Determination of Homonuclear $^{13}C-^{13}C$ *J* Couplings between Aliphatic Carbon Atoms in Perdeuterated Proteins

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One of the major limitations in high-resolution NMR spectroscopy is the increase of line widths for large macromolecules which define an upper limit to the size of molecules that can be studied by NMR. The line width of carbons is reduced by incorporation of deuterium in proteins[1] and RNA.[2] Due to the magnetogyric ratio of ^{2}H, which is 6.5 smaller than that of ^{1}H, the C_α transverse relaxation in a $C_\alpha-D$ moiety is reduced by a factor of 16 compared to that in a $C_\alpha-H$ moiety. The sensitivity of certain heteronuclear triple-resonance NMR experiments is improved by deuteration of all but the exchangeable hydrogen atoms usually bound to carbon atoms.[3]

A number of strategies have been developed to compensate the lack of structural information due to the reduced number of protons: Fractional labeling with various degrees of deuteration[4] and type-specific protonation within an otherwise deuterated protein[5] as well as selective deuteration of H_α hydrogen atoms.[6]

In a perdeuterated sample distance restraints can only be derived between exchangeable hydrogen atoms which usually leads to low-resolution protein structures, for which determination of global folds is difficult.[7] Measurement of backbone and side chain angles holds the promise to improve the structure. We propose a method to measure homonuclear ^3J(C,C) coupling constants between C_α and C_δ aliphatic atoms to define χ_2 in aliphatic side chains.

The proposed pulse sequence relies on quantitative correlations[8] of C_α atoms with aliphatic side chain carbon atoms for the determination of the torsion angle χ_2. The experiment, an "out and back" type quantitative HN(CO)CAC$_{ali}$ (Figure 1),[9] creates transverse C_α magnetization that defocuses due to long-range homonuclear couplings, i.e., between C_α and C_{ali} during a period of $2\tau'' = (2/^1J_{CC})$. The transfer amplitude for the autocorrelation peaks that remain on C_α magnetization is proportional to $\cos(2\pi J_{C_\alpha C_\delta}\tau'')\prod_k \cos(2\pi J_{C_\alpha C_k}\tau'')$ [$k \neq \alpha, \delta$ covers all other *J*-coupled aliphatic carbons], while the transfer amplitude for the cross-peak is proportional to $\sin(2\pi J_{C_\alpha C_\delta}\tau'')\prod_k \cos(2\pi J_{C_\alpha C_k}\tau'')$. After chemical shift evolution of aliphatic carbon magnetization during t_2, the same fractions are transferred back following the reverse pathway. Thus, the ratio of transfer amplitudes for the autocorrelation and cross peaks is given by $-\tan^2(2\pi J_{C_\alpha C_\delta}\tau'')$. As τ'' is known and the line shapes of the autocorrelation and the cross peaks are assumed to be identical in the ^{15}N (t_1) and $^1H^N$ (t_3) dimensions and within the digital resolution of the ^{13}C (t_2) dimension, the intensity ratio of these peaks provides a measure of the magnitude of J_{CC}: $I^{cross}/I^{auto} = -\tan^2(2\pi J_{C_\alpha C_\delta}\tau'')$.

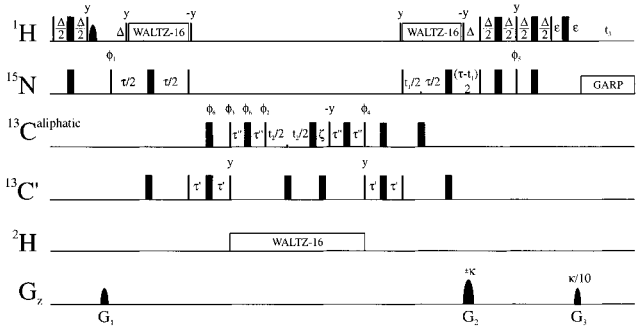

Figure 1. Pulse sequence for the HN(CO)CAC$_{ali}$ experiment. Narrow and wide bars denote 90° and 180° pulses, respectively, and unless indicated the phase is *x*. Phase cycling: $\phi_1 = x, -x, \phi_2 = 2(y), 2(-y), \phi_3 = 4(x), 4(-x), \phi_4 = 8(x), 8(-x), \phi_5 = -y, \phi_6 = x$, receiver = $x, -x, x, -x, x, -x, x, -x, x, -x, x, -x, x, -x, x, -x$. Quadrature detection in the t_2 dimension is obtained by altering ϕ_2, ϕ_3, and ϕ_6 in the states-TPPI manner.[17] For each t_1 value, echo and antiecho coherences are obtained by recording data sets where the phase ϕ_5 and the second gradient are inverted.[18] Delay durations: $\Delta = 5.5$ ms, $\tau = 35$ ms, $\tau' = 4.55$ ms, $\tau'' = 28.2$ ms, $\zeta = t_2(0)$, and $\epsilon = 1.2$ ms. Carrier positions: ^{1}H, 4.65 ppm; ^{13}C, 44.3 ppm; ^{15}N, 118.3 ppm; ^{2}H, 4.65 ppm. Proton pulses are applied using a 20.8 kHz rf field with the exception of the 2 ms rectangular water−selective 90° flip back pulse,[19] the 4.2 kHz WALTZ-16[20] decoupling pulses, and the 4.2 kHz 90° ± *y* pulses flanking the decoupling intervals. The ^{15}N pulses are at a field of 8.1 kHz, and GARP[21] decoupling during acquisition is applied with a 1 kHz field. ^{2}H decoupling is performed with a 1 kHz field. All carbon pulses are Gaussian cascades;[22] G3 and G4 pulses had durations of 256 and 409.6 μs, respectively. Every second G4 pulse was time-reversed to avoid phase errors. Carbonyl-selective pulses were implemented as phase-modulated pulses. Gradients (sine bell shaped): G1 = (2 ms, 25 G/cm), G2 = (1 ms, 40 G/cm), and G3 = (1 ms, 4.05 G/cm). Sixteen scans per t_1 (32 complex points, spectral width 1824.8 Hz) and t_2 (50 complex points, spectral width 1000 Hz) experiment were recorded with 1024 complex points in t_3 (spectral width 7936.5 Hz). A repetition delay of 1.7 s was used between scans, giving rise to a total measurement time of 52 h.

Experiments were applied to a (>95%) deuterated sample of $^{13}C/^{15}N$-enriched calmodulin, complexed with Ca^{2+} (5 mM $CaCl_2$), in a 220 μL Shigemi microcell. Experiments were performed on a Bruker DRX-600 MHz spectrometer in 90:10 H_2O/D_2O (pH 6.5, 100 mM KCl) at 309 K.

Figure 2 shows the results obtained; the carbon resonance frequencies in ω_2 can be inferred from strip plots of the CC-

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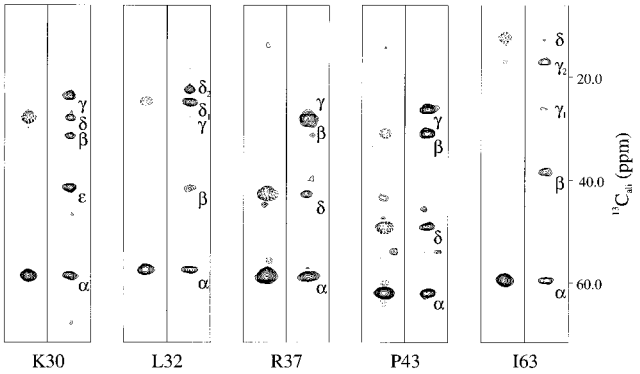

Figure 2. (ω_2, ω_3) strips from the HN(CO)CAC$_{ali}$ experiment (left strips) and the CC-TOCSY-(CO)NH experiment (right strips) of perdeuterated calmodulin in H_2O, taken at the $^1H^N$ (ω_3) and ^{15}N (ω_1) frequencies of Glu31, Gly33, Ser38, Thr44, and Asp64. Autocorrelation (solid contours) and cross (dashed contours) peaks in the HN(CO)-CAC$_{ali}$ strips as well as in the CC-TOCSY-(CO)NH strips correspond to the amide frequency of the preceding residue in the protein. The side chain ^{13}C resonances are assigned in the figure. The sample of calmodulin was 0.6 mM.

Table 1. Values of $^3J_{C_\alpha C_\delta}$ for the Calmodulin Residues Shown in Figure 2[a]

residue	$^3J_{C_\alpha C_\delta}$ (Hz)	rotamer	residue	$^3J_{C_\alpha C_\delta}$ (Hz)	rotamer
Lys30	3.8	trans	Ile63	3.3	trans
Arg37	5.0	trans	Pro43	3.7[b]	
Leu32	3.5	trans			

[a] Values of $^3J_{C_\alpha C_\delta}$ were determined from the ratios of cross-peak and diagonal peak intensities as described in the text. Values are given for the first of the two independent folding domains of calmodulin. [b] Sum of ^2J and ^3J coupling.

TOCSY-(CO)NH experiment.[10] In the HN(CO)CAC$_{ali}$ experiment, each strip shows the positive autocorrelation peak, originating from the $^{13}C_\alpha$ being active in t_1 and the negative cross-peaks originating from long-range coupled aliphatic carbon atoms. The strips for the representative residues are obtained at the ^{15}N and $^1H^N$ shifts of the following amino acid in the protein. All measured values of $^3J_{C_\alpha C_\delta}$ (2.6−3.8 Hz) fall between the values reported for trans (4.0 Hz) and gauche (0.9 Hz) aliphatic $^3J_{CC}$ couplings in proteins,[11] with the exception of Arg37 for which a coupling of 5.0 Hz is measured (see Table 1). Large values for $^3J_{C_\alpha C_\delta}$ (>3.2 Hz) correspond to trans conformations, whereas intermediate couplings are expected if rotamer averaging takes place or if the torsion angles do not correspond to either the trans or gauche rotamer position. We have not found evidence for a gauche χ_2 rotamer position since all measured three-bond couplings are larger than 2.5 Hz. For some residues we observed additional cross peaks such as the two-bond $C_\alpha-C_\gamma$ correlation for Ile63. The $^1J_{C_\alpha C_\beta}$ coupling of Pro43 is larger than the "tuned" value of 35 Hz (37.5 Hz); therefore, we observed a correlation with intensities corresponding to those of a long-range correlation via 37.5−35 = 2.5 Hz coupling.

The average rotational correlation time of calmodulin at 309 K is 6.7 ns.[12] To determine whether the proposed HN(CO)-CAC$_{ali}$ experiment will work on larger proteins, for which deuteration is indispensable, the relaxation losses of heteronuclear transverse operators in the periods 2τ, $4\tau'$, and $4\tau''$ for overall isotropic correlation times of 6.7 and 15 ns will be compared. For average transverse relaxation times for ^{15}N, $^{13}C'$, and $^{13}C_\alpha$ of 120 ms (45 ms),[12] 94 ms (45 ms),[13] and 284 ms (130 ms)[14] for 6.7 ns (15 ns), respectively (see the Supporting Information), the signal-to-noise ratio for larger proteins is reduced by approximately a factor of 4. The ^{15}N transverse relaxation has the biggest contribution, and τ should be adjusted for larger proteins.

In conclusion, we have introduced a sensitive method for the measurement of homonuclear $^{13}C-^{13}C$ couplings in perdeuterated proteins. The assignment of χ_2 rotamers derived from $^3J_{C_\alpha C_\delta}$ is of considerable importance because of the limited NOE contacts in deuterated side chains. The HN(CO)CAC$_{ali}$ experiment can be used to improve the structure elucidation of large deuterated proteins as the side chains of Arg, Lys, Leu, Ile, and Pro residues are often involved in intra- and intermolecular interactions. Further experiments based on the E.COSY principle to measure couplings to the amide protons as well as experiments based on quantitative *J* correlation experiments to measure couplings to the carbonyl carbons and nitrogens have successfully been performed in our laboratory[15] and others[16] and will provide additional conformational restraints for high-resolution structure determination of perdeuterated proteins.

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Supporting Information Available: A table containing all measured J_{CC} couplings in calmodulin, a figure showing the corresponding strip plots, and a paragraph explaining the assumed heteronuclear relaxation rates (4 pages). See any current masthead page for ordering and Internet access instructions.

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