Protein Side-Chain Rotamers from Dipolar Couplings in a Liquid Crystalline Phase

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One-bond backbone dipolar couplings can readily be measured for proteins dissolved in a dilute liquid crystalline phase.[1−3] They can be used to refine NMR structures determined by conventional methods or, in favorable cases, be sufficient to determine a structure de novo.[4,5] They also provide an invaluable tool for quality evaluation.[6,7] Nearly all focus thus far has been on the measurement of backbone dipolar couplings. However, side-chain conformations determine the critical details of the protein surface, and their characterization is therefore of the utmost importance.

Here, we demonstrate a simple and sensitive method for measuring $C^{\beta}-H^{\beta}$ dipolar couplings. Because for staggered rotamers the $C^{\beta}-H^{\beta}$ bonds are parallel to either $C^{\alpha}-H^{\alpha}$, $C^{\alpha}-N$, or $C^{\alpha}-C'$ bonds, comparison of the dipolar couplings frequently can identify the side chain rotamer directly, even in the absence of a backbone structure. As shown below, the $C^{\alpha}-H^{\alpha}$ coupling is obtained from the same experiment as the $C^{\beta}-H^{\beta}$ coupling. The $C^{\alpha}-N$ dipolar coupling is not easily measured at high accuracy,[8] but to a good approximation this bond is parallel to the $C^{\alpha}-C'$ bond of the preceding residue (rmsd = 6.5°, as derived from high-resolution crystal structures). For residues with a C^{β} methylene site, our method measures the sum of the two $C^{\beta}-H^{\beta}$ couplings. For a staggered rotamer this sum must be equal to the normalized sum of $C^{\alpha}-H^{\alpha}$ and $C^{\alpha}-N$ ($\chi_1 = -60°$), $C^{\alpha}-H^{\alpha}$ and $C^{\alpha}-C'$ ($\chi_1 = 180°$) or $C^{\alpha}-N$ and $C^{\alpha}-C'$ couplings ($\chi_1 = +60°$).

The $C^{\alpha}-H^{\alpha}$ and $C^{\beta}-H^{\beta}$ couplings are measured with the CB(CA)CONH pulse scheme[9] of Figure 1. It has been slightly modified from its original implementation by the use of adiabatic pulses, which decrease effects of RF inhomogeneity and offset, thereby significantly increasing sensitivity.[10] The experiment is performed three times, in an interleaved manner, with the ^{1}H 180° pulse during t_1 evolution switched between positions *a*, *b*, and *c*. The J_{CH} rephasing of $^{13}C^{\beta}$ (and $^{13}C^{\alpha}$) at the end of the constant-time t_1 evolution period depends on the duration of Δ_1. Ignoring cross-correlated relaxation during the short constant-time evolution period, the Δ_1-dependence of the methine and methylene C^{α} and C^{β} signal intensities is given by

$$S(\Delta_1) = A \sin[\pi \textstyle\sum J_{CH}(2\Delta_1 + \Delta_{eff})] \qquad (1)$$

where A is a constant, Δ_{eff} accounts for dephasing during the various pulses as defined in the legend to Figure 1, and $\sum J_{CH}$ is the sum of the $^1J_{CH}$ scalar coupling(s) and the residual one-bond dipolar coupling(s).

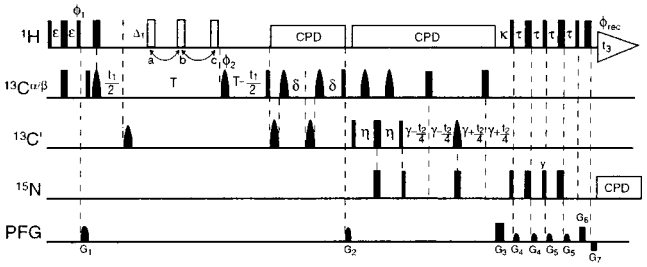

Figure 1. Pulse scheme of the 3D CB(CA)CONH quantitative J_{CH} experiment. Narrow and wide pulses correspond to flip angles of 90° and 180° respectively. All pulse phases are *x*, unless specified otherwise. The first three $^{13}C'$ 180° pulse has the shape of the center lobe of a sin *x*/*x* function, and a duration of 150 μs (at 150 MHz ^{13}C frequency). All other shaped $^{13}C'$ and $^{13}C^{\alpha/\beta}$ pulses (46 ppm) are of the hyperbolic secant adiabatic inversion type (500 μs), with a squareness level, μ, of 3.[17] The open ^{1}H pulses, applied at time point *a*, *b*, or *c*, is composite ($90°_x-180°_y-90°_x$). Delay durations: $\epsilon = 1.3$ ms; $\Delta_1 = 0.77$, 1.67 and 3.42 ms in the three interleaved experiments; T = 3.4 ms; $\delta = 3.4$ ms; $\eta = 11.0$ ms; $\gamma = 6.8$ ms; $\kappa = 5.4$ ms; $\tau = 2.7$ ms. For the reference spectrum, the first 180° ^{1}H pulse (open shape) is applied at position *a*, which gives a total J_{CH}-modulation duration of $2\Delta_1 + \Delta_{eff} = 1.93$ ms, where Δ_{eff} accounts for the effect of J_{CH} dephasing during the adiabatic ^{13}C inversion pulse, during the ^{13}C 90° pulses, and during short switching delays. The average rate of J_{CH} dephasing during the ^{13}C hyperbolic secant pulse, when the ^{1}H 180° pulse is applied at its center, equals ca. $J_{CH}/1.25$. Phase cycling: $\phi_1 = y,-y$; $\phi_2 = x,x,y,y$; Receiver = $x,-x,-x,x$. Rance-Kay t_2 quadrature detection is used,[18] alternating the phase of the first ^{15}N 90° pulse after gradient G_3 between *x* and $-x$, in concert with the polarity of gradient G_3. States-TPPI quadrature selection is used in the t_1 dimension. Pulsed field gradients $G_{1,2,4,5}$ are sine-bell shaped (18 G/cm for $G_{1,2}$, 28 G/cm for $G_{4,5}$), and $G_{3,6,7}$ are rectangular (30 G/cm). Durations: $G_{1,2,3,4,5,6,7}$ = 2, 1.4, 2.705, 0.4, 0.4, 0.2, 0.074 ms, with respective gradient axes: *xy*, *y*, *z*, *x*, *y*, *z*, *z*.

With two unknowns and intensities measured for three values of Δ_1, the best-fitted value of $\sum J_{CH}$ is derived using a SIMPLEX minimization routine.[11] Noise with an amplitude equal to the spectral noise is added to estimate the random error in the derived values of $\sum J_{CH}$. The first experiment utilizes $2\Delta_1 + \Delta_{eff} = 1.93$ ms and is essentially the same as the regular CB(CA)CONH. If the S/N in this "reference" spectrum for a given peak equals *Q*, the error in the coupling (or the sum of the couplings for $C^{\beta}H_2$ or Gly $C^{\alpha}H_2$) derived from the intensities in the three interleaved spectra equals approximately 50/*Q* Hz.

Figure 2 shows an example of strips taken through the 3D spectra of Ca^{2+}-calmodulin. Strips A−C are taken for the isotropic sample, for $2\Delta_1 + \Delta_{eff}$ values of 1.93, 3.73, and 7.22 ms. Strips D−F are the corresponding traces for the sample in liquid crystalline Pf1 (18 mg/mL).[12] The strips are taken at the (^{15}N,^1H) coordinates of Asp[93], and show the correlations to C^{β} and C^{α} of Phe[92]. Methine carbons show highest intensity in strip B and near zero intensity in strip C, where $2\Delta_1 + \Delta_{eff} \approx 1/J_{CH}$. The small negative residual C^{α} intensity in strip C indicates that $J_{C\alpha H\alpha}$ (150 Hz) is larger than 1/(7.22 ms) = 138.5 Hz. Non-Gly residues with positive ϕ angles (none in calmodulin, but clearly identifiable in several other proteins) are the only ones with negative intensity, caused by the characteristically small $J_{C\alpha H\alpha}$ in such residues.[13]

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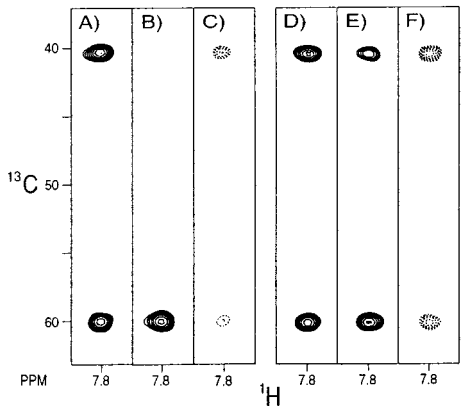

Figure 2. $^{13}C^{\alpha/\beta}$ strips of the interleaved 3D CB(CA)CONH spectra of mammalian Ca^{2+}-calmodulin, taken at the (^{15}N,^{1}H) shifts of Asp^{93}, recorded for different J_{CH} dephasing delays of (A,D) 1.93 ms, (B,E) (3.73 ms), and (C,F) 7.22 ms. Spectra A−C and D−F correspond to isotropic and Pf1-aligned (18 mg/mL) sample, respectively. Dashed contours denote negative intensity. Spectra were recorded at 600 MHz ^{1}H frequency for 0.4 mM Ca^{2+}-calmodulin, pH 6.8, 100 mM KCl, 25 °C. Each spectrum originates from a 61* × 45* × 512* data matrix with acquisition times of 6.8 (t_1), 27 (t_2), and 60 (t_3) ms, 4 scans per FID, and a total measuring time of 40 h for the three interleaved spectra.

Table 1. Normalized One-Bond Dipolar Couplings in Calmodulin

residue	$D_{C\alpha N}$[a,b] (Hz)	$D_{C'C\alpha}$[b] (Hz)	$D_{C\alpha H\alpha}$[c] (Hz)	$\sum D_{C\beta H\beta}$[c] (Hz)	χ_1[d] (deg)
Asp^{93}	−4	39	−48	−5	180
Ser^{101}	−52	8	15	−44	69
Val^{108}	−23	18	2	3	179
Lys^{115}	−20	−5	9	−9	−56
Glu^{127}	13	−23	17	7	−76
Tyr^{138}	−26	39	14	54	176

[a] Obtained from $D_{C'C\alpha}$ of the preceding residue. [b] Values normalized to $D_{C\alpha H\alpha}$ by multiplication by 10. Random errors in the normalized values are ±3 Hz for $D_{C\alpha N}$ and $D_{C'C\alpha}$. [c] Average estimated random error ±3 Hz. [d] Averaged over two X-ray structures.[15,16]

As expected on the basis of eq 1, the C^{β} intensity in strips B and C is near zero, and corresponds to $J_{C\beta H\beta 2} + J_{C\beta H\beta 3} = 263$ Hz. In the aligned state (strips D−F) the intensities yield splittings of $\sum J_{C\alpha H\alpha} = 157$ Hz, and $\sum J_{C\beta H\beta} = 230$ Hz.

Table 1 presents measured dipolar couplings for a selected subset of residues. For the complete set, the dipolar couplings are compatible with ideal staggered rotamers for about 50% of the non-Gly/Ala residues. These only include residues for which the sum of the two normalized $^{13}C^{\alpha}-X$ (X = $^{1}H^{\alpha}$, $^{13}C'$, ^{15}N) dipolar couplings differs by less than 6 Hz from $D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$, or for Val, Thr, and Ile residues, $^{13}C^{\alpha}-X$ differs less than 6 Hz from $D_{C\beta H\beta}$. The total range of experimental normalized couplings spans from −52 to +45 Hz. The 6 Hz threshold corresponds to the rms change in $D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$ and $D_{C\beta H\beta}$ for simulated calmodulin data, where the rotamers are twisted by +10° or −10° from ideal staggered positions, using an alignment tensor magnitude of $D_a^{CH} = -22$ Hz, and rhombicity $R = 0.47$, as applies for calmodulin. The vast majority of the 50% of residues for which the dipolar couplings identify near-ideal staggered rotamers are located in regular secondary structure, or are relatively shielded from solvent. Stereospecific H^{β} and rotamer assignments using a grid search and homonuclear *J* coupling and NOE data yields comparable results,[14] but it is important to note that the two approaches are complementary and fail in different cases. For example, the conventional analysis usually fails if the two H^{β} protons overlap; the dipolar coupling method fails if for a given C^{α} the three normalized backbone couplings coincidentally are similar to one another.

Examples shown in Table 1 are selected to illustrate the different cases encountered. For the Ca^{2+}-coordinating side chain of Asp^{93}, $D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$ is closest to $D_{C'C\alpha} + D_{C\alpha H\alpha}$ (indicating $\chi_1 \approx 180°$), in agreement with X-ray structures.[15,16] For Ser^{101}, $D_{C\alpha N} + D_{C'C\alpha}$ matches $D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$, indicating $\chi_1 = +60°$, but $D_{C'C\alpha} \approx D_{C\alpha H\alpha}$ and the possibility of a $\chi_1 \approx -60°$ rotamer therefore cannot be excluded without additional data. For Val^{108}, $D_{C\alpha H\alpha} = D_{C\beta H\beta}$, indicating $\chi_1 = 180°$. For Lys^{115}, $D_{C\alpha N} + D_{C\alpha H\alpha} = D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$, indicating $\chi_1 \approx -60°$. However, the reference spectral intensity (for $2\Delta_1 + \Delta_{eff} = 1.93$ ms) is much higher than average, indicative of substantial internal backbone motion of this loop residue. Although the $D_{C\beta H\beta}$ data suggest that the $\chi_1 \approx -60°$ rotamer is prevalent, absolute values of the couplings are too small to exclude small populations of the other rotamers. For Glu^{127}, $D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$ is intermediate between $D_{C\alpha N} + D_{C\alpha H\alpha}$ ($\chi_1 \approx -60°$) and $D_{C'C\alpha} + D_{C\alpha H\alpha}$ ($\chi_1 \approx 180°$), suggestive of rotameric averaging but not excluding the possibility of a skewed rotamer. Finally, for Tyr^{138} $D_{C\beta H\beta 2} + D_{C\beta H\beta 3} = 54$ Hz has a larger value than the maximum allowed value of 44 Hz for an ideal tetrahedral carbon and an alignment tensor magnitude $D_a^{CH} = 22$ Hz. Small distortions from idealized geometry and random error in the measurement are likely causes of this discrepancy. The $\chi_1 \approx +180°$ rotamer is the only one compatible with the large observed value of $D_{C\beta H\beta 2} + D_{C\beta H\beta 3}$. The other rotamers predict much smaller values of −12 ($\chi_1 = -60°$) or +13 Hz ($\chi_1 = +60°$).

Calculations show that the amplitude of dipolar couplings is little affected by harmonic oscillations about the average rotamer orientation. Using a Gaussian distribution of rotamers with rmsd of 15° relative to the average position causes a rms change in dipolar coupling that is less than 3 Hz (averaged over all residues in calmodulin, using $D_a^{CH} = -22$ Hz, and $R = 0.47$). This confirms that dipolar couplings are rather insensitive to small amplitude motions. In contrast, rotameric averaging involves large reorientations and usually has a pronounced effect on the dipolar coupling. Details regarding the effect of motional averaging on dipolar couplings will be presented elsewhere.

The CB(CA)CONH experiment presented here is a sensitive method for simultaneous measurement of $C^{\alpha}H^{\alpha}$ and $C^{\beta}H^{\beta}$ couplings. Together with $C'C^{\alpha}$ couplings derived from a $^{13}C^{\alpha}$-coupled HNCO experiment, these provide χ_1 rotamer information for a significant fraction of residues in a protein, even in the absence of backbone structure. CB(CA)CONH and HNCO are among the most sensitive triple resonance experiments and can be applied to proteins of substantial size. This indicates that the approach will be applicable to a wide range of proteins.

Software available: For pulse sequence code and analysis software, see http://spin.niddk.nih.gov/bax/pp.

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