

# Measurement of Two- and Three-Bond Proton to Methyl-Carbon $J$ Couplings in Proteins Uniformly Enriched with $^{13}\text{C}$

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Three-bond  $^1\text{H}$ - $^{13}\text{C}$   $J$  couplings are valuable carriers of structural information (1, 2). To date, these couplings have not been widely used for protein structure studies because they tend to be small compared to the natural linewidth. Furthermore, since the sensitivity in proteins with natural-abundance  $^{13}\text{C}$  is low, quantitative measurement is virtually impossible. However, with the recent advances in NMR methodology and cloning techniques, the study of proteins uniformly enriched with  $^{13}\text{C}$  and  $^{15}\text{N}$  has surged. For such isotopically enriched proteins, the problem of low sensitivity of the  $^{13}\text{C}$ - $^1\text{H}$   $J$  coupling measurement is greatly alleviated and numerous techniques, mostly based on the E.COSY principle (3), have been proposed for measuring small heteronuclear coupling constants in proteins (4–13). Recently, a number of techniques based on a different principle have been proposed for measuring an array of  $J$  couplings, varying from  $^3J_{\text{HNH}\alpha}$  (14),  $^3J_{\text{CN}}$  (15),  $^3J_{\text{CC}}$  (16), to  $^3J_{\text{CH}}$  (17). In this latter approach, referred to as quantitative  $J$  correlation, the  $J$  value is derived from the fraction of magnetization transferred “out and back” to its  $J$ -coupled partner relative to the fraction that does not get transferred. Because of sensitivity and linewidth considerations, the approach is most suitable for measurement of couplings to methyl carbons. Rapid methyl-group rotation about the threefold symmetry axis greatly reduces the dipolar broadening of the  $^{13}\text{C}$  resonance and its transverse relaxation is highly nonexponential due to cross-correlation effects (18, 19). The slowly decaying transverse component of the methyl-carbon magnetization typically has a  $T_2$  of over 100 ms, even for proteins as large as 20 kDa. This contrasts with  $T_2$  values of about 15 ms for the backbone  $\text{C}^\alpha$  resonances.

In one application of quantitative  $J$  correlation we demonstrated its use for measuring  $^{13}\text{C}^\delta$ - $\text{H}^\beta$   $J$  couplings in the protein staphylococcal nuclease selectively labeled with [5,5- $^{13}\text{C}$ ]Leu (17). Here we present an analogous experiment that can be applied to uniformly  $^{13}\text{C}$ -enriched proteins and is therefore more generally applicable.

The pulse scheme for the long-range  $^{13}\text{C}$ - $^1\text{H}$  correlation experiment (LRCH) is shown in Fig. 1. Since a detailed description of the analogous experiment, applicable to se-

lectively  $^{13}\text{C}$ -labeled proteins, has been discussed elsewhere (17), only a brief qualitative description of the new scheme is presented below. The presence of undesirable antiphase  $^{13}\text{C}$ - $\{^1\text{H}\}$  terms associated with polarization-transfer techniques is avoided by starting with NOE-enhanced  $^{13}\text{C}$  magnetization at time a. This does not present any serious loss in sensitivity as the NOE for methyl carbons tends to be large (about 2.5) and  $^{13}\text{C}$   $T_1$  relaxation times are relatively short ( $\sim 0.5$  s) (20). The pulse scheme between time points a and d is essentially a reverse HMQC experiment (21, 22): The antiphase transverse  $^{13}\text{C}$  magnetization is converted into  $^{13}\text{C}$ - $^1\text{H}$  multiple-quantum coherence at time b and converted back into  $^{13}\text{C}$  antiphase magnetization at time c. In order to eliminate the effect of  $^{13}\text{C}$  dephasing caused by one-bond  $^{13}\text{C}$ - $^{13}\text{C}$   $J$  couplings, the interval between time points a and d must be an integral multiple of  $1/{}^1J_{\text{CC}}$ , independent of  $t_2$ . Moreover, in order to avoid generation of multiple-quantum coherence between the  $^{13}\text{C}$  and its directly attached protons, the interval during which  $^1\text{H}$ - $^{13}\text{C}$  dephasing is active (between time points a and b) must be an integral multiple of  $1/{}^1J_{\text{CH}}$ . At time d the  $^{13}\text{C}$  magnetization is transferred to its attached protons via an inverse INEPT scheme and therefore the  ${}^1J_{\text{CH}}$  coupling must be active between time points c and d for an integral multiple of  $1/{}^1J_{\text{CH}}$  minus 2 ms (23).

All the above-mentioned conditions are met by setting  $2T$  equal to 29.6 ms,  $\zeta$  equal to 3 ms (resulting in a 23.6 ms  $J_{\text{CH}}$ -dephasing time between time points a and b), and  $\xi$  equal to 4 ms (yielding a 21.6 ms  $J_{\text{CH}}$ -rephasing period between time points c and d). These choices limit the maximum acquisition time in the  $t_2$  dimension of the 3D experiment to 12 ms. For the application to medium-sized  $^{13}\text{C}$ -labeled proteins this  $t_2$  acquisition time is only slightly shorter than the transverse relaxation time of the protons involved. Because of sensitivity considerations, a much longer  $t_2$  acquisition time would be undesirable, and this maximum duration of  $t_2$  is therefore not a serious limitation.

In the resulting 3D spectrum, the correlation between a  $^{13}\text{C}$  nucleus and a proton  $k$  has a cross-peak volume proportional to  $\sin[\pi J_{\text{CH}k}2(T - \zeta)]\sin[\pi J_{\text{CH}k}2(T - \xi)] \times \prod_{j \neq k} \cos[\pi J_{\text{CH}j}2(T - \zeta)]\cos[\pi J_{\text{CH}j}2(T - \xi)]$ , where  $j$  refers

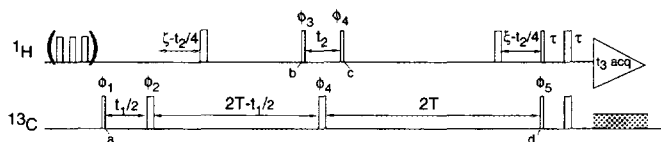


FIG. 1. Pulse sequence of the 3D  $^1\text{H}$ -detected quantitative long-range [ $^{13}\text{C}$ - $^1\text{H}$ ] correlation experiment for proteins uniformly enriched in  $^{13}\text{C}$ . Narrow and wide pulses correspond to  $90^\circ$  and  $180^\circ$  flip angles, respectively. Unless indicated otherwise, all pulses are applied along the  $x$  axis. The phase cycle is as follows:  $\phi_1 = x$ ;  $\phi_2 = 4(x), 4(y), 4(-x), 4(-y)$ ;  $\phi_3 = x, -x$ ;  $\phi_4 = 2(x), 2(-x)$ ;  $\phi_5 = 16(y), 16(-y)$ ; receiver =  $P, -P, P, -P, -P, P, -P, P$ , with  $P = (x, -x, -x, x)$ . The 2D reference spectrum was recorded by setting  $t_2 = 0$  and using  $P = 4(x)$  for the receiver phase cycle. Quadrature detection in  $t_1$  and  $t_2$  is obtained by the States-TPPI technique incrementing phases  $\phi_1$  and  $\phi_3$ , respectively. The pulse train on the  $^1\text{H}$  channel for building up the heteronuclear NOE during the relaxation delay consists of low-power  $135^\circ$  pulses, spaced by 10 ms delays. Delay durations are  $\tau = 1.7$  ms,  $2T = 29.6$  ms,  $\zeta = 3$  ms, and  $\xi = 4$  ms, permitting a maximum acquisition time in the  $t_2$  dimension of 12 ms.

to all other protons coupled to the  $^{13}\text{C}$ . Using the same pulse scheme but fixing  $t_2$  at zero, a 2D reference spectrum is recorded using the alternative phase cycle indicated in the legend to Fig. 1. The methyl-group  $^{13}\text{C}$ - $^1\text{H}_3$  2D cross-peak volume is, to a good approximation, proportional to  $\prod_j \cos[\pi J_{\text{CH}_j} 2(T - \zeta)] \cos[\pi J_{\text{CH}_j} 2(T - \xi)]$ , where  $j$  now extends over all protons coupled to the methyl carbon. Consequently, after appropriate scaling of the 3D cross-peak volume to compensate for the effect of the additional Fourier transformation (17), the ratio of the 3D cross-peak volume ( $V_{3\text{D}}$ ) to the 2D reference-peak volume ( $V_{2\text{D}}$ ) can be expressed as

$$V_{3\text{D}}/V_{2\text{D}} = \tan[\pi J_{\text{CH}_k} 2(T - \zeta)] \\ \times \tan[\pi J_{\text{CH}_k} 2(T - \xi)] \approx \tan^2[\pi J_{\text{CH}_k} (2T - \zeta - \xi)]. \quad [1]$$

The value of the  $J_{\text{CH}_k}$  coupling can be extracted in a straightforward way using Eq. [1]. The LRCH experiment is illustrated for a  $\text{D}_2\text{O}$  solution containing 1 mM of a 1:1 complex of uniformly  $^{13}\text{C}/^{15}\text{N}$ -enriched calmodulin and a 26-amino-acid peptide fragment of skeletal muscle myosin light-chain kinase (CaM/M13) at  $p^2\text{H}$  6.8. The 3D spectrum and its 2D reference spectrum were recorded at  $35^\circ\text{C}$  on a Bruker AMX600 spectrometer using the pulse scheme in Fig. 1 in 63 and 1 h, respectively.

Figure 2 shows strips along the  $F_2$  axis taken from the 3D LRCH spectrum at the methyl-group  $^{13}\text{C}/^1\text{H}$  ( $F_1/F_3$ ) resonance frequencies of Ile52, Leu48, and Val142. The magnitudes of the long-range coupling constants are reflected in the intensities of the corresponding cross peaks. For example, Fig. 2B shows correlations from the  $\text{H}^\beta$  and  $\text{H}^\gamma$  resonances to the  $\text{C}^{\delta 2}$  (left strip) and  $\text{C}^{\delta 1}$  (right strip) resonances of Leu48.  $J$  couplings, measured from these intensities using Eq. [1], are  $J_{\text{C}^{\delta 2}\text{H}^\beta} = 3.7$  Hz,  $J_{\text{C}^{\delta 2}\text{H}^\gamma} < 2.5$  Hz, and  $J_{\text{C}^{\delta 1}\text{H}^\beta}$

$= 5.6$  Hz and  $J_{\text{C}^{\delta 1}\text{H}^\beta} < 2.1$  Hz,  $J_{\text{C}^{\delta 1}\text{H}^\gamma} = 3.9$  Hz, and  $J_{\text{C}^{\delta 1}\text{H}^\beta} = 2.6$  Hz. The absence of  $\text{C}^{\delta 2}$ - $\text{H}^\gamma$  and  $\text{C}^{\delta 1}$ - $\text{H}^{\beta 2}$  correlations for Leu48 defines the upper limit for the corresponding  $J$  couplings. Together with the stereospecific assignments of the  $\text{H}^\beta$  methylene protons previously made on the basis of  $^3J_{\text{NH}\beta}$  (8) and  $^3J_{\text{COH}\beta}$  (9) couplings and a large (3 Hz)  $J_{\text{C}^{\delta 1}\text{C}^\alpha}$  coupling (16), the present results yield the  $\chi_2$  angle ( $180^\circ$ ) and the stereospecific assignments of the Leu48  $\text{C}^\delta$  resonances. Both methyl carbons also show very intense corre-

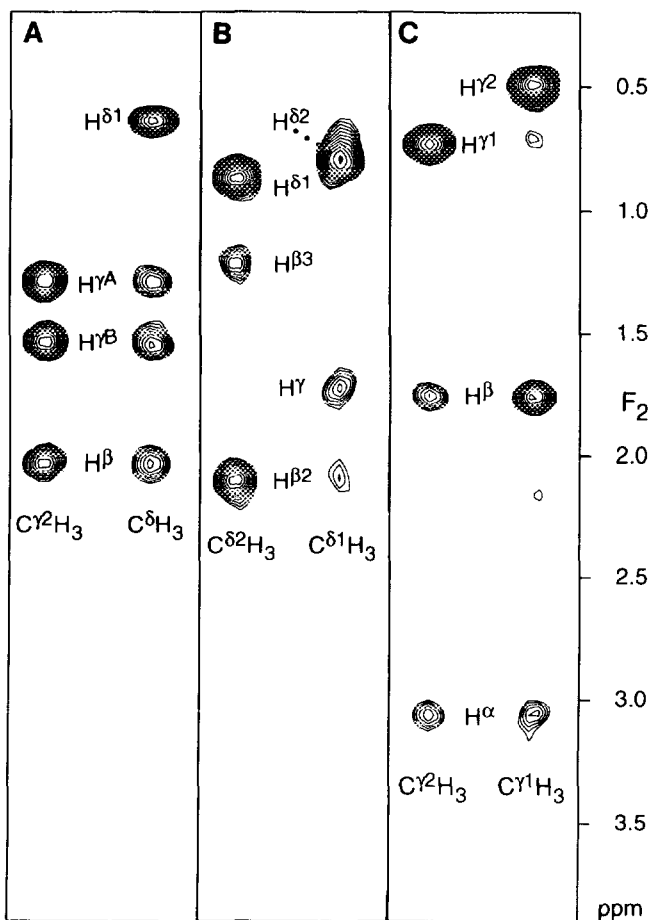


FIG. 2. Six  $F_2$  strips from the 3D  $^1\text{H}$ -detected [ $^{13}\text{C}$ - $^1\text{H}$ ] long-range spectrum of calmodulin complexed with a 26-residue peptide. The strips are taken at the ( $F_1, F_3$ ) resonance frequencies of  $\text{C}^{\gamma 2}\text{H}_3$  and  $\text{C}^{\delta 1}\text{H}_3$  of Ile52 (A),  $\text{C}^{\delta 2}\text{H}_3$  and  $\text{C}^{\delta 1}\text{H}_3$  of Leu48 (B), and  $\text{C}^{\gamma 2}\text{H}_3$  and  $\text{C}^{\gamma 1}\text{H}_3$  of Val142 (C). The spectrum was recorded with the pulse scheme in Fig. 1 and results from a  $56^*(t_1) \times 32^*(t_2) \times 384^*(t_3)$  data matrix ( $n^*$  denotes  $n$  complex points) with acquisition times of 24.6 ( $t_1$ ), 11.9 ( $t_2$ ), and 53 ( $t_3$ ) ms, using 64 scans per complex increment. A  $60^\circ$ -shifted, squared sine-bell window was used in  $t_3$  prior to zero filling to  $1024^*$  and Fourier transformation. After Fourier transformation and phasing in the  $t_2$  domain, the length of the  $t_1$  time domain was doubled by mirror-image linear prediction (28). A squared cosine-bell window was applied before zero filling to  $128^*$  and Fourier transformation. Finally, Hilbert transformation followed by inverse Fourier transformation and backward-forward linear prediction (29) doubled the length of the  $t_2$  domain to  $64^*$ . Subsequently, the  $t_2$  domain was filtered with a squared cosine-bell window, zero filled to  $256^*$ , and Fourier transformed.

lations to the vicinal methyl protons due to the  $\sim 5.2$  Hz  $^3J_{C\delta H\beta}$  coupling. These correlations are three times more intense than expected for a 5.2 Hz  $J$  coupling to a single proton because the cross peak represents the superposition of three correlations to the three equivalent methyl protons.

As noted previously (17), the 5.6 and 3.7 Hz  $^3J_{C\delta H\beta}$  values, corresponding to gauche and trans couplings, are considerably less extreme than the values reported by Sattler *et al.* (13) for a leucine residue in a cyclic peptide. In part, the smaller difference between the gauche and trans couplings is caused by  $H^{\beta 2}$ - $H^{\beta 3}$  spin flips during the delays between time points a and b and between c and d. Consider, for example, the case where the  $C^\delta$  coupling to one of the two  $H^\beta$  spins, denoted  $I_1$ , equals zero whereas the  $J$  coupling to the second  $H^\beta$  spin,  $I_2$ , equals  $J$ . In the absence of relaxation, the transverse magnetization of carbon spin,  $S$ , evolves between time points a and b according to

$$S_y \rightarrow \cos[2\pi J(T - \zeta)]S_y + \sin[2\pi J(T - \zeta)]2S_x I_{2z}. \quad [2]$$

$I_{1z}I_{2z}$  spin flips during this interval have the effect of decreasing the  $S_x I_{2z}$  term and creating an  $S_x I_{1z}$  term, despite the absence of  $J$  coupling between  $S$  and  $I_1$ . If a second  $I_{1z}I_{2z}$  spin flip occurs between time points c and d, a spurious  $S$ - $I_1$  correlation will appear in the LRCH spectrum. Hence, spin flips tend to equalize the measured  $J$  values to these geminal protons in much the same way as spin diffusion tends to equalize the NOE interactions to geminal methylene protons in NOESY spectra.

As was shown previously (17), the error caused by these spin flips depends, to first order, linearly on the duration of the de- and rephasing periods. For the relatively short de- and rephasing periods used in the present experiment, these effects are calculated to be relatively small. They decrease the difference between the two couplings, as measured from the LRCH spectrum, by approximately 30% relative to the difference of their true values (17). Spin flips between  $I_2$  and protons other than its geminal neighbor occur at much slower rates and have the effect of decreasing the size of the  $S$ - $I_2$  correlation, likewise decreasing the measured value of the  $J$  coupling. It should be noted that such an effect of spin flips on the measurement of  $J$  is not unusual. As pointed out by Harbison (24), spin flips affect all types of  $J$  measurements, including E.COSY-based measurements and direct observation of in-phase or antiphase  $J$  splittings.

Figure 2A shows the strips for the methyl carbons of Ile52. In this case,  $C^{\gamma 2}$  shows approximately equal  $J$  couplings (4.3 and 3.9 Hz) to the  $C^{\gamma 1}$  methylene protons, labeled  $H^{\gamma A}$  and  $H^{\gamma B}$ , indicating rotameric averaging of the  $\chi_2$  angle. This is supported by an intermediate value (2.1 Hz) of the  $^3J_{C\delta C\alpha}$  coupling<sup>1</sup> (16). The correlation between  $C^{\gamma 2}$  and  $H^\alpha$  (outside

the region shown in Fig. 2) is vanishingly weak, indicating a  $J$  coupling smaller than 1.7 Hz, in agreement with a rigid  $\chi_1$  rotamer assignment of  $-60^\circ$  (15). The  $C^\delta$  of Ile52 also shows a rather intense resonance to its directly attached protons, indicating that  $^1J_{CH}$  differs substantially from its "tuned" value of 127 Hz. A much weaker one-bond correlation is also observed in Fig. 2C for the  $C^{\gamma 1}$  methyl group of Val142, indicating a very small ( $\sim 1.5$  Hz) deviation from 127 Hz. The  $J_{C^{\gamma 1}H^\alpha}$  and  $J_{C^{\gamma 2}H^\alpha}$  couplings for Val142 are 2.3 and 3.0 Hz, respectively, in agreement with the previously determined  $\chi_1$  angle of  $180^\circ$  (15).

As demonstrated here, the LRCH experiment can be used to measure the  $^{13}C$ - $^1H$  couplings to methyl carbons in proteins as large as 20 kDa. The data presented above were collected before pulsed field gradients were available on our spectrometer. Considering that the sensitivity of the experiment for this 20 kDa protein at a concentration of 1 mM is sufficient to detect even small gauche couplings, it is anticipated that the measuring time can be shortened significantly by suitable use of pulsed field gradients (25-27). For valine, isoleucine, and threonine residues, the values of the  $^3J_{CH}$  couplings to methyl carbons complement  $\chi_1$  information obtainable from  $^{13}C^{\gamma}$ - $^{13}CO$  (16),  $^{13}C^{\gamma}$ - $^{15}N$  (15),  $^{15}N$ - $H^\beta$  (8), and  $^{13}CO$ - $H^\beta$  (9)  $J$  coupling measurements. Such an overdetermined set of  $J$  couplings is important to establish unambiguously the presence or absence of rotamer averaging. For leucine and isoleucine residues, the LRCH experiment also provides information on the  $\chi_2$  angle and the data make it possible to stereospecifically assign the leucine  $C^\delta$  methyl groups and the isoleucine  $C^{\gamma 1}$  methylene protons in cases where no  $\chi_2$  rotamer averaging is taking place.

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<sup>1</sup> The " $<2.1$ " value reported for  $J_{C^{\gamma 2}CO}$  in Ref. (16) is a typographical error and should read "2.1 Hz."

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