A Sensitive Method for the Measurement of Three-Bond C′,H$^{\alpha}$ *J* Couplings in Uniformly ^{13}C- and ^{15}N-Enriched Proteins

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Vicinal spin−spin coupling constants can provide direct information about local conformation. A variety of NMR methods have been developed to determine the magnitude of homonuclear and heteronuclear couplings in isotopically labeled proteins.[1] Due to the ambiguity inherent to Karplus-type relations,[2] accurate torsion angles can only be derived from a combination of several coupling constants. For the polypeptide backbone angle ϕ, the measurement of ^3J(C,H) coupling constants[3−5] supplements the structural information available from $^3J(H^N,H^{\alpha})$. Recently, we have introduced an E.COSY-type[6] experiment for the quantitative determination of $^3J(C'_{i-1},H^{\alpha}_i)$.[4] A drawback of this (H)NCAHA pulse sequence is that α-protons are detected during acquisition while the use of H_2O as the solvent is mandatory. Hence, the evaluation of coupling constants is impossible for residues with $^1H^{\alpha}$ resonances in the vicinity of the intense water signal. In the HCAN-[C′]E.COSY described by Wang and Bax,[5] this difficulty is circumvented by a magnetization transfer pathway suitable for samples dissolved in D_2O. Thus, accurate values for $^3J(C'_{i-1},H^{\alpha}_i)$ can, in principle be obtained for all residues in a protein. However, as a consequence of the long periods of $^{13}C^{\alpha}$ transverse magnetization, this pulse sequence is expected to be relatively inefficient for proteins with fast $R_{2,C\alpha}$ relaxation rates. In this paper, we propose a sensitive E.COSY-type experiment, H$^{\alpha}$-coupled H(N)CA,CO, in which the $^3J(C'_{i-1},H^{\alpha}_i)$ displacement occurs in an indirectly detected ^{13}C′ dimension, thereby avoiding difficulties caused by the solvent resonance.

The new method is based on the COHNNCA,[7] a dimensionality reduced sequence which simultaneously correlates amide protons and nitrogens with carbonyl carbons of the preceding and α-protons of the same and the preceding residue. In the pulse scheme depicted in Figure 1, $^{13}C^{\alpha}$ and ^{13}C′ chemical shifts are sampled in two independent dimensions. Since no proton decoupling is applied during t_1 and t_2, the large $^1J(C^{\alpha},H^{\alpha})$ coupling leads to a splitting of the signals along the F_1 ($^{13}C^{\alpha}$) dimension. Vicinal and geminal ^{13}C′,$^1H^{\alpha}$ couplings evolving in the subsequent t_2(^{13}C′) domain can be extracted from the displacement of the two components of the intra- and interresidual correlations, respectively (Figure 2A). It might be argued that the assignment of the role of the passive spin to α-protons leads to an underestimation of the ^{13}C′,$^1H^{\alpha}$ coupling constants due to different relaxation rates of antiphase terms compared to those of in-phase terms.[8] This effect is, however, minimized as the time between $^{13}C^{\alpha}$ and ^{13}C′ evolution periods, in which $^1H^{\alpha}$ spin flips can occur, is essentially zero owing to the synchronous buildup of ^{15}N antiphase magnetization with respect to both carbon species.

Sufficient resolution in the ^{13}C′ domain requires a large number of t_2 increments, leading to a relatively long measuring time. Although extensive aliasing would afford relief, we prefer to employ chemical shift scaling[9] to avoid the accidental introduction of signal overlap from different spectral regions. In version B of the H$^{\alpha}$-coupled H(N)CA,CO pulse sequence, the evolution of two-bond and three-bond ^{13}C′,$^1H^{\alpha}$ couplings during t_2 is scaled up by a factor of 2 with respect to ^{13}C′ chemical shifts (Figure 2B). As a result, the number of increments can be substantially decreased, while retaining the

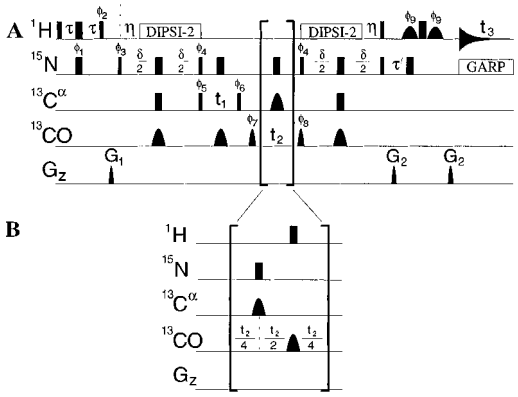

Figure 1. Pulse scheme of the H$^{\alpha}$-coupled H(N)CA,CO experiment without (version A) and with (version B) chemical shift scaling in the ^{13}C′ dimension. Narrow and wide pulses denote 90 and 180° flip angles, respectively. The proton carrier frequency is placed on the water resonance and is shifted to the center of the amide region for the DIPSI-2[12] decoupling sequences. The carbon carrier position is 58 ppm until completion of the t_1 evolution and changed to 176 ppm following the pulse with the phase ϕ_6. The shaped $^{13}C^{\alpha}$ pulse is a G3 Gaussian cascade[13] with a duration of 0.5 ms. Carbonyl 90 and 180° pulses have a duration of 124 μs and an amplitude profile of the center lobe of a sinc function. GARP ^{15}N decoupling[14] during acquisition is performed using a 0.75 kHz radiofrequency field. Solvent suppression is achieved with the WATERGATE method,[15] applied during the ^{1}H,^{15}N reverse INEPT step.[16] The width of the 90° Gaussian-shaped pulses for selective excitation of the water resonance is 2.5 ms. Gradient durations and strengths are the following G_1, 1 ms and 10 G cm^{-1}; G_2, 0.8 ms and 35 G cm^{-1}. Phase cycling is as follows: $\phi_1 = x, -x$; $\phi_2 = y, -y$; $\phi_3 = 2(x), 2(-x)$; $\phi_4 = y$; $\phi_5 = 4(x), 4(-x)$; $\phi_6 = x + 25°$; $\phi_7 = 8(x), 8(-x)$; $\phi_8 = x + 47°$; $\phi_9 = -x$; receiver x, $2(-x)$, x, $-x$, $2(x)$, $2(-x)$, $2(x)$, $-x$, x, $2(x)$, x. Nonlabeled pulses are applied along the x-axis. The phases ϕ_6 and ϕ_8 are adjusted to compensate for zero-order Bloch−Siegert phase errors as indicated. Quadrature in the t_1 and t_2 domains is obtained by changing the phases ϕ_5 and ϕ_7, respectively, in the States−TPPI manner.[17] Delay durations are $\tau = 2.3$ ms, $\delta = 28.6$ ms, $\eta = 5.4$ ms, and $\tau' = 2.7$ ms. Acquisition times were 14.1 ms in t_1 and 106.5 ms in t_3. In t_2, 82 ($t_{2,max} = 75.4$ ms) and 52 ($t_{2,max} = 95.7$ ms) complex increments were recorded for A and B, respectively. Accumulation of 16 scans per FID resulted in measuring times of 72 h (A) and 41 h (B).

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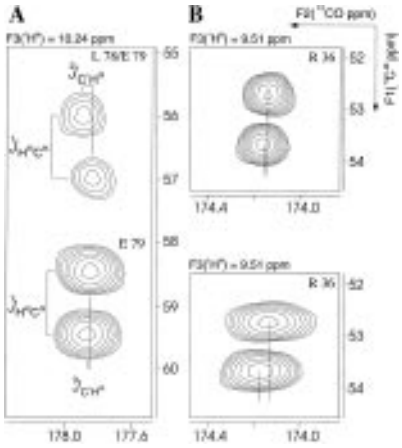

Figure 2. (A) Expansion of a F_1, F_2 plane of the H^{α}-coupled H(N)CA,CO spectrum (version B) at the F_3 ($^1H^N$) chemical shift of residue Glu 79 from *D. vulgaris* flavodoxin. The horizontal displacements of the two doublet components are due to $^3J(C'_{i-1},H^{\alpha}_i)$ and $^2J(C'_{i-1},H^{\alpha}_{i-1})$ couplings for intraresidual and sequential crosspeaks, respectively. (B) Effect of chemical shift scaling. The intraresidual crosspeaks of Arg 36 in the spectra recorded with versions A (top) and B (bottom) of the H(N)CA,CO pulse sequence are shown. Since identical ppm scales are used, the line width and the E.COSY splitting appear doubled in the second case.

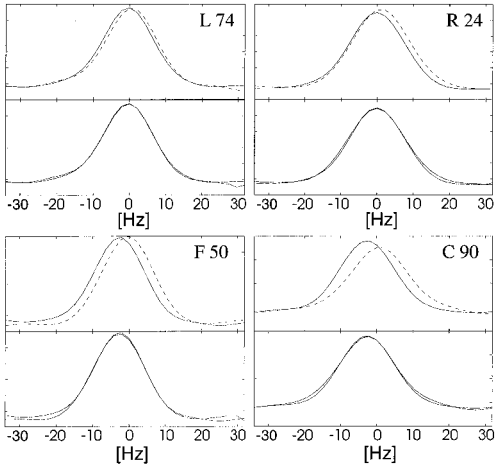

Figure 3. Traces from the H^{α}-coupled H(N)CA,CO spectrum (version B) of flavodoxin, obtained by summation over the spectral points in F_1 and F_3. The upper panels represent the original traces through the low-field (solid line) and high-field (dashed line) multiplet halves. In the lower panels, a superposition of the fitted traces is shown. A continuous frequency shift is achieved by inverse Fourier transformation, followed by multiplication of the t_2 time domain data with a complex phase factor.[10] The $^3J(C'_{i-1},H^{\alpha}_i)$ values resulting from the fitting procedure are 0.83±0.12 Hz (Leu 74), 1.22±0.1 Hz (Arg 24), 2.41±0.16 Hz (Phe 50), and 3.39±0.14 Hz (Cys 90).

spectral width and the accuracy of the frequency determination of the individual crosspeak components.

The two versions of the novel pulse sequence were applied to a 1.4 mM uniformly $^{13}C/^{15}N$-enriched sample of oxidized *Desulfovibrio vulgaris* flavodoxin (MW 16.3 kDa). Both experiments were performed at a Bruker DMX 600 spectrometer equipped with a pulsed field gradient unit and an actively shielded gradient triple-resonance probe. For the evaluation of *J*-coupling constants, a least-squares fit was carried out in the t_2 time domain as described by Schmidt et al.[10] Representative examples are shown in Figure 3.

Three-bond $^{13}C',^1H^{\alpha}$ couplings were determined for 103 residues of flavodoxin. The root mean square pairwise difference for the coupling constants obtained with versions A and B was 0.54 Hz. A graphical representation of the dependence on the ϕ-angle together with the available Karplus curves is given in Figure 4. These values are taken from the spectrum recorded with the chemical shift scaled version of the H(N)CA,CO sequence, unless overlap resulting from the broader lines in F_2 prevented the evaluation, as was the case for nine residues. The Karplus parameters of Wang and Bax[5] yielded the best agreement between measured and expected coupling constants although for residues with ϕ-angles around −120° slightly higher values are obtained in the present study. A relatively large scattering of ^3J couplings is observed for some residues with similar ϕ-angles. Apart from experimental errors, this might be due to a varying amplitude of fluctuations leading to *J* averaging[11] or due to differences between the backbone conformation in the crystal and the solution structure. The majority of residues exhibiting large deviations of experimental $^3J(C'_{i-1},H^{\alpha}_i)$ coupling constants with respect to the Karplus curve[5] is located outside regions of regular secondary structure elements. Sequential correlations yielding $^2J(C'_{i-1},H^{\alpha}_{i-1})$ values could be detected for all residues unless obscured by stronger, overlapping signals. Since the intensities are considerably smaller compared to those of intraresidual peaks, a lower precision can be expected for the geminal coupling constants.

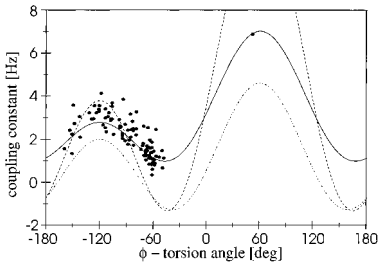

Figure 4. Experimental $^3J(C'_{i-1},H^{\alpha}_i)$ coupling constants versus ϕ-angles derived from the crystal structure of oxidized *D. vulgaris* flavodoxin at 1.7 Å resolution.[18] The corresponding Karplus curves are taken from Wang and Bax[5] (—), Bystrov et al.[19] (- - -), and Solkan and Bystrov[20] (— ·· —).

In conclusion, a method has been introduced which allows the determination of $^3J(C'_{i-1},H^{\alpha}_i)$ for all non-glycine and non-proline residues in isotopically labeled proteins. The high sensitivity of the pulse sequence should enable its application to larger proteins than the one investigated in this work.

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