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Measurement of three-bond, $^{13}C'$-$^{13}C^{\beta}$ J couplings in human ubiquitin by a triple resonance, E. COSY-type NMR technique

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Abstract

A [CO]HN(CA)CB-E.COSY pulse scheme is described for measurement of three-bond couplings, $^3J_{C'C^{\beta}}$, between carbonyl and aliphatic C^{β} carbons in ubiquitin, uniformly enriched with ^{13}C and ^{15}N. A Karplus relation, $^3J_{C'C^{\beta}} = 1.28\cos^2(\phi - 120°) - 1.02\cos(\phi - 120°) + 0.30$ Hz, is obtained by correlating the $^3J_{C'C^{\beta}}$ values measured for human ubiquitin with backbone ϕ angles from its crystal structure. As predicted, the new Karplus parametrization yields $^3J_{C'C^{\beta}}$ values slightly larger than previously obtained by quantitative J correlation [Hu, J.-S. and Bax, A. (1997) *J. Am. Chem. Soc.*, **119**, 6360–6368], but considerably smaller than what has been reported on the basis of other E.COSY-type measurements carried out on flavodoxin.

The protein backbone torsion angle ϕ is characterized by six three-bond J couplings,$^3J_{HNH\alpha}$, $^3J_{HNH\beta}$, $^3J_{HNC'}$, $^3J_{C'H\alpha}$, $^3J_{C'C^{\beta}}$ and $^3J_{C'C'}$ All these couplings have been measured in human ubiquitin by quantitative J correlation methods (Hu and Bax, 1996, 1997; Wang and Bax, 1996) whereas E. COSY-type techniques have also been used to measure three of them: $^3J_{HNH\alpha}$, $^3J_{HNC'}$, and $^3J_{H\alpha C'}$ (Wang and Bax, 1996). For these latter J couplings, excellent agreement is observed between the results obtained with the two different measuring techniques. Karplus-type equations (Karplus, 1959) have been parametrized or reparametrized which correlate these couplings with the intervening dihedral ϕ angle (Hu and Bax, 1996, 1997; Wang and Bax, 1996). For any given residue, there is a strong correlation between the differences between the six measured J couplings and values predicted by their Karplus curves on the basis of the X-ray structure. Indeed, any one of these J couplings can be predicted more accurately on the basis of the remaining five J couplings than on the basis of the ϕ angle obtained from the crystal structure (Hu and Bax, 1997). This suggests that the methods used for measuring these ^3J couplings are highly accurate and self-consistent.

Recently, Löhr et al. (1997) described an elegant new E. COSY-type experiment for measurement of three-bond $^3J_{C'C^{\beta}}$ couplings in the oxidized flavodoxin from *Desulfovibrio vulgaris.* Surprisingly, the magnitude of the $^3J_{C'C^{\beta}}$ couplings and the corresponding Karplus coefficients measured for flavodoxin are considerably (∼40%) larger than those from our quantitative J measurements. We predicted (Hu and Bax, 1997) that the values obtained from the HN(CO)C quantitative J correlation experiment would be smaller by only 5–10% relative to the true J values, as no correction for the $^{13}C^{\beta}$ T_1 value had been made. In order to eliminate the possibility of a systematic error in our previous measurement of $^3J_{C'C^{\beta}}$, we use a technically straightforward E. COSY-type experiment to remeasure, $^3J_{C'C^{\beta}}$ couplings in human ubiquitin. Although the precision obtainable with this new measurement is lower compared to that of the original HN(CO)C experiment, the new data confirm that the HN(CO)C experiment underestimates $^3J_{C'C^{\beta}}$ by only ∼7%.

Supplementary material available from the authors: one table with $^3J_{C'C^{\beta}}$ coupling constants in ubiquitin, measured with the [CO]HN(CA)CB-E.COSY experiment.

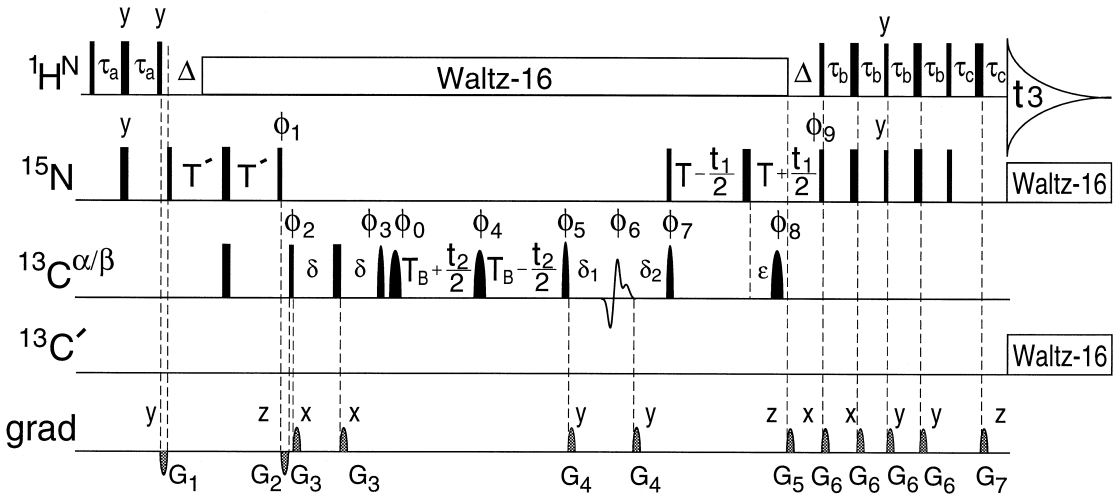

Figure 1. Pulse scheme for the 3D [CO]HN(CA)CB-E.COSY experiment. Narrow and wide pulses denote 90° and 180° flip angles, respectively, and unless indicated the phase is *x*. ^{1}H decoupling is applied using the WALTZ-16 modulation scheme ($\gamma_H B_1 = 3.3$ kHz), with the carrier at the H_2O resonance. $^{13}C^{\alpha/\beta}$ (46 ppm) rectangular pulses have durations of 49 μs for 90° and 43.8 μs for 180°, with a null in the excitation profile at the $^{13}C'$ frequency at 151 MHz ^{13}C frequency. The $^{13}C^{\alpha/\beta}$ $180^\circ_{\phi 6}$ pulse is G_3-shaped (Emsley and Bodenhausen, 1990) and has a duration of 370 μs. The $^{13}C^{\alpha/\beta}$ $90^\circ_{\phi 3}$, $90^\circ_{\phi 5}$ and $90^\circ_{\phi 7}$ pulses are sine-bell shaped and have durations of 130 μs, while $^{13}C^{\alpha/\beta}$ (40 ppm) $180^\circ_{\phi 0}$, $180^\circ_{\phi 4}$ and $180^\circ_{\phi 8}$ pulses (600 μs) have hyperbolic secant envelopes with squareness levels, μ, of 3 (Silver et al., 1984), and an inversion bandwidth (>90%) of ±5.8 kHz. The $180^\circ_{\phi 0}$ pulse compensates for the phase distortion introduced by the $180^\circ_{\phi 4}$ refocusing pulse. $^{13}C'$ (177 ppm) decoupling is applied using a WALTZ16 modulation scheme with $\gamma_C B_2 = 1.0$ kHz. For ^{15}N (116.5 ppm), $\gamma_N B_2 = 5.3$ kHz (pulses), or 1.0 kHz (decoupling). Phase cycling: $\phi_1 = x, -x$; $\phi_2 = x$; $\phi_3 = 2(y), 2(-y)$, $\phi_4 = 4(x), 4(y)$; $\phi_5 = -y$; $\phi_6 = 83^\circ$ (to compensate for the change in phase at the different power level used); $\phi_7 = x$; $\phi_8 = x$; $\phi_9 = -x$; receiver = x, −x, x, 2(−x), x, −x, x. Quadrature detection in the t_1 dimension is obtained by inverting the polarity of G_5 together with ϕ_9, with data stored separately, in order to obtain Rance–Kay–type data (Kay et al., 1992). Quadrature detection in the t_2 dimension is obtained by altering the phase ϕ_2 and ϕ_3 in the States-TPPI manner. Delay durations: $\tau_a = 2.25$ ms; $\tau_b = 2.65$ ms; $\tau_c = 0.4$ ms; $\Delta = 5.4$ ms; T = 45 ms; $T_B = 14.25$ ms; $T' = 14.0$ ms; $\delta = 7.14$ ms; $\delta_1 = \delta + 20$ μs; $\delta_2 = \delta - 20$ μs; $\epsilon = 30.0$ ms. Gradients (sine-bell shaped; 25 G/cm at center): $G_{1,2,3,4,5,6,7} = 1.0,\ 0.5,\ 0.35,\ 0.35,\ 2.0,\ 1.0$, and 0.2065 ms. The directions of the gradients are marked in the figure.

The pulse sequence shown in Figure 1 is an E. COSY-type variant of the HN(CA)CB experiment (Wittekind and Mueller, 1993), aimed at measurement of $^{3}J_{C'C^\beta}$ couplings. The $^{13}C'$ preceding the observed amide group is the passive spin in this E. COSY experiment, and the experiment is therefore referred to as [CO]HN(CA)CB-E.COSY. A detailed description of the magnetization transfer in this experiment has been presented by Wittekind and Mueller (1993) and therefore will not be repeated here. The scheme correlates the intraresidue $^{1}H^N$ (F_3), ^{15}N (F_1), and $^{13}C^\beta$ (F_2) resonances in the three orthogonal dimensions of the 3D spectrum. The pulse scheme of Figure 1 is fully analogous to the original scheme and differs only in the following details. (i) Special care is taken that the $^{13}C'$ spins are not affected by any of the ^{13}C pulses following the start of the constant-time t_2 evolution period until the end of the ^{15}N evolution period. This is accomplished by using highly selective 90° and 180° $^{13}C^{\alpha/\beta}$ pulses. (ii) The $^{13}C^\alpha$ de- and rephasing delays, 2δ are set to $1/(2J_{C\alpha C\beta})$ instead of $1/(4J_{C\alpha C\beta})$, such that transfer to $^{13}C^\beta$ is large and $^{13}C^\alpha$ has vanishingly weak intensity. (iii) A constant-time $^{13}C^\beta$ evolution period of duration $1/(J_{C\alpha C\beta})$ is used to optimize resolution in the $^{13}C^\beta$ dimension. (iv) a Rance–Kay gradient enhancement scheme (Palmer et al., 1991; Kay et al., 1992) is used for increasing the sensitivity of the experiment.

The [CO]HN(CA)CB-E.COSY experiment is recorded with a relatively long ^{15}N constant-time evolution period (2T = 90 ms), yielding high resolution in the ^{15}N dimension with a well-resolved one-bond $^{13}C'$-^{15}N splitting. The two ^{15}N doublet components correspond to $|\ \alpha\ >$ and $|\ \beta\ >$ spin states of $^{13}C'$, and their displacement in the $^{13}C^\beta$ dimension therefore corresponds to $^{3}J_{C'C\beta}$. Decoupling of $^{13}C'$ during H^N detection (t_3) is employed to ensure that no displacement of the doublet components occurs in the H^N dimension, which conceivably could interfere with accurate determination of the splitting in the $^{13}C^\beta$ di-

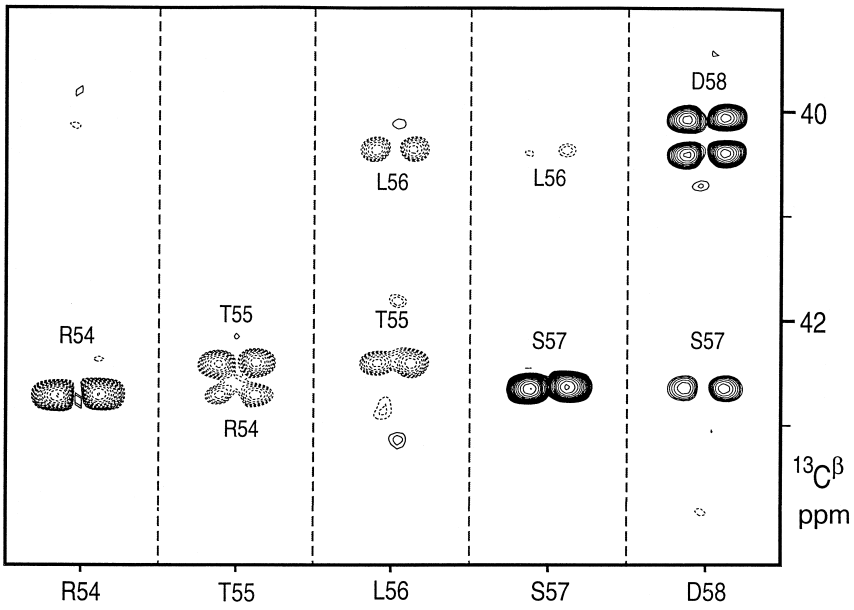

Figure 2. Regions of (F_1,F_2) strips from the 600 MHz 3D [CO]HN(CA)CB-E.COSY spectrum of 3.5 mg $^{13}C/^{15}N$-enriched ubiquitin (pH 4.7; 30 °C) in a 220 μl Shigemi microcell (1.8 mM) at 600 MHz, taken orthogonal to the F_3 ($^1H^N$) axis at the $^1H^N$ and ^{15}N (F_1) frequencies of Arg^{54}-Asp^{58}. The horizontal displacement for each doublet corresponds to $^1J_{C'N}$, the small vertical displacement to $^3J_{C'C\beta}$ (intraresidue), or $^2J_{C'C\beta}$ for cases where $^{13}C^{\beta}_{i-1}$ is observed (strips of Thr^{55}-Asp^{58}). The full $^{13}C^{\beta}$ spectral window is 10 ppm, and extensive aliasing occurs in this dimension.

mension. The applicability of the experiment is limited to relatively small proteins; for larger proteins rapid transverse ^{13}C relaxation during the relatively long $^{13}C^{\alpha}$ de- and rephasing periods and the long $^{13}C^{\beta}$ constant-time evolution period will result in very low sensitivity.

The experiment is applied to 1.5 mM $^{13}C/^{15}N$-enriched human ubiquitin, pH 4.7, at 30°, using a Bruker DMX-600 spectrometer, equipped with a triple-resonance probehead containing a self-shielded three-axis gradient coil. The 3D [CO]HN(CA)CB-E. COSY spectrum of human ubiquitin was recorded at 600 MHz as a $88^* \times 43^* \times 512^*$ (n^* refers to n complex points) data matrix with acquisition times of 90.0 (t_1, ^{15}N), 28.5 (t_2, ^{13}C), and 61.0 ms (t_3, $^1H^N$). The spectral windows were 16 ppm (^{15}N), 10 ppm ($^{13}C^{\beta}$) and 13.9 ($^1H^N$) ppm, and extensive aliasing in both the ^{15}N and $^{13}C^{\beta}$ dimensions was used to maximize the digital resolution. The total measuring time was 48 h. The experiment was carried out twice, and the averages of the two measurements are used for fitting the Karplus curve. The pairwise root-mean-square difference between the two sets of $^3J_{C'C^{\beta}}$ values was 0.5 Hz. The excitation profile of each of the $180°_{\phi 4}$, $90°_{\phi 5/\phi 7}$, $180°_{\phi 6}$ and $180°_{\phi 8}$ pulses in the $^{13}C^{\alpha/\beta}$ pulses was checked to ensure that these pulses would not cause spurious partial inversion of the $^{13}C'$ pulse. These tests confirmed the lack of any detectable inversion of the $^{13}C'$ spin, and only minimal conversion of longitudinal to transverse $^{13}C'$ magnetization (corresponding to a $\leq 4°$ flip angle for each of the $^{13}C^{\alpha/\beta}$ pulses).

Acquired data were apodized with a 60°-shifted squared sine-bell in the H^N dimension, truncated at 10%, with a cosine-bell in the ^{15}N dimension, truncated at 10%, and with a 55°-shifted sine-bell in the $^{13}C^{\beta}$ dimension, truncated at 5%. Data were zero-filled to yield a digital resolution of 1.9 (F_1), 2.9 (F_2), and 8.2 Hz (F_3). Data were processed using the package NMRPipe (Delaglio et al., 1995). Resonance assignments for human ubiquitin follow those reported previously (Di Stefano and Wand, 1987; Weber et al., 1987; Schneider et al., 1992; Wang et al., 1995).

Figure 2 shows F_1/F_2 ($^{15}N/^{13}C$) strips for residues Arg^{54}-Asp^{58} taken orthogonal to the F_3 (H^N) axis of the 3D spectrum. Each amide shows an intense correlation to its intraresidue C^{β} and a weaker one to the C^{β} of the preceding residue, resulting from magnetization transfer via $^2J_{NC^{\alpha}}$. The splitting in the horizontal dimension (^{15}N) corresponds to the $^1J_{C'N}$ coupling whereas the relative displacement of the intraresidue

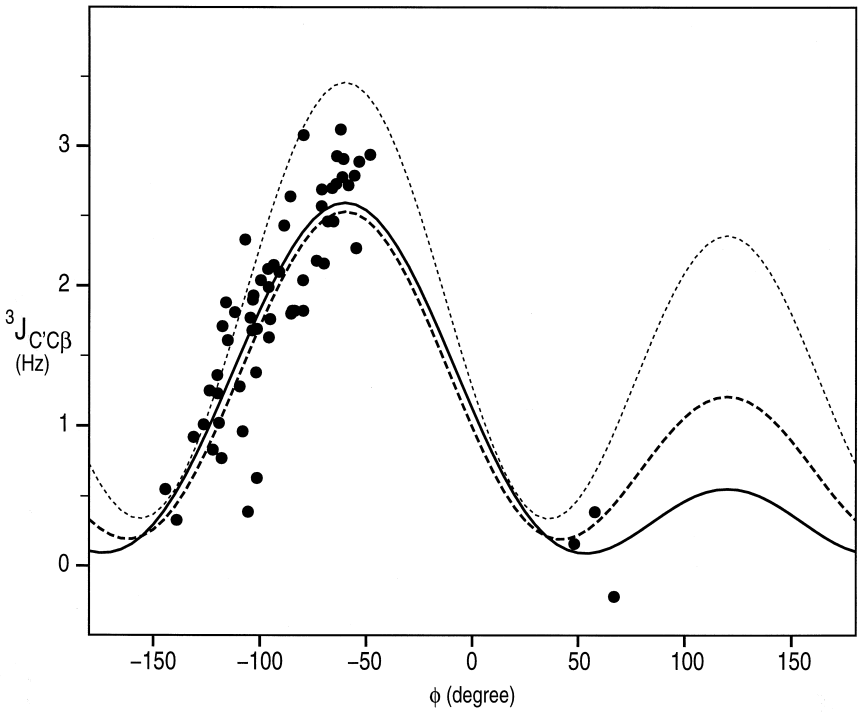

Figure 3. Relation between $^3J_{C'C^\beta}$ values measured with the [CO]HN(CA)CB-E.COSY experiment and protein backbone ϕ angles in human ubiquitin. Lines correspond to the Karplus curve $^3J_{C'C^\beta} = A\cos^2(\phi - 120°) + B\cos(\phi - 120°) + C$ Hz. The thick solid line (A = 1.28; B = −1.02; C = 0.30) represents the best fit between [CO]HN(CA)CB-E.COSY-derived $^3J_{C'C^\beta}$ values and ϕ angles derived from the crystal structure. The values of the Karplus curve for $\phi > 0°$ are ill determined because of a lack of data points. The thick dashed line is the curve obtained with HN(CO)C-derived values (Hu and Bax, 1997) (A = 1.61; B = −0.66; C = 0.26). The thin dashed line is the curve reported by Löhr et al. (1997) (A = 2.54; B = −0.55; C = 0.37).

C^β doublet components in the $^{13}C^\beta$ dimension (F_2) equals $^3J_{C'C^\beta}$. The displacement of the C^β doublet components of the preceding residues corresponds to $^2J_{C'C^\beta}$. Correlations to C^β of Asp (e.g., Asp^{58} in Figure 2), Asn, and aromatic residues exhibit an additional splitting in the $^{13}C^\beta$ dimension, resulting from $^1J_{C\beta C\gamma}$. Note that the difference in the ^{15}N dimension of these pairs of doublets corresponds to $^3J_{NC\gamma}$.

For ubiquitin, $^{13}C'T_1$ values at 151 MHz fall in the 1–1.4 s range (N. Tjandra and A. Bax, unpublished results). Considering that this T_1 is much longer than the $^{13}C^\beta$ constant-time evolution period and the $^{13}C^\alpha$ rephasing period, 2δ, this finite $^{13}C'T_1$ value has a negligible effect on the relative displacement of the multiplet components in the $^{13}C^\beta$ dimension. No correction for the finite $^{13}C'$ T_1 is therefore necessary (Wang and Bax, 1996). Peak positions were determined using two different approaches: the commonly used polynomial interpolation method and the average position of ellipsoids best fitted to the calculated contours between 45 and 75% of the peak maximum (Wand and Bax, 1996), using the program PIPP (Garrett et al., 1991). The two methods yield small (pairwaise rmsd 0.21 Hz) random differences and the average of the two $^3J_{C'C^\beta}$ values measured in this manner is used for all further analysis. Results are available as Supplementary Material.

$^3J_{C'C^\beta}$ couplings measured in ubiquitin with the [CO]HN(CA)CB-E.COSY experiment fall in the −0.2 to 3.1 Hz range. Figure 3 shows the relation between these couplings and the corresponding crystal structure ϕ angles (Vijay-Kumar et al., 1987). A best fit of the measured values to a Karplus equation, $^3J = A\cos^2\theta + B\cos\theta + C$, is calculated using singular value decomposition (SVD), yielding

$$^3J_{C'C^\beta} = 1.28\cos^2(\phi - 120°) - 1.02\cos(\phi - 120°) + 0.30 \text{ Hz}. \quad (1)$$

For $\phi > 0°$, the new Karplus curve (thick solid line in Figure 3) is ill-parametrized because of a lack of data. For $-180° < \phi < 0°$, the new parametrization is very similar to the curve obtained previously with the HN(CO)C experiment (thick dashes in Fig-

ure 3), but predicts $^3J_{C'C\beta}$ values slightly larger than our earlier curve. This agrees with our previous estimate (Hu and Bax, 1997) that the HN(CO)C-derived $^3J_{C'C^\beta}$ values are smaller by 5–10% relative to the true values, because no correction for the finite $^{13}C^\beta$ T_1 values was made. The parametrization by Löhr et al. (thin dashes in Figure 3) deviates substantially from both our earlier and our present parametrization, and predicts substantially larger *trans* $^3J_{C'C^\beta}$ values ($\phi \approx -60°$).

The rmsd between $^3J_{C'C^\beta}$ values measured by [CO]HN(CA)CB-E. COSY and those predicted by Equation 1 is 0.4 Hz when using ϕ angles from the crystal structure. This rmsd is considerably poorer than that calculated with $^3J_{C'C\beta}$ values measured with the HN(CO)C experiment (0.24 Hz). This larger rmsd reflects the higher random uncertainly in the $^3J_{C'C\beta}$ values measured by [CO]HN(CA)CB-E. COSY, owing to the inherently lower sensitivity of the experiment, and the difficulty to measure peak positions in the $^{13}C^\beta$ dimension with an accuracy higher than 0.4 Hz. Note that the acquisition of the time domain data in this dimension is limited to $1/^1J_{CC} \approx 28$ ms.

The [CO]HN(CA)CB-E.COSY data reported here indicate the absence of large systematic errors in our previous measurement of $^3J_{C'C\beta}$ values in ubiquitin, and confirm that the previously reported Karplus parameters are quite accurate. Our original parametrization also agrees very well with values previously measured for C′-N-C-C cis-form model compounds (Kao and Barfield, 1985). Although $^3J_{C'C\beta}$ values measured with the original HN(CO)C slightly underestimate the true values (by ∼7% for ubiquitin, and somewhat less for slower tumbling proteins with longer $^{13}C^\beta$ T_1 values), this is accounted for in the original parametrization of this Karplus curve. The precision obtainable for $^3J_{C'C\beta}$ is considerably better for the original HN(CO)C experiment than for the [CO]HN(CA)CB-E.COSY experiment and the HN(CO)C experiment is therefore recommended for routine measurement of this coupling. Although the original and present parametrizations are virtually indistinguishable for negative ϕ angles, for positive ϕ angles the new parametrization is essentially undetermined and deviates very significantly. However, it is interesting to note that both in our original HN(CO)C derived Karplus parametrization and in Figure 3 the average of the *trans* $^3J_{C'C\beta}$ coupling ($\phi \approx -60°$) falls slightly above the best-fit Karplus curve. This raises the intriguing question whether the assumption underlying the empirical Karplus curve, namely that $^3J_{C'C\beta}$ is dominated by contributions from valence **s** orbitals to the Fermi contact term, is strictly valid for this type of coupling (Edison et al., 1993; Fukui, 1994). If not, Karplus parameters obtained for a given protein will depend on the fraction of residues with a *trans* $^3J_{C'C\beta}$ coupling, and therefore could vary from one protein to another.

Acknowledgements

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