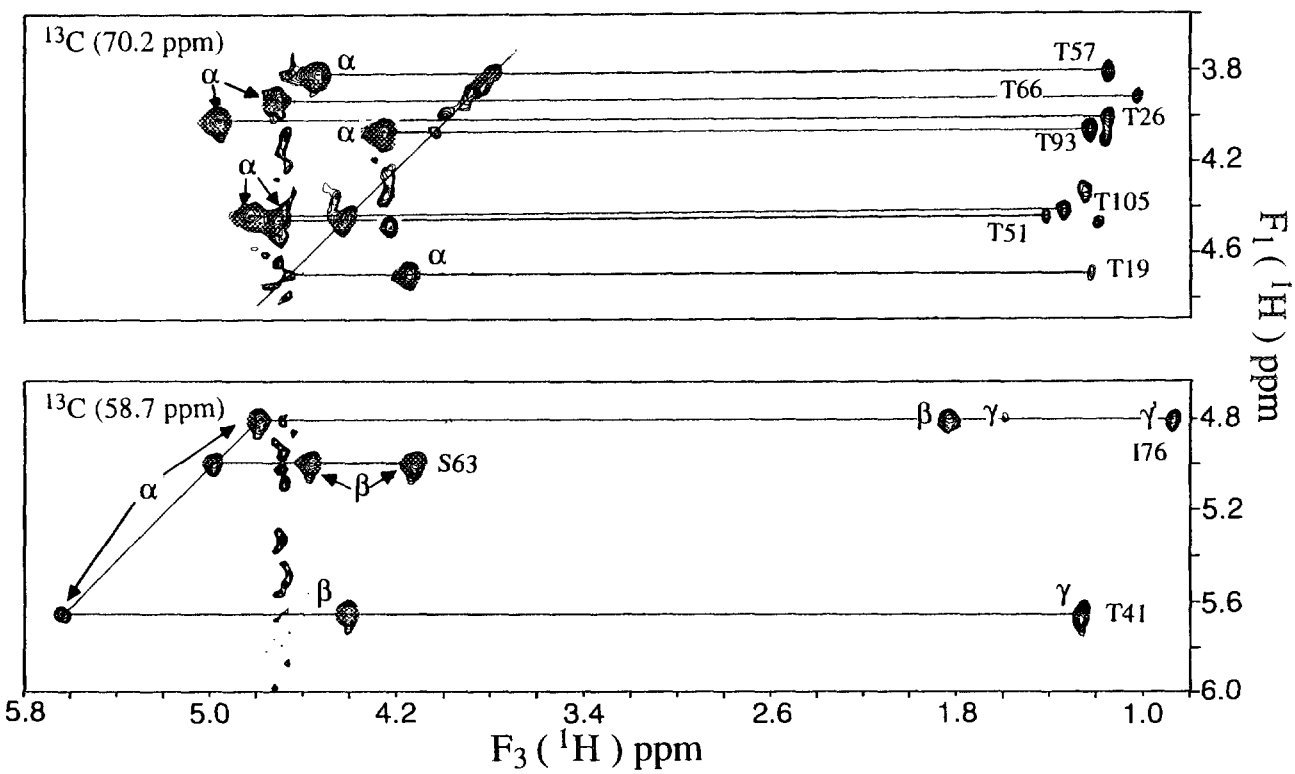

FIG. 2. F_1–F_3 slices of the gd-HCCH-TOCSY spectrum of 1.5 mM CBD, 90% H_2O, 10% D_2O, pH 7.0, T = 37°C at ^{13}C chemical shifts of 70.2 (top) and 58.7 ppm (bottom). The small residual water does not hinder the observation of cross peaks resonating at or near the water line. No presaturation or postacquisition data massaging to remove the residual water was employed.

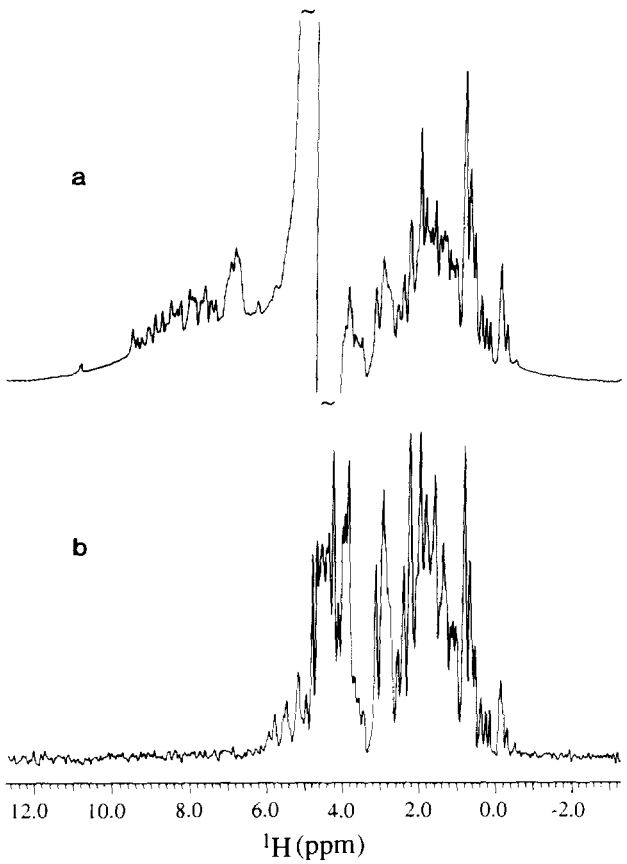

FIG. 3. (a) One-dimensional presaturation spectrum of a 2.2 m*M* sample of the ^{15}N, ^{13}C-labeled C-terminal SH2 domain from PLC-γ_1, 90% H_2O, 10% D_2O, pH 6.0, 0.1 *M* sodium phosphate, T = 30°C. A presaturation field of 30 Hz was used. The spectrum was recorded with 16 scans and a repetition delay of 2 s. (b) The first block of the gd-HCCH–TOCSY spectrum is shown for comparison. No presaturation or postacquisition data massaging to remove the residual water was employed.

of the receiver phase to eliminate ^{1}H magnetization that remains in the transverse plane after this pulse (*13*). In addition, application of the gradient pulse g_3 also aids in the suppression of the water signal. If the carrier is positioned on the water resonance, then for $\phi_1 = x$, the on-resonance component of the water signal is unaffected by the application of the 90$^\circ_y$ ^{1}H pulse and therefore remains in the transverse plane after this pulse. The subsequent application of g_3 acts to dephase this water signal. In contrast, for $\phi_1 = y$, the on-resonance component is rotated to the z axis by the action of the 90$^\circ_y$ ^{1}H pulse and is thus unaffected by g_3. The water suppression is therefore worse for the $\phi_1 = y$ component of the signal.

In principle, it is possible to improve the water suppression by substitution of

$$\begin{array}{lcccc} ^1\text{H:} & 90^\circ_x\text{—}\tau_a\text{—}180^\circ\text{—}\tau_a\text{—}SL_x\text{—}t_1/2 & & t_1/2\text{—}90^\circ_{\phi_1} \\ ^{13}\text{C:} & & 180^\circ & & 180^\circ \end{array}$$

for the portion of the gd-HCCH–TOCSY sequence of Fig. 1 up to and including the ^{1}H 90$^\circ_y$ pulse. In the sequence above, SL_x is a spin-lock pulse applied along the x axis and $\tau_a = 1/(4J_{HC})$. At the point of application of the spin-lock pulse the desired magnetization is of the form I_xS_z and hence will not be affected by SL_x, while water magnetization is along the y axis and therefore will be dephased by SL_x. This approach should assist in the suppression of water in a manner independent of the phase ϕ_1 (*14*). On our spectrometer, however, we find a negligible improvement in water suppression at the expense of an additional ^{13}C 180° pulse and a spin-lock delay and therefore we have not made this substitution.

Immediately after the DIPSI mixing period, a 90° ^{13}C pulse rotates carbon magnetization to the z axis. This is followed by 10 kHz ^{1}H x- and y-purge pulses of durations 7 and 4.3 ms, respectively, applied at the frequency of the water resonance, which greatly suppress the water signal. Water magnetization remaining in the transverse plane is then dephased by the action of the gradient pulse g_8. Any residual water along the z axis that would be unaffected by g_8 is subsequently brought into the transverse plane by a ^{1}H 90° pulse and acted on by a second gradient pulse g_9. It is important to recognize that during this period the decay of magnetization is governed by the T_1 values of the individual ^{13}C spins, and thus relaxation effects, although not completely negligible, are quite small.

The ^{1}H/^{13}C 90° pulses immediately following the $2\tau_c$ delay convert antiphase ^{13}C magnetization into antiphase transverse ^{1}H magnetization. Any residual water that is along the z axis is rotated into the transverse plane by the action of the ^{1}H pulse. Following a period of duration $2\tau_a$, during which the proton magnetization is refocused, the (small) water component is rotated to the z axis by the final 90$^\circ_x$ ^{1}H pulse and hence is not detected. Note that the desired signal is along the x axis and is thus unaffected by this final pulse.

The gd-HCCH–TOCSY experiment is illustrated on a 1.5 m*M* sample of ^{15}N, ^{13}C-labeled *Cellulomonas fimi* cellulose binding domain (CBD), 90% H_2O, 10% D_2O, pH 7.0, T = 37°C, and a 2.2 m*M* sample of the ^{15}N, ^{13}C-labeled C-terminal SH2 domain from human phospholipase C-γ_1 (PLC-γ_1), 90% H_2O, 10% D_2O, pH 6.0, 0.1 *M* sodium phosphate, T = 30°C. Mixing times of 16 ms were employed in the recording of both spectra. Experiments were performed on a Varian UNITY 500 MHz spectrometer equipped with a gradient probe and a gradient amplifier unit. Spectra were obtained from 128 × 32 × 512 complex time-domain matrices with acquisition times of 36.6 ms (t_1), 10.7 ms (t_2), and 64 ms (t_3). A repetition delay of 0.8 s was employed, giving rise to a total acquisition time of ~65 hours/spectrum. No water presaturation was used.

Figure 2 illustrates portions of two slices from the CBD data set at ^{13}C chemical shifts of 70.2 and 58.7 ppm. As can be seen, the signal from the small amount of residual water

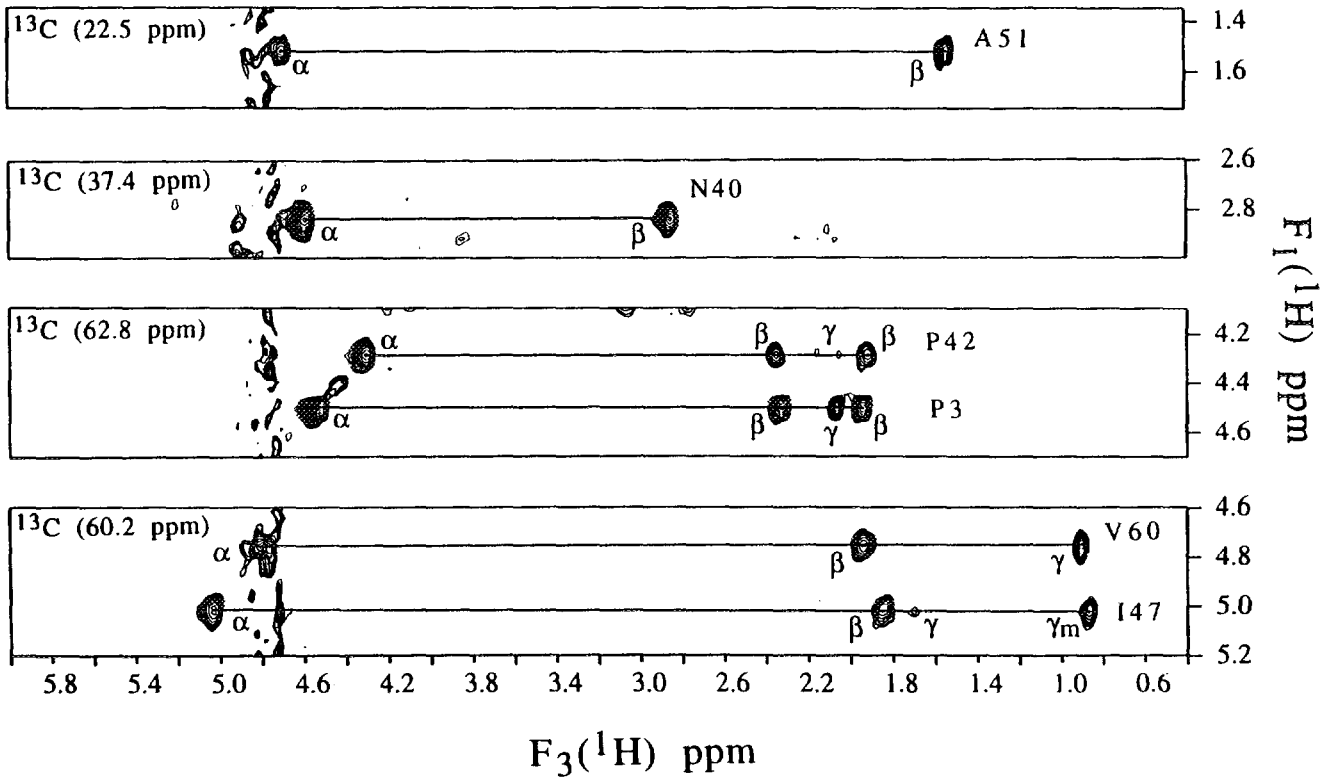

FIG. 4. F_1–F_3 slices of the gd-HCCH–TOCSY spectrum of 2.2 m*M* PLC-γ_1 SH2, 90% H_2O, 10% D_2O, pH 6.0, 0.1 *M* sodium phosphate, T = 30°C. No presaturation or postacquisition data massaging to remove the residual water was employed.

does not pose a problem for the identification of peaks resonating at or very close to the water line. For example, although the CαH protons of both T66 and T51 resonate at the water line, it is nevertheless straightforward to identify the $\beta \rightarrow \alpha$ cross peaks in the slice at a ^{13}C chemical shift of 70.2 ppm. A comparison of H_2O and D_2O data sets revealed that in all but one or two cases, it was possible to observe cross peaks associated with resonances having the same chemical shift as water.

Figure 3a shows the one-dimensional presaturation spectrum of PLC-γ_1 SH2 recorded using a saturation field of 30 Hz. This particular sample has the distinction of giving the worst presaturation spectrum of any protein currently under study in our laboratories and represents a particularly challenging case. The residual water linewidth at the average height of the protein peaks, for example, is on the order of 350 Hz. The spectrum from the first block of the gd-HCCH–TOCSY experiment is illustrated in Fig. 3b for comparison and shows the excellent level of water suppression that is possible with the gd-HCCH–TOCSY sequence. Figure 4 illustrates several portions of slices from the PLC-γ_1 SH2 data set. The small amount of residual water has not posed a problem in the analysis of the data set.

A concern associated with the substitution of gradient experiments recorded in H_2O for D_2O experiments deals with the relative sensitivities of the two classes of experiments. In order to quantitate potential sensitivity differences, a comparison of H_2O spectra recorded using the gd-HCCH–TOCSY sequence with D_2O spectra recorded using the nongradient version of the sequence with a more comprehensive phase-cycling scheme was made. For two proteins for which both H_2O and D_2O samples were available, CBD and *Xenopus laevis* calmodulin, a decrease of ~15% in signal intensity was noted for spectra recorded with the gd-HCCH–TOCSY sequence after normalization for (slightly) different amounts of protein present in the H_2O and D_2O samples.

In summary, in this Communication we have presented a gd-HCCH–TOCSY experiment that allows the assignment of aliphatic side-chain ^{1}H and ^{13}C chemical shifts using an H_2O sample. The use of a single H_2O sample for recording experiments to assign both backbone (*11*) and side-chain resonances of ^{15}N,^{13}C-labeled proteins should help reduce ambiguities caused by isotope shifts and differences in sample conditions between H_2O and D_2O samples, as well as minimize the cost of sample production.

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