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SUMMARY

A simple E.COSY type technique is described for measurement of two-bond $J_{COH\alpha}$ coupling constants in proteins that are uniformly enriched with ^{13}C. The method has been used to measure $^2J_{COH\alpha}$ for 132 residues in the proteins calmodulin and staphylococcal nuclease having non-overlapping H_α-C_α correlations. Measured $^2J_{COH\alpha}$ coupling constants fall in the 0 to -9.5 Hz range. A separate experiment, measuring the accuracy of these values, indicates a root-mean-square error of 1 Hz. Comparison of the J couplings with the dihedral backbone angles from crystallographic studies confirms a weak but statistically significant correlation between the dihedral angle ψ and the magnitude of $^2J_{COH\alpha}$, but also indicates that parameters other than ψ have a significant effect on the value of the coupling.

It has long been recognized that spin-spin coupling constants provide very useful information regarding polypeptide structure (Bystrov, 1976). Mainly for sensitivity reasons, the use of J coupling information in proteins has long been restricted to homonuclear $^3J_{HNH\alpha}$ couplings and $^3J_{H\alpha H\beta}$ couplings. However, recent advances in molecular biology have greatly facilitated isotopic enrichment with ^{13}C and ^{15}N for many proteins of biological interest. A large array of new experiments has been proposed in recent years which make it possible to measure both homo- and heteronuclear multiple- and single-bond J couplings in such isotopically enriched proteins (Montelione et al., 1989; Wider et al., 1989; Chary et al., 1991; Delaglio et al., 1991; Edison et al., 1991; Wagner et al., 1991; Griesinger and Eggenberger, 1992; Vuister et al., in press). Many, but not all, of these methods are based on the E.COSY principle (Griesinger et al., 1986) where the coupling to a nucleus of interest, X, can be measured from the relative displacement of the cross peaks between two other nuclei that are both coupled to X. Here we report the first application of a hetero-

*Supplementary material available from the authors: One table containing ψ angles and the measured $^2J_{COH\alpha}$ coupling constants for the proteins calmodulin and staphylococcal nuclease.

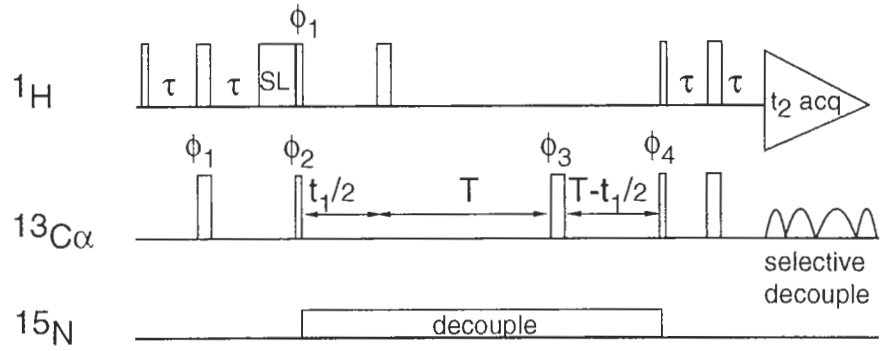

Fig. 1. Pulse sequence of the ^{13}CO-coupled CT-HSQC experiment. Narrow and wide pulses denote 90° and 180° flip angles, respectively. The ^{13}C carrier was positioned at 57 ppm and the power of the 90° and 180° pulses was adjusted such that these pulses have a null in their excitation profile at the ^{13}CO frequency. Each of the 90°, 180°, and 270° pulse elements of the selective WALTZ-16 decoupling scheme has the shape of the center lobe of a $\sin x/x$ function and a duration of 101, 202, and 303 μs, respectively. SL denotes a short spinlock pulse (500 μs) to suppress the residual HDO resonance (Messerle et al., 1989). Unless indicated otherwise, all pulses are applied along the x-axis. The phase cycle is as follows: $\phi_1 = y, -y$; $\phi_2 = x$; $\phi_3 = 2(x), 2(-x), 2(y), 2(-y)$; $\phi_4 = 8(x), 8(-x)$; receiver $= 2(x,-x), 4(-x,x), 2(x,-x)$. Quadrature detection in t_1 is obtained by the States-TPPI technique, incrementing phase ϕ_2 (Marion et al., 1989). The delay τ was set to 1.7 ms.

nuclear E.COSY-type experiment, where the passive spin X is a non-protonated ^{13}C, in order to measure $^2J_{COH\alpha}$. This work was stimulated by a recent report (Hansen, 1991) which suggests a correlation between $^2J_{COH\alpha}$ and the backbone angle ψ.

The pulse scheme used herein is sketched in Fig. 1 and is a simple modification of the constant-time HSQC experiment (Palmer et al., 1992; Santoro and King, 1992; van de Ven and Philippens, 1992; Vuister and Bax, 1992). In the present scheme, decoupling of the $^1J_{COC\alpha}$ interaction during the constant-time evolution period is omitted, and during data acquisition a WALTZ-16 decoupling scheme is used (Shaka et al., 1983) in which each of the 90°, 180°, and 270° pulse elements have the shape of the center lobe of a $\sin x/x$ function. A similar shaped decoupling scheme has recently been described in detail by McCoy and Müller (1992). The modified WALTZ scheme has the advantage that it does not significantly affect resonances at offsets more than $4\nu_{RF}$, where ν_{RF} is the average decoupler RF field strength. Therefore, this shaped WALTZ scheme effectively decouples the $^1J_{C\alpha H\alpha}$ interaction without affecting the spin state of its adjacent ^{13}CO nucleus. This means that the spin state of the ^{13}CO nucleus remains unchanged during the entire pulse sequence and two correlations for each $^1H_\alpha$-$^{13}C_\alpha$ pair are observed, corresponding to the $|\alpha>$ and $|\beta>$ spin states of the ^{13}CO nucleus. The F_1 displacement of the two components corresponds to $^1J_{COC\alpha}$, and the F_2 displacement corresponds to $^2J_{COH\alpha}$. The pulse scheme has been applied to Ca^{2+}-ligated calmodulin (CaM) (1.0 mM; p^2H 6.3; 100 mM KCl; 99.8% D_2O) and to staphylococcal nuclease ligated with Ca^{2+} and thymidine 3′,5′-biphosphate (1.5 mM, p^2H 7.0; 100 mM NaCl; 99.8% D_2O). All experiments were carried out on a Bruker AMX-600 spectrometer, operating at 35 °C.

Figure 2 shown a small region of the 2D H_α-C_α shift correlation spectrum of CaM. It is clear from the cross peaks shown that the signs of $^1J_{COC\alpha}$ and $^2J_{COH\alpha}$ are opposite because the doublet components are displaced from the doublet center in directions that are opposite in the F_1 and F_2 dimensions (Bax and Freeman, 1981). Thus, a $|\beta>$ ^{13}CO spin state causes an increase in the $^{13}C_\alpha$

resonance frequency and a decrease in the H_α frequency, assuming a positive $^1J_{COC\alpha}$ (Bystrov, 1976), which confirms $^2J_{COH\alpha}$ to be negative (Hansen et al., 1975). For the two proteins combined, 132 pairs of cross peaks were sufficiently resolved for reliable measurement of the coupling. For 125 of these pairs, accurate backbone angles are available from the X-ray crystal structures. The peak positions for each pair of cross peaks were determined using a constrained peak picking procedure (Delaglio, unpublished), which utilizes the information that both components of the doublet must have identical shape and amplitude. The accuracy of this method for measuring coupling constants was tested by applying the method to data recorded with the scheme of Fig. 1, but with the selective WALTZ replaced by a non-selective GARP sequence which collapses the $^2J_{COH\alpha}$ splitting. The average displacement measured this way was -0.05 ± 1.05 Hz; hence, 1 Hz is a good estimate for the root-mean-square (rms) error in our measurement of $^2J_{COH\alpha}$.

The one-bond $^1J_{COC\alpha}$ coupling constants measured for the 125 residues show a quite uniform value of 53.0 ± 1.2 Hz. Two-bond $^2J_{COH\alpha}$ coupling constants are expected to vary as a function of the backbone angle ψ (Bystrov, 1976). Figure 3 shows values of the measured 125 $^2J_{COH\alpha}$ coupling constants vs. the angles ψ obtained from the X-ray crystal structures of CaM (Babu et al., 1988) and staphylococcal nuclease (Loll and Lattman, 1989). It is evident from Fig. 3 that the $^2J_{COH\alpha}$ couplings span a considerable range (-9.5–0 Hz), significantly larger than the 4.2–7.1 Hz

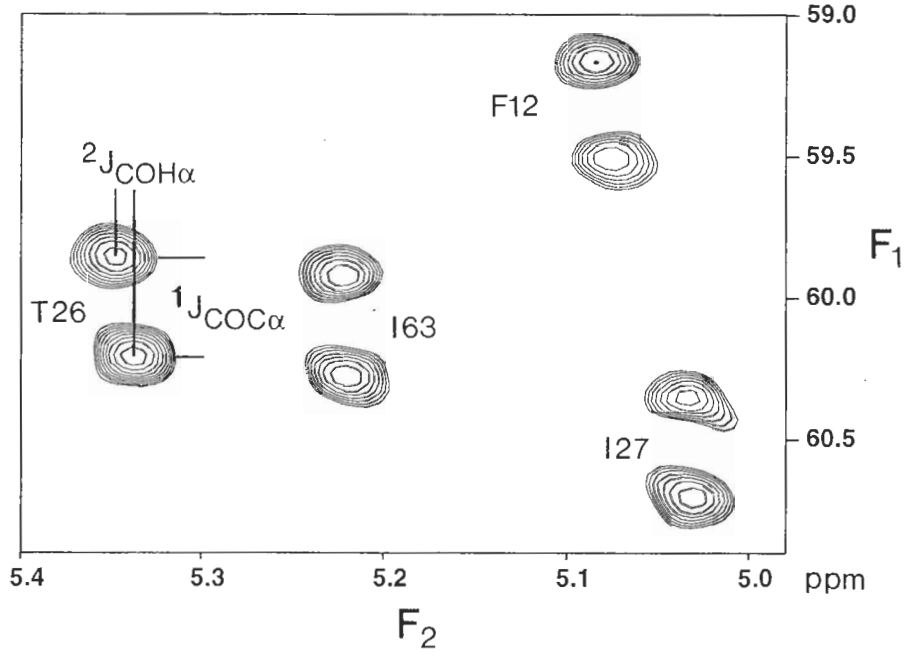

Fig. 2. Small section of the C_α-H_α region of the ^{13}CO-coupled CT-HSQC spectrum of CaM, recorded with the pulse scheme of Fig. 1. F_1 and F_2 displacements of the doublet components correspond to $^1J_{COC\alpha}$ and $^2J_{COH\alpha}$, respectively. The spectrum results from a $256^*(t_1) \times 512^*(t_2)$ point data matrix, recorded with acquisition times of 51 (t_1) and 71 (t_2) ms, using 256 scans per complex t_1 increment and a total measuring time of 18 h per spectrum. Squared sine-bell windows, shifted by 60° in t_1 and 30° in t_2 were used. Data were zero-filled to $1024^*(t_1) \times 2048^*$ prior to Fourier transformation, resulting in a digital resolution of 4.9 Hz (F_1) and 3.5 Hz (F_2).

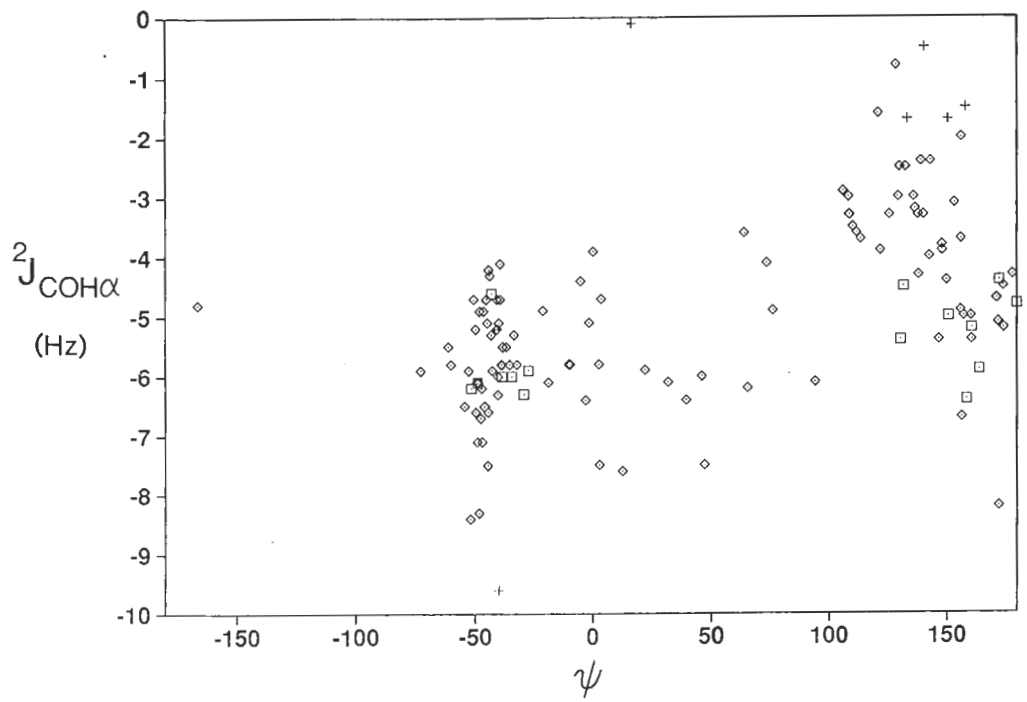

Fig. 3. Measured $^2J_{COH\alpha}$ coupling constants vs. the backbone angle ψ, obtained from the X-ray crystal structures of CaM (Babu et al., 1988) and staphylococcal nuclease (Loll and Lattman, 1989). $^2J_{COH\alpha}$ values measured for proline residues are marked +, threonine and serine residues are marked by squares, and all other residues are represented by diamonds.

reported earlier by Hansen et al. (1975) for peptides. The average $^2J_{COH\alpha}$ value for residues with a ψ angle of ca. $-60°$ is more negative than for residues with a ψ angle of $\sim 120°$. This is in accordance with the earlier observation that the intensities of CO-Hα cross peaks in the HMBC spectrum of BPTI are smaller for residues with a dihedral angle ψ of ca. 120° (Hansen, 1991).

The 1-Hz rms error in our measurement is considerably smaller than the spread in $^2J_{COH\alpha}$ observed for a given ψ angle, particularly for $\psi \sim 120°$. This indicates that factors other than ψ have a significant influence on $^2J_{COH\alpha}$. These may include the ϕ angle, the χ_1 angle, the ω angle, and the amino acid type (Hansen, 1991). Figure 3 suggests that prolines (marked + in Fig. 3) with a large positive ψ angle tend to have near-zero values for $^2J_{COH\alpha}$. Three proline residues in BPTI with large positive ψ angles also have unusually small couplings (Hansen, 1991). The only *cis* proline in our database (staphylococcal nuclease Pro^{117}) shows the smallest of all observed couplings ($^2J_{COH\alpha} = -0.1$ Hz, $\psi = 16°$). The largest coupling in our data set (−9.5 Hz) was also measured for a proline residue (staphylococcal nuclease Pro^{56}). This residue is located at the beginning of an α-helix and has a substantially different ψ angle ($-40°$). In contrast to the large variation observed for proline residues, the $^2J_{COH\alpha}$ coupling constants in threonine and serine residues all show quite uniform values of ~ -5 Hz, with no noticeable dependence on ψ.

We have shown that $^2J_{COH\alpha}$ coupling constants can be measured in a straightforward way from E.COSY type doublet patterns in a CT-HSQC spectrum which is optimized to avoid excitation of

the ^{13}CO nucleus. Although the observed magnitudes of the $^{2}J_{COH\alpha}$ coupling constants correlate with the backbone angle ψ, the high degree of scatter in this correlation prevents the direct use of $^{2}J_{COH\alpha}$ for protein structure determination until a better understanding has been obtained of other factors influencing this coupling. The method presented here is easily extended to measurement of $^{3}J_{COH\beta}$ by combining the present experiment with the 3D HCCH-COSY technique (Kay et al., 1990; Ikura et al., 1991).

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