Constant-Time Through-Bond 13 C Correlation Spectroscopy for Assigning Protein Resonances with Solid-State NMR Spectroscopy

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Constant-Time Through-Bond ^{13}C Correlation Spectroscopy for Assigning Protein Resonances with Solid-State NMR Spectroscopy

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While protein structural folds provide architectural templates for enzymatic function, side-chain conformation and dynamics ultimately determine mechanism. Subtle differences in structure and activity among closely related molecules often arise from amino acid mutations or changes in chemical environment. NMR spectroscopy provides exquisite insight into this chemistry, including hybridization, protonation state, hydrogen-bond lengths, bond order, and rates of chemical exchange. Solid-state NMR in particular offers unique access to macroscopically disordered states, such as fibrils, integral membrane proteins, and trapped reaction intermediates. In several cases now, complete site-specific assignments of solid-state proteins have been reported using through-space, dipolar-driven correlation spectroscopy.[1−3] In contrast, there have been relatively few reports of ^{13}C scalar coupling driven correlation in solid-state proteins.[3,4] Yet through-bond, scalar-coupling-driven correlation offers several unique features that make it an important complementary tool to through-space methods. In particular, scalar couplings are relatively insensitive to global molecular motion, which can compromise sensitivity in dipolar methods. As well, scalar methods provide a means to delineate through-bond and through-space connectivity, a critical step for establishing structure. Here we show that an important additional benefit is that through-bond correlation in solids can be implemented in an efficient "constant time" manner, with the indirect evolution and transfer periods combined into a single constant time interval.[5] This provides substantially increased spectral resolution without compromising sensitivity, which we find to be comparable to or better than the sensitivity of dipolar methods. In the $\beta 1$ immunoglobulin binding domain of protein G (GB1), this allows us to resolve peaks that are otherwise unresolved and to make assignments in the absence of multibond transfers.

Our approach makes use of a constant-time, refocused COSY framework (Figure 1a,b). This sequence is closely related to the previously introduced UC2QF COSY.[6] Differences, however, include a modified phase cycle that passes both zero and double quantum coherences, resulting in cross-peaks with twice the intensity compared to that of the original DQ-filtered version. Although the sequence no longer filters out uncoupled spins, the combined constant-time evolution and mixing period improves resolution through homonuclear decoupling in the indirect dimension. This further increases sensitivity when the scalar couplings are partially or fully resolved. We refer to this pulse sequence as the *c*onstant-*t*ime *u*niform-sign *c*ross-peak (CTUC) COSY.

Figure 1c shows the application of the aliphatic-selective CTUC COSY (Figure 1b) to uniformly-^{13}C,^{15}N-enriched GB1. Based on the previously assigned spectra,[2] all directly bonded carbons are observed in the aliphatic region. Under 25 kHz magic angle spinning (MAS) and 150 kHz proton decoupling (conditions where T_2' is maximized[7] and residual dipolar couplings are suppressed[8]), we find superior side-chain correlation, with sensitivity comparable to or surpassing that of dipolar-driven correlation using spin diffusion. In particular, compared to experiments using dipolar-assisted rotational resonance (DARR) under similar conditions (albeit at necessarily lower MAS rates), we find larger cross-peak intensities (ranging from a factor of 2 to 4) and significantly improved resolution (reduction in line widths in the indirect dimension of 30−50 Hz at primary carbons and 90 Hz at tertiary carbons). The higher resolution is a direct consequence of the removal of homonuclear couplings during the constant-time evolution period and is observed when comparing the CTUC COSY to any directly evolved (nonconstant-time) single-quantum or double-quantum experiment. Indeed, we have performed such comparisons with the nonconstant-time version of the UC COSY, the refocused INADEQUATE, and DARR (spectra in Supporting Information). In addition to the improved sensitivity and resolution, the CTUC COSY diagonal intensity is ideally zero for a two-spin system and, in all cases, is significantly reduced relative to DARR. This allows closer scrutiny, for example, of Leu and Lys Cγ-Cδ side-chain correlations that often arise close to the diagonal. We also note that all cross-peaks are single-bond transfers, except for an extremely weak cross-peak between Val39 Cα−Cγ1. This is in contrast to DARR, where for the intermediate length mixing time of 5 ms, multibond transfers are prevalent.

The aliphatic-selective sequence is just one of several variations that can be constructed under the CTUC COSY framework. For example, the aliphatic-carbonyl correlation shown in Figure 1d is effected by replacing the aliphatic-selective π pulses with hard πs and removing the carbonyl-selective π. The multispin dynamics (formalized in the Supporting Information) result in strong cross-peaks between the carbonyl and the aliphatic regions, with only correlations not involving alpha carbons in the aliphatic region. Again, the increases in resolution and sensitivity are significant, while the constraint of single-bond transfers diminishes spectral congestion and aids in assignment, especially among Asp and Glu side-chain carboxyl sites, where the protonation state is of great importance to enzymatic function. The same cross-peak region of the DARR experiment is shown in Figure 1e and illustrates the dramatically increased resolution of the CTUC COSY.

Even as available magnetic fields for NMR continue to increase, resolution remains one of the most critical limitations in assigning and solving structures of larger biomolecules. The constant-time UC COSY offers superior resolution, by a factor of 2 to 3, compared to that of directly evolved experiments. The sensitivity is also enhanced and the sequence performs as well or better than the

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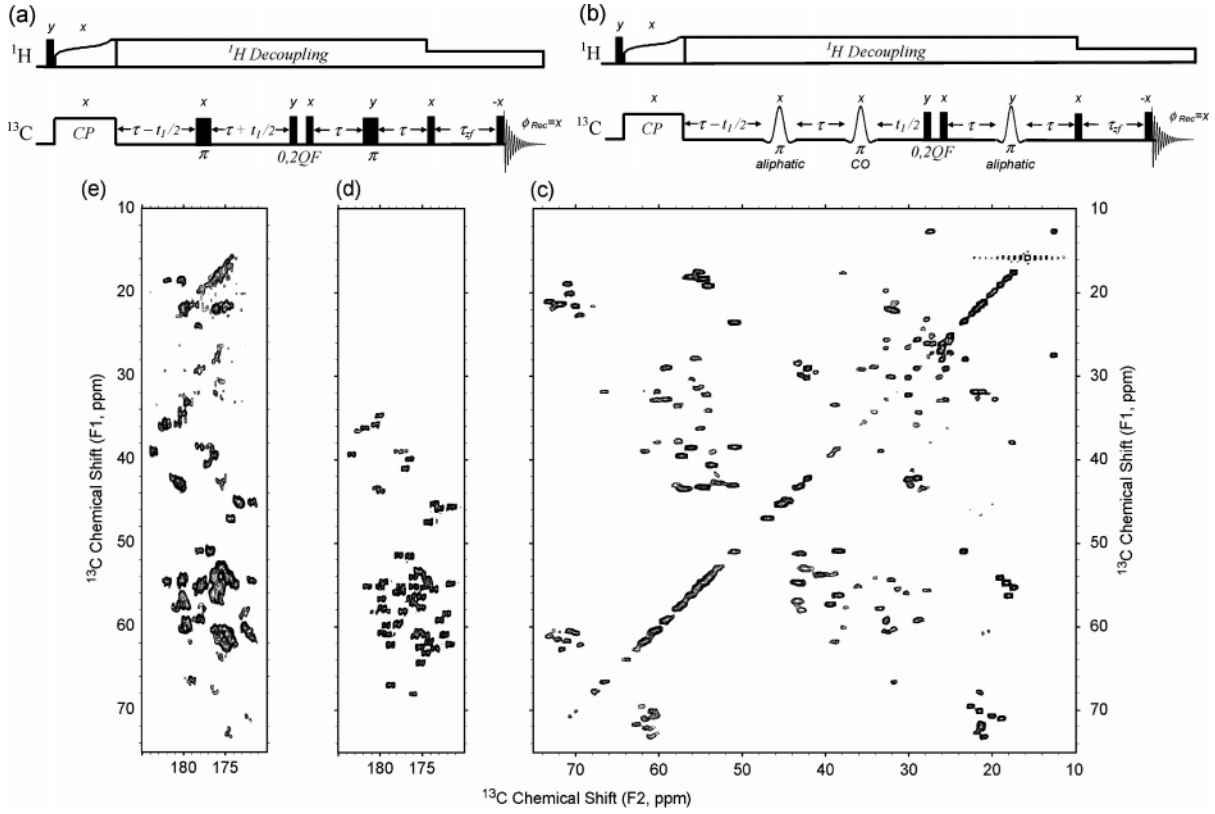

Figure 1. Pulse sequence for the constant-time uniform-sign cross-peak (CTUC) COSY, shown optimized for (a) CA−CO correlation and (b) aliphatic correlation. In these pulse sequences, thin vertical lines indicate $\pi/2$ pulses, wide vertical lines indicate π pulses, and selective π pulses are shown as amplitude modulated waveforms. 80 kHz ^{13}C pulses were used throughout for the hard pulses along with 150 kHz SPINAL64 ^{1}H decoupling[9] during the τ and constant-time intervals. 100 kHz decoupling was used during the *z*-filter (τ_{zf}, typically 16 ms) and acquisition (t_2). For the selective π pulses, 180 μs and 420 μs r-SNOB pulses were used for selective aliphatic and carbonyl pulses, respectively. These were centered in the respective spectral regions and rotor synchronized as described in ref 10. The rotor-synchronized refocusing delay τ was set to 5 ms. The first three carbon pulses were phase cycled together for zero and double quantum excitation. The CTUC COSY of GB1 shows superior resolution in both the aliphatic−carbonyl region (d) and the aliphatic region (c). Data were acquired on a 9.4 T Bruker DSX spectrometer (^{1}H frequency 400.13 MHz) equipped with a double resonance 2.5 mm MAS probe spinning at a MAS rate of 25 kHz. 3.5 mg of GB1 (prepared as described in ref 2) was center-packed in the rotor, and the drive gas was cooled to 5 °C. For (c), 125 complex-valued t_1 points and 256 complex t_2 points were acquired with a spectral width of 12.5 kHz in each dimension. 128 scans per t_1 increment were co-added with a relaxation delay of 2 s. For (d), 250 complex-valued t_1 points and 512 complex t_2 points were acquired with a spectral width of 25 kHz in both dimensions; 64 scans per t_1 increment were co-added with a relaxation delay of 2 s. In (e), the proton-driven spin diffusion spectrum of GB1 is shown for comparison. Acquisition was carried out similar to (d), with 9 kHz MAS (chosen to bracket the aliphatic region between the first- and second-order spinning sidebands of the carbonyl) and a 9 kHz proton spin lock during the 5 ms mixing time. All spectra were linear predicted once in the indirect dimension and zero-filled to 4096 points in both dimensions before Fourier transformation.

commonly used dipolar methods, especially for side-chain sites exhibiting molecular motion. The improved sensitivity of this scalar-coupling driven method is comparable to results found by Meier using the isotropic-mixing TOBSY sequence,[3] although the CTUC COSY offers the additional resolution advantage of a constant-time implementation. Because the indirect evolution and transfer periods can be combined into a single constant time interval, the increased resolution is not obtained at the cost of sensitivity, a distinguishing feature of COSY-type sequences compared to that of strong-coupling or INADEQUATE sequences. For GB1, this allows for multiple peaks to be resolved that are otherwise obscured.

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Supporting Information Available: Full CTUC COSY phase cycle; multispin dynamics simulations; and a comparison of 2D methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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