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A Three-Dimensional NMR Experiment for the Separation of Aliphatic Carbon Chemical Shifts via the Carbonyl Chemical Shift in $^{15}N,^{13}C$-Labeled Proteins

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The assignment of the proton spectrum of a protein is the first step in its structure determination by NMR methods (*1*). For small proteins, with molecular weights less than approximately 10 kDa, this can be successfully accomplished using homonuclear 2D experiments. For larger proteins, NOE-based assignment strategies often fail due to spectral overlap, while experiments which correlate chemical shifts via ^1H–^1H scalar couplings become insensitive. The development of triple-resonance multidimensional spectroscopy has significantly extended the molecular weight limit of proteins that can be studied with NMR techniques (*2, 3*). Using a variety of 3D (*2–7*) and 4D (*8–10*) experiments, it is possible to assign the backbone resonances in $^{15}N,^{13}C$ uniformly labeled proteins using only through-bond correlations. The assignment of side-chain chemical shifts is accomplished using the HCCH-COSY (*11, 12*) and the HCCH-TOCSY (*13, 14*) experiments, which transfer magnetization via the large and uniform one-bond ^{13}C–^{13}C couplings. Recently, experiments have been developed which separate side-chain correlations via the backbone carbonyl (C′) chemical shift (*15*). One such experiment, the HCACO-COSY, provides correlations linking the C′, Hα, and Hβ chemical shifts, while the HCACO-TOCSY experiment links the C′ chemical shift with all the ^1H shifts along a given side chain. A third experiment correlates the C′, Cβ, and Hβ as well as the C′, Cα, and Hα chemical shifts. Grzesiek and Bax have developed experiments which link ^{15}N and NH chemical shifts with intraresidue and sequential Cα and Cβ chemical shifts (*16, 17*). In this Communication, an experiment is presented which separates side-chain carbon chemical shifts via the intraresidue C′ chemical shift. This experiment is closely related to the HCACO-TOCSY scheme (*15*), and these two experiments together provide a powerful approach for the assignment of side-chain ^1H and ^{13}C resonances.

Figure 1 illustrates the pulse sequence used to correlate the carbon side chain and C′ chemical shifts. Magnetization transfer follows the pathway

$$H_i \xrightarrow{J_{H_iC_i}} C_i(t_1) \xrightarrow[\text{TOCSY}]{J_{C_iC_j}} C\alpha \xrightarrow{J_{C_\alpha C'}} C'(t_2) \xrightarrow{J_{C_\alpha C'}} C\alpha \xrightarrow{J_{H_\alpha C_\alpha}} H\alpha(t_3),$$

where H_i and C_i are side-chain proton and carbon spins, respectively, and the active couplings responsible for magnetization transfer are indicated above the arrows. The pulse sequence can be understood in more detail by considering a product-operator description (*18*) of the magnetization-transfer process. In what follows such a description is provided. For simplicity, the effects of relaxation are neglected, only the terms of interest are retained, and numerical factors preceding these terms are omitted. The side-chain ^1H and ^{13}C spins are denoted by I′ and S′, respectively, the Hα, Cα, and C′ spins are denoted by I, S, and C′, and J_{ij} is the scalar coupling constant between spins i and j. ^1H longitudinal magnetization at time a is transferred via an INEPT transfer (*19*) to the side-chain carbons. At time b in the sequence, the magnetization is given by

$$\rho_b = I'_z S'_y \sin(2\pi J_{I'S'}\tau_a). \qquad [1]$$

During τ_b, carbon magnetization which is antiphase with respect to the directly coupled proton spin(s) refocuses, and the carbon chemical shift is recorded in a constant-time manner (*20, 21*) during the period $2T$. Because each carbon spin can be attached to one, two, or three protons, a compromise value of $\tau_b \sim 0.3/J_{I'S'}$ is chosen. In order to enhance the sensitivity of the experiment, carbon magnetization is maintained in-phase with respect to the directly coupled proton spin(s) by the application of coherent decoupling on the proton channel (*5, 6*). The effects of C′–C and N–C scalar couplings are refocused by the application of C′ and N 180° pulses at $t_1/2$. At time c, the magnetization of interest is given by

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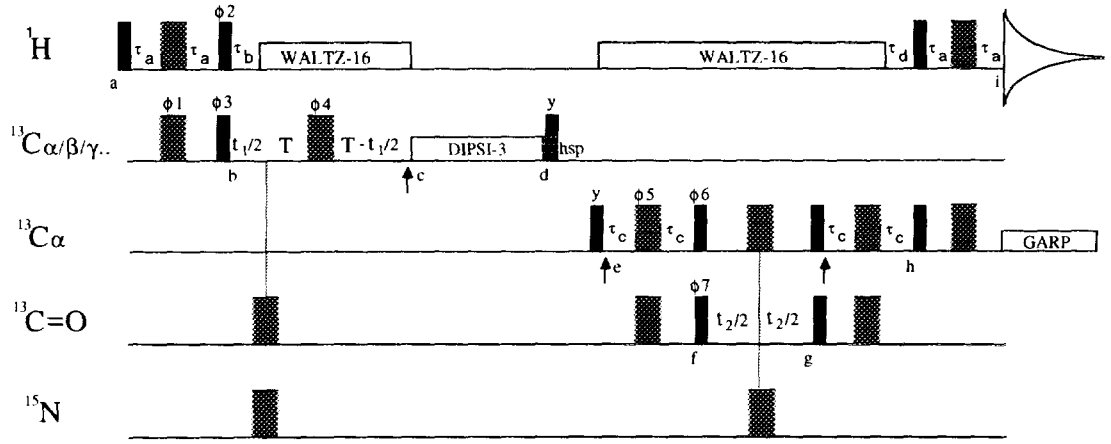

FIG. 1. Pulse sequence used to correlate aliphatic carbon shifts with the intraresidue C′ shift. All narrow pulses have a flip angle of 90° with wider pulses having a flip angle of 180°. Pulses for which the phases are not indicated are applied along the x axis. The C$\alpha/\beta/\gamma$ · · · pulses are applied at 43 ppm with an RF field strength of 4.4 kHz for the 90° pulses and a field strength of 9.8 kHz for the 180° pulses. The Cα pulses are applied at 54 ppm with an RF field strength of 4.0 kHz for the 90° pulses and a field strength of 9.0 kHz for the 180° pulses. This ensures that the application of the aliphatic pulses produces minimal effects in the C′ region of the spectrum and vice versa. All C′ pulses are implemented as phase-modulated pulses. A DIPSI-3 mixing scheme (*22*) of 16 ms is employed, preceded by a 1 ms trim pulse and followed by a trim pulse of 0.5 ms. Because the carbon synthesizer on our spectrometer does not maintain a constant phase during a frequency jump, magnetization is transferred to the z axis prior to shifting the frequency from 43 to 54 ppm. A 10 ms homospoil (hsp) pulse is applied to eliminate magnetization not along the z axis during this interval. Carbon decoupling during acquisition is achieved via GARP decoupling (*27*) with a 2 kHz RF field. A WALTZ (*28*) decoupling field of 6 kHz was employed to maintain in-phase carbon magnetization throughout the course of much of the sequence. The delays used are τ_a = 1.78 ms, τ_b = 2.2 ms, τ_c = 3.6 ms, τ_d = 3.5 ms, and T = 3.0 ms. The phase cycle used is $\phi_1 = x, -x$; $\phi_2 = y, -y$; $\phi_3 = x$; $\phi_4 = 2(x), 2(y), 2(-x), 2(-y)$; $\phi_5 = 8(x), 8(-x)$; $\phi_6 = 8(x), 8(-x)$; $\phi_7 = 16(x), 16(-x)$; Rec = $x, 2(-x), 2(x), 2(-x), x, -x, 2(x), 2(-x), 2(x), -x$. The receiver is inverted for the last 16 scans. Quadrature in t_1 and t_2 is obtained using the STATES-TPPI method (*29*) by incrementing the phases ϕ_3 and ϕ_7. The arrows indicate the points at which the C′ compensation 180° pulses are applied.

$$\rho_c = S'_x \cos(\omega_{S'} t_1)\sin(\pi J_{1'S'}\tau_b) \times \cos^n(\pi J_{1'S'}\tau_b) \prod_{K \neq S'} \cos(2\pi J_{S'K} T), \quad [2]$$

where $\omega_{S'}$ is the Larmor frequency of spin S′, n = 0, 1, 2 for methine, methylene, and methyl carbons, respectively, and $J_{S'K}$ is a homonuclear ^{13}C–^{13}C coupling constant. Subsequently ^{13}C magnetization is relayed among aliphatic side-chain carbons during the DIPSI-3 (*22*) mixing scheme. Magnetization that resides on the Cα carbon after the DIPSI mixing period (point d) is then transferred to the coupled C′ spin to give magnetization at time f of

$$\rho_f = S_y C'_z \sin(2\pi J_{SC'}\tau_c)\cos(2\pi J_{SS'}\tau_c). \quad [3]$$

After the simultaneous 90° pulses on the Cα and C′ spins, the C′ shift is recorded during t_2 while the C′–Cα and C′–N scalar couplings are refocused by the application of Cα and N 180° pulses in the middle of the t_2 evolution. At time g, the magnetization is given by

$$\rho_g = S_z C'_y \cos(\omega_{C'} t_2). \quad [4]$$

The magnetization is subsequently transferred from the C′ spin to the Cα spin. The Cα magnetization is refocused due to the C′–Cα scalar coupling during $2\tau_c$ as it dephases due to the Hα–Cα coupling during the period τ_d. Immediately prior to detection, the magnetization is given by

$$\rho_g = I_x \cos(\omega_{S'} t_1)\cos(\omega_{C'} t_2) \prod_{K \neq S'} \cos(2\pi J_{S'K} T) \times \sin(2\pi J_{1'S'}\tau_a)\sin(\pi J_{1'S'}\tau_b)\cos^n(\pi J_{1'S'}\tau_b)\Gamma(\text{DIPSI}) \times \sin^2(2\pi J_{SC'}\tau_c)\cos^2(2\pi J_{SS'}\tau_c) \times \sin(\pi J_{IS}\tau_d)\cos^k(\pi J_{IS}\tau_d)\sin(2\pi J_{IS}\tau_a), \quad [5]$$

where Γ(DIPSI) denotes the efficiency of magnetization transfer from spin S′ to spin S, k = 1 for glycine, and k = 0 for all other residues. Bax and co-workers have presented figures showing the transfer of magnetization between particular carbons for different amino acid side chains as a function of mixing time (*13, 23*). Fourier transformation of the resulting signal gives rise to peaks at coordinates ($\omega_{S'}$, $\omega_{C'}$, ω_1). Therefore, the side-chain carbon chemical shifts are displayed along a line which intersects the F_3 axis at the intraresidue Hα chemical shift.

The carbon pulse widths in this experiment are adjusted so that the application of a C′ pulse produces minimal effects in the Cα (54 ppm) or C$\alpha/\beta/\gamma$ · · · (43 ppm) regions of the spectrum and vice versa. The C′ pulses are applied 15.6 kHz

(17.0 kHz) downfield from the Cα (Cα/β/γ · · ·) carrier by phase modulation of the RF source. Despite careful adjustments of the pulse widths, the off-resonance effects of the pulses cannot be neglected (*24, 25*). For example, consider the evolution of aliphatic y magnetization during the application of a C′ x pulse centered ν_0 hertz downfield from the carbon carrier. It can be shown that

$$S'_y \rightarrow S'_y \cos 2\pi\{\Delta + 0.5(\nu_1^2/\nu_0)\}\tau_p - S'_x \sin 2\pi\{\Delta + 0.5(\nu_1^2/\nu_0)\}\tau_p, \quad [6]$$

where S'_x and S'_y are the x and y components of aliphatic magnetization, ν_1 is the strength of the applied RF field, τ_p is the duration of the pulse, and Δ is the chemical-shift offset of the aliphatic magnetization from the carbon carrier. In the derivation of Eq. [6] it is assumed that $\nu_1/\nu_0 \ll 1$ and that $\Delta \ll \nu_0$. At a spectrometer frequency of 500 MHz and for Cα magnetization with the carbon carrier centered at 54 ppm, $|\Delta| < \sim 1.3$ kHz, $\nu_1 = 4.0$ kHz, and $\nu_0 = 15.6$ kHz.

Equation [6] indicates that the C′ 180° pulse applied between points *b* and *c* in Fig. 1 produces a phase shift, $\phi \sim \pi(\nu_1^2/\nu_0)\tau_p$, of the transverse aliphatic magnetization that cannot be corrected by simply inserting a delay equal to the C′ pulse width on the opposite side of the aliphatic 180° pulse (ϕ^4). Following the lead of Bax and co-workers (*16, 17, 26*), the phase error introduced by the application of the C′ 180° pulse is compensated by the application of a second C′ 180° pulse immediately prior to the DIPSI mixing period. This ensures that carbon magnetization is modulated by a pure cosine (or sine) function in t_1. In a similar fashion, the application of the C′ 180° pulse between points *e* and *f* and between points *g* and *h* results in a phase shift of the transverse Cα magnetization. Although not indicated in the figure, in both cases the C′ 180° pulse is applied immediately after the Cα 180° pulse. Unless properly corrected, the off-resonance effects of these C′ pulses result in a decrease in the overall sensitivity of the experiment since the Cα 90° pulses that follow the Cα/C′ 180° pulse pairs require a particular phase of the magnetization for optimal transfer. In the present experiment, the C′ 180° pulses are applied with a strength of 4.0 kHz for a duration of 124.1 μs. This creates an off-resonance phase shift of ~25°, giving rise to a decrease in sensitivity of ~10% for each occasion where the C′ pulse is compensated only by a delay equal to its length. The arrows in Fig. 1 indicate the points at which the C′ compensation pulses are applied.

The sequence described is illustrated on a 1.5 m*M* sample of ^{15}N,^{13}C-labeled calmodulin from *Xenopus laevis*, complexed with four molar equivalents of calcium, 0.1 *M* KCl, pH 6.3 (uncorrected), and dissolved in 0.5 ml D_2O. The experiment was performed on a Varian UNITY-500 spectrometer at 37°C. The spectrum was obtained from a 42 × 42 × 512 complex matrix with acquisition times of 6.0 ms (t_1), 28.0 ms (t_2), and 85.0 ms (t_3). A 32-step phase cycle was used with a repetition delay of 0.8 s, giving rise to a total acquisition time of 2.5 days.

Figures 2 and 3 illustrate the quality of the data obtained. For the 16 ms mixing time employed, many of the side chains show complete correlations. For example, the Cα, Cβ, Cγ, and Cδ peaks of R106 are clearly illustrated in the plane with a C′ shift of 178.9 ppm and, although the maximum intensities for the cross peaks from K115 are on an adjacent slice, it is still possible to obtain all the carbon side-chain chemical shifts from the slice at 176.1 ppm.

In summary, a pulse sequence has been presented in this Communication for the correlation of the side-chain carbon chemical shifts with the backbone C′ and Hα chemical shifts. A related set of useful experiments consists of those which separate side-chain chemical shifts via the backbone ^{15}N and NH shifts (*16, 17*). The motivation for separating side-chain shifts based on the backbone C′ resonances in this and other studies (*15*) is based on the fact that experiments which correlate both side-chain and backbone shifts are, in general, less sensitive than the corresponding experiments which correlate side-chain or backbone resonances exclusively. In the

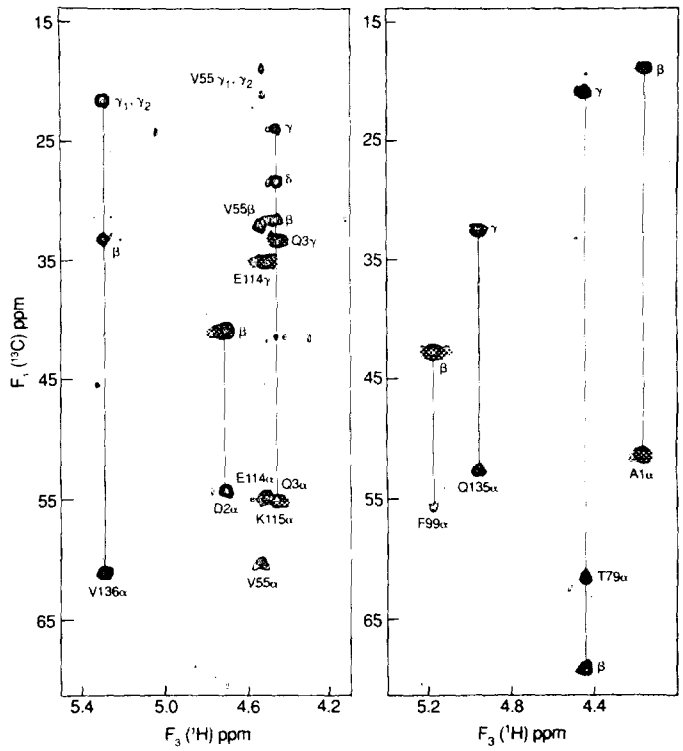

FIG. 2. Sections of slices (F_1/F_3) from the C′-edited ^{13}C–^{13}C TOCSY experiment at C′ shifts of 174.9 and 176.1 ppm. The experiment was recorded on a 1.5 m*M* sample of *Xenopus laevis* calmodulin at 500 MHz, 37°C, pH 6.8. The spectrum was recorded as a 42 × 42 × 512 complex data matrix. After processing, including linear prediction of the t_1 time domain, the absorptive part of the final 3D spectrum was 128 × 128 × 1024. (Left) $F_2(^{13}C') = 176.1$ ppm; (right) $F_2(^{13}C') = 174.9$ ppm.

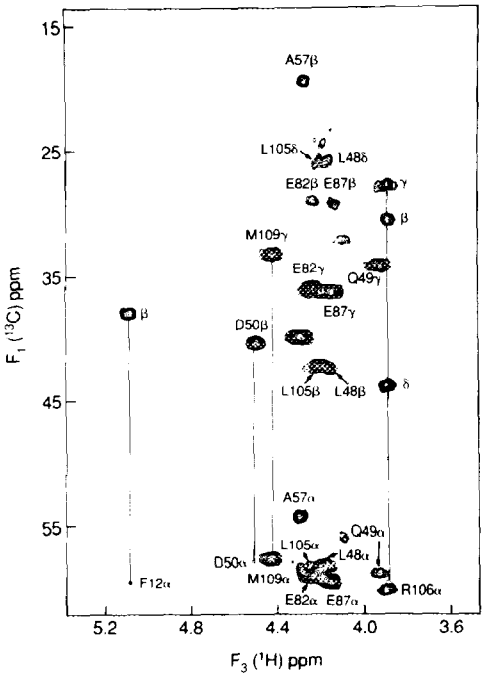

FIG. 3. Expanded region of the F_1/F_3 slice of the C′-edited TOCSY experiment at a C′ shift of 178.9 ppm. $F_2(^{13}C') = 178.9$ ppm.

construction of backbone-edited side-chain experiments it is important, therefore, to ensure that each magnetization-transfer step is as efficient as possible. In this regard, HCACO-based side-chain experiments are a particularly good choice because of the very high intrinsic sensitivity of the HCACO experiment. The experiment presented here, when used in conjunction with the HCCH–COSY/TOCSY and HCACO–COSY/TOCSY schemes, should enable the assignment of aliphatic side-chain ^1H and ^{13}C shifts in a straightforward fashion.

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Note added in proof. Slightly better sensitivity can be obtained by setting $\tau_c \sim 2.7$ ms. In this case, the second ^1H WALTZ-16 decoupling period should end at position g in the sequence and an additional ^1H 180°_x pulse is inserted 1.8 ms after position g. In this case τ_d has no meaning.

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