Measurement of Three-Bond $^{13}$C-$^{13}$C J Couplings between Carboxyl and Carbonyl/Carboxyl Carbons in Isotopically Enriched Proteins

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Three-bond $^{13}$C-$^{13}$C J couplings have long been recognized as a valuable source of structural information in the study of organic, organometallic, and biological compounds. For $^{13}$C-enriched proteins, two different approaches have been proposed for measurement of these couplings: ECOSY and quantitative J coupling. Both approaches benefit from the exceptionally narrow line widths of methyl $^{13}$C resonances, and in proteins these measurements therefore have largely been restricted to $^{3}$J$^{13}$C couplings involving $^{13}$CH$_3$. Here we demonstrate that the quantitative J correlation approach can also be used for measurement of $^{3}$J$^{13}$C coupling between carboxyl (C') and carbonyl/carboxyl carbons. $^{3}$J$^{13}$C provides direct information on the $\phi$ backbone angle, and $^{3}$J$^{13}$CC in Asn and Asp residues relates to $\gamma$.

Previously, a Karplus curve appropriate for peptides has been proposed on the basis of FPT-INDO calculations. Here we present the first empirical Karplus relation for this coupling based on $^{3}$J$^{13}$CC couplings measured in ubiquitin (76 residues) and $\phi$ angles from its X-ray structure. Application to apocalmodulin (apo-CAm, 148 residues) confirms the backbone geometry of the first Ca$^{2+}$-binding site and adds new information on the orientations of its three Asp side chains which ligate Ca$^{2+}$ in the bound state.

The experiment (Figure 1) for measuring $^{1}$J$^{13}$CC starts with transfer of magnetization from the amide proton (H$^\beta$) through the $^{15}$N to the preceding $^{13}$C and is followed by a $^{13}$C-$^{13}$C$^{2}$ dephasing period prior to magnetization transfer to its long-range coupled $^{13}$C$^{2}$ which subsequently evolves during $t_2$ and is finally transferred back along the reverse pathway for H$^\gamma$ detection. The pulse scheme is very similar to that of the long-range carbon-carbon correlation experiment and details regarding the magnetization transfer in this class of experiments have been discussed elsewhere. In the present case, after magnetization has been transferred from $^{15}$N to its adjacent $^{13}$C$^{2}$ (referred to as source-C, or sC$^{2}$), it dephases during the time $2\tau$ with respect to carbonyls $J$ coupled to sC$^{2}$. Exactly analogous to the homonuclear HAB experiment, a fraction

\[
\sin(2\pi J_{13CC}z) \Pi_{k} \cos(2\pi J_{13CC}2k) \text{ of the source-C' magnetization is transferred to the destination C', dC', where the product}
\]

$\Pi_{k} \cos(2\pi J_{13CC}2k)$ remains in-phase with $sC'$ and results from the reverse transfer of C' magnetization between the end of $t_1$ and the end of the second $2\tau$ period, and the diagonal-cross peak ratio therefore equals $\tan^2(2\pi J_{13CC}2k)$. As $\zeta$ is known, $J_{13CC}$ follows directly from the intensity ratio. Strictly speaking, this ratio applies to the volume integrals of the $sC'$ and dC' resonances, as well as the line widths of the two resonances in the 3D spectrum are identical in the $^{15}$N ($t_1$) and $^1H^\beta$ ($t_2$) dimensions, and the same to within the digital resolution in the C' ($t_2$) dimension, peak heights are used instead for deriving $J_{13CC}$.

Experiments were carried out for samples containing 3.5 mg of $^{13}$C$^{15}$N-enriched ubiquitin (pH 4.7; 30 °C) in a 220 µl Shigemi microcell (1.8 mM) at 600 MHz and 10 mg of apoCAm (pH 6.3; 23 °C) in 450 µL (1.3 mM) at 500 MHz. Experiments on ubiquitin were carried out twice, once with 4 scans (Figure 2A) and once with 16 scans per FID (12 h and 2 ms measuring time, respectively). Each strip shows the negative 1H$^\gamma$ “diagonal” peak, corresponding to the $^{13}$C of the preceding residue, and positive cross peaks to its long-range coupled $^{13}$C$^{2}$. For most backbone carbonyls, the value of $J_{13CC}$ was measured twice, once with $sC'$ = C$^{2}$-$d$ and dC' = C', and once vice versa. The pairwise root-mean-square (rms) difference between these measurements was smaller than 0.1 Hz, and the rms pairwise difference between measured J values in the 4-scan and 16-scan experiments was also less than 0.1 Hz (supporting information). Figure 3 shows the averages of these two measurements as a function of the intervening $\phi$ angles, taken from the ubiquitin X-ray structure. The data were fit to a Karplus curve, yielding

\[
J_{13CC} = 1.33 \cos^2 \phi - 0.88 \cos \phi + 0.62 \text{ Hz (1)}
\]

and a rms difference of 0.18 Hz between measured $J_{13CC}$ values and those predicted by eq 1 when using crystallographic $\phi$ angles.


Figure 1. Pulse scheme for the 3D HN(CO)CO experiment. Narrow and wide pulses denote 90° and 180° flip angles (except for low power 90°-1H pulses), respectively, and unless indicated the phase is x. Phase cycling: $\phi_1 = x; \phi_2 = 4(\pi_1, 4(\pi), 4(-\pi), 4(-\pi)); \phi_3 = 2(\pi_2, 2(-\pi_2)); \phi_4 = x; \phi_5 = x, -x; \phi_6 = 2(\pi_2, 2(-\pi_2), 2(\pi_2), 2(-\pi_2)).$ Quadrature detection in the $t_1$ dimension is obtained by altering $\phi_1$ in the States-TPPI manner, quadrature in $t_2$ by States-TPPI phase incrementation of $\phi_4$, $\phi_5$, and $\phi_6$. RF power: $^1H, \gamma B_1 = 27$ kHz (high-power pulses), 220 Hz (low-power pulses), 3.1 kHz (Waltz-16); $^{13}N, \gamma B_2 = 5.3$ kHz, or 1.0 kHz (Waltz-16); $^{13}C, \gamma B_3 = 4.5$ and 4.0 kHz at 151 and 126 MHz; $^{13}CO$, G1 180° pulses$^5$ of 400 ms (126 MHz) or 333 µs (151 MHz). Carrier position: $^1H, H_2O (4.79 ppm); ^13C, 177 ppm; ^15N, 117 ppm. Delay durations: $\tau_1 = 2.5$ ms; $\Delta = 5.3$ ms; $\tau_2 = 14.5$ ms; $\tau_3 = 12.5$ ms; $\delta = 32$ ms; $\zeta = 45–55$ ms. Gradients (sine bell shaped): 25 G/cm at center: G1,2,3,4,5,6 = 1.75, 0.8, 0.35, 0.165, 1.35, and 0.5 ms.

Asp 58 in ubiquitin. On the basis of the measurement of 3 mM ubiquitin (600 MHz, $\bar{\omega}$ $\sim$ 33$^\circ$ angles. This difference decreases to 0.16 Hz when using Figure 3. Relation between measured 3$^\circ$ vs 4.1 ns), i.e., 2-fold shorter transverse relaxation times. 

angles. Positions of expected 3$^\circ$ cross peaks, but which are below the noise threshold, are marked by (F 2 ,F 3 ) strips from the 3D HN(CO)CO spectra of (A) 1.8 mM ubiquitin (500 MHz, $\bar{\omega}$ = 45 ms, 12 scans, 48 h), taken at the 1H N /15 N frequencies of Lys 21 , Gly 23 , Gly 25 , Thr 26 , Ile 27 , and Thr 28 . The diagonal $C^{\prime}$ resonance is negative (dashed contours) and corresponds to the $C^\circ$ preceding the amide in the polypeptide. Positions of expected 3$^\circ$ values and crystallographic $\phi$-sheet. 

This is confirmed by 3$^\circ$CC values of 2.0 (between residues 25 and 26), 1.8 (26 and 27), and $<1.2$ Hz (27 and 28). 

The present report provides the first extensive study of 3$^\circ$CC couplings between carbonyls. Results indicate that 3$^\circ$CC is useful for determining backbone geometry, particularly in combination with 3$^\circ$HNHa values, as it removes the ambiguity around $\phi$ = $-120^\circ$ in the 3$^\circ$HNHa Karplus curve. Other types of J couplings could also be used for this, 12,14 but as 3$^\circ$CC has its steepest $\phi$ dependence near $\phi$ = $-120^\circ$ it is particularly well suited for this purpose. Therefore, the HN(CO)CO experiment is a useful addition to the host of recent experiments for determining backbone $\phi$ angles. 12,14 

Asp and Asn residues frequently play critical roles in intermolecular interactions, and the $\chi_1$ angle information derived from 3$^\circ$CC can be invaluable. 

The HN(CO)CO experiment is reasonably sensitive and can be carried out in a few days or less. For proteins with relatively long rotational correlation times, such as apo-CaM (t $\sim$ 8 ns), 15 the experiments are best carried out at frequencies of 500 MHz or below, as at higher field strengths the 13C$^{\circ}$ T2, which is dominated by chemical shift anisotropy, 16 decreases rapidly, adversely affecting sensitivity.

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Supporting Information Available: One table, containing the 3$^\circ$CC couplings measured in human ubiquitin, and one figure showing the correlation between 3$^\circ$CC couplings measured from a 4-scan and a 16-scan HN(CO)CO spectrum (4 pages). See any current masthead page for ordering and Internet access instructions.

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