

## A simple and sensitive experiment for measurement of $J_{CC}$ couplings between backbone carbonyl and methyl carbons in isotopically enriched proteins

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### SUMMARY

A simple 2D difference experiment is described that allows quantitative measurement of  $^{13}\text{C}$ – $^{13}\text{C}$  J couplings between backbone carbonyl and side-chain carbons. Precise  $^3J_{CC}$  values were measured from data recorded in just 2 h for a 1-mM solution of the 20-kD complex between the protein calmodulin and a 26-residue synthetic peptide. The J couplings aid in determining the  $\chi_1$  angles of valine, isoleucine and threonine residues, and in making stereospecific assignments of the Val  $C^\gamma$  methyl groups. Error analysis indicates that the uncertainty in the derived J couplings is generally less than ca. 0.3 Hz.

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Three-bond  $^{13}\text{C}$ – $^{13}\text{C}$  J couplings are valuable carriers of structural information (Bystrov, 1976; Krivdin and Della, 1991). A recent report demonstrated the feasibility for measuring these small  $^3J_{CC}$  couplings in proteins uniformly enriched with  $^{13}\text{C}$  (Bax et al., 1992). In this so-called quantitative long-range  $^{13}\text{C}$ – $^{13}\text{C}$  (LRCC) correlation experiment, the size of the J coupling is obtained from the fraction of magnetization that can be transferred ‘out-and-back’ from a (methyl) carbon to its long-range coupling partner. The carbon from which magnetization originates and on which it returns must have a  $^{13}\text{C}$  line width that is not much larger than the value of the  $J_{CC}$  coupling. Hence, for larger proteins (> ~20 kD) the method is only useful for measuring J couplings to methyl carbons.

Quantitative interpretation of the long-range correlation intensities in the 2D or 3D LRCC spectrum requires that the RF pulses applied to the J-coupled  $^{13}\text{C}$  nuclei are not severely affected by off-resonance effects. For the purposes of obtaining  $^3J_{CC}$  values between methyl carbons and backbone carbonyl nuclei this is not easily accomplished in practice. Moreover, the carbonyl ( $^{13}\text{CO}$ ) chemical-shift information obtained from the LRCC experiment is generally redundant, except for alanine  $C^\beta$  methyl carbons which can experience both a two- and a three-bond coupling to  $^{13}\text{CO}$ . Here, we demonstrate the use of a simple and sensitive 2D  $^{13}\text{C}$ – $\{^{13}\text{CO}\}$  spin-echo differ-

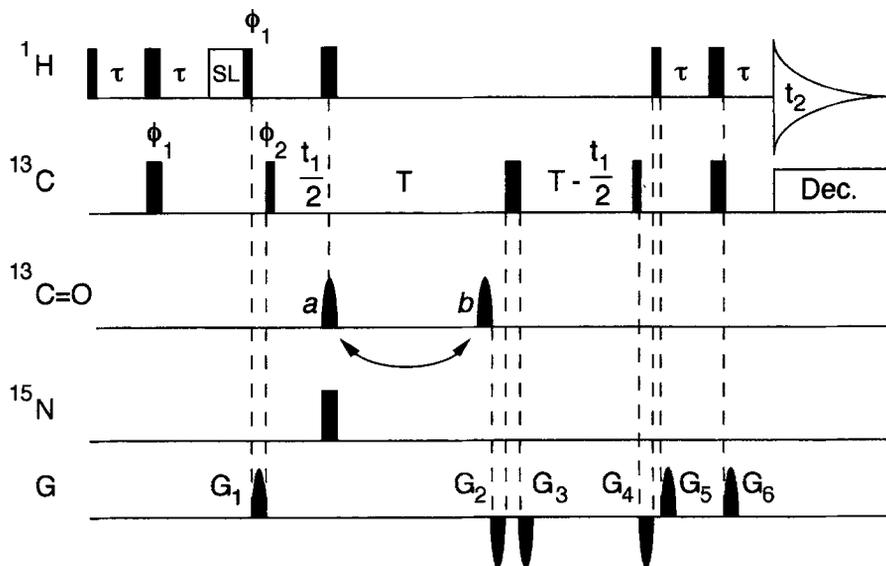


Fig. 1. Pulse scheme for the  $\{^{13}\text{CO}\}$  spin-echo difference CT-HSQC experiment. Narrow and wide pulses denote  $90^\circ$  and  $180^\circ$  flip angles, respectively. The reference CT-HSQC spectrum is recorded with the shaped  $^{13}\text{CO}$  pulse in position *a*, omitting the pulse labeled *b*, whereas the attenuated CT-HSQC experiment is recorded using pulse *b* and omitting pulse *a*. The  $^{13}\text{C}$  carrier is positioned at 46 ppm and all rectangular  $^{13}\text{C}$  pulses are applied using an RF field strength of 17 kHz, except for the  $^{13}\text{C}$  pulse immediately following pulsed field gradient  $G_2$ . The power of the latter pulse is set to cause a null in its excitation profile in the center of the  $^{13}\text{CO}$  region (177 ppm), requiring an RF field strength of 11.7 kHz for a  $^{13}\text{C}$  frequency of 151 MHz. As this  $^{13}\text{C}$  refocusing pulse and the shaped  $^{13}\text{CO}$  pulse in the attenuated spectrum are not exactly coincident,  $J_{\text{CCO}}$  dephasing is not effective for the entire period  $2T$ , but must be reduced by the duration of the shaped  $180^\circ$   $^{13}\text{CO}$  pulse and by two times the duration of  $G_2$ . The  $^{13}\text{CO}$  pulses have an envelope corresponding to the center lobe of a  $\sin(x)/x$  function, with a duration of  $300 \mu\text{s}$ . Durations of the pulsed field gradient pulses are 5 ms ( $G_1$ ), 1.5 ms ( $G_2, G_3$ ),  $700 \mu\text{s}$  ( $G_4$ ) and  $100 \mu\text{s}$  ( $G_5, G_6$ ). Other delay durations are:  $\tau = 1.7$  ms;  $T = 28.7$  ms. The pulse labeled SL is a 0.5-ms spin-lock pulse and serves to scramble the HDO signal. Unless indicated otherwise, all pulses are applied along the *x*-axis. The phase cycle is as follows:  $\phi_1 = y, -y$ ;  $\phi_2 = 2(x), 2(-x)$ ; receiver =  $x, -x, -x, x$ . Quadrature detection in  $t_1$  is obtained with the States-TPPI technique by incrementing  $\phi_2$ .

ence constant-time heteronuclear single-quantum correlation (CT-HSQC) experiment (Santoro and King, 1992; Van de Ven and Philippens, 1992; Vuister and Bax, 1992) from which the  $J_{\text{CC}}$  couplings to  $^{13}\text{CO}$  carbons can be extracted in a simpler and more sensitive manner. The experiment is technically analogous to the recently proposed  $^{13}\text{C}\text{-}\{^{15}\text{N}\}$  spin-echo difference CT-HSQC experiment, which provides a measurement for the complementary  $^{13}\text{C}\text{-}\{^{15}\text{N}\}$   $J$  coupling (Vuister et al., 1993). Conceptually, the experiment is also closely related to the 1D  $^1\text{H}\text{-}\{^{113}\text{Cd}/^{199}\text{Hg}\}$  spin-echo difference experiment used for the measurement of very small  $J$  couplings between backbone amide protons and  $^{113}\text{Cd}$  or  $^{199}\text{Hg}$  in the metal-binding protein rubredoxin (Blake et al., 1992). Factors affecting precision and accuracy of this type of measurement are discussed.

The pulse scheme for the  $^{13}\text{C}\text{-}\{^{13}\text{CO}\}$  spin-echo difference CT-HSQC experiment is sketched in Fig. 1. When the  $^{13}\text{CO}$   $180^\circ$  pulse is applied in position *a* the scheme is identical to the constant-time  $^1\text{H}\text{-}\{^{13}\text{C}\}$  correlation experiment (CT-HSQC) described elsewhere (Vuister and Bax, 1992). The effect of one-bond  $^{13}\text{C}\text{-}\{^{13}\text{C}\}$   $J$  couplings during the constant-time evolution period is suppressed by

adjusting the duration ( $2T$ ) of the constant-time evolution period to  $2/J_{CC}$  (58 ms). The effect of  $^{13}\text{C}$ - $^{15}\text{N}$  and  $^{13}\text{C}$ - $^{13}\text{CO}$  J couplings is eliminated by the  $180^\circ$  pulses applied at position  $a$ , and the acquired signal is denoted  $S_a$ . In the second experiment the  $180^\circ$   $^{13}\text{CO}$  pulse is shifted to position  $b$ , causing the  $J_{CCO}$  coupling to be active during the entire  $2T$  period and thereby resulting in a weaker signal,  $S_b$ , attenuated by  $\cos(\pi J_{CCO}T)$ .

The two experiments are executed in an interleaved manner and the signals,  $S_a$  and  $S_b$ , are stored separately. The value of the  $J_{CCO}$  coupling is calculated from:

$$(S_a - S_b)/S_a = 1 - \cos(2\pi J_{CCO}T) = 2\sin^2(\pi J_{CCO}T) \quad (1)$$

As the duration of the constant-time evolution period ( $2T$ ) is relatively long and  $^{13}\text{C}$  magnetization does not decay for increasing  $t_1$  durations (Bax et al., 1979), excellent resolution can be obtained in the 2D  $^{13}\text{C}$ - $\{^{13}\text{CO}\}$  spin-echo difference CT-HSQC spectrum. The scheme incorporates the use of pulsed field gradients in the standard manner in order to reduce the required phase cycling and the minimum measuring time (Bax and Pochapsky, 1992). The method is demonstrated for uniformly  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched calmodulin (CaM) (1.0 mM) complexed with a 26-residue synthetic peptide known as M13 (Klevit et al., 1984) and 4 molar equivalents of  $\text{Ca}^{2+}$ , dissolved in 99.8%  $\text{D}_2\text{O}$ ,  $p^2\text{H}$  6.8. Spectra were recorded at  $35^\circ\text{C}$  on a Bruker AMX600 spectrometer.

Figure 2 compares the methyl region of the 2D  $^{13}\text{C}$ - $\{^{13}\text{CO}\}$  spin-echo difference CT-HSQC spectrum of the CaM/M13 complex (B) with the corresponding region of the reference spectrum (A) (recorded with the  $180^\circ$   $^{13}\text{CO}$  pulse in position  $a$ ). The lowest contour level for the difference spectrum is set 6 times lower than for the reference spectrum and falls just above the thermal noise level. A fraction of the methyl carbons shows intense resonances in the difference spectrum, indicating significant J couplings to the backbone carbonyl. Values for the  $J_{CC}$  couplings, derived from Eq. 1, are listed in Table 1. These values are in fair agreement with those obtained previously from the quantitative LRCC correlation spectrum, but are systematically ca. 25% larger. As noted in the supplementary table to Bax et al. (1992), the values measured from the LRCC spectrum were not corrected for RF inhomogeneity or offset effects, which are particularly severe for correlations to the far downfield-shifted  $^{13}\text{CO}$  resonances. The present data are more reliable because  $^{13}\text{CO}$  pulses are applied with the carrier shifted to the center of the  $^{13}\text{CO}$  region, and offset effects are therefore much smaller. Nevertheless, it is useful to consider the accuracy and precision of the present measurements, i.e. the effect of systematic and random errors, and these will be briefly discussed below.

There are three potential sources of systematic errors: (a) incomplete  $^{13}\text{C}$  labeling; (b) faster relaxation of  $^{13}\text{C}$ - $\{^{13}\text{CO}\}$  antiphase terms relative to in-phase  $^{13}\text{C}$  magnetization; and (c) incomplete inversion of the  $^{13}\text{CO}$  resonances by the shaped  $180^\circ$   $^{13}\text{CO}$  pulse. All other factors affect the reference and difference spectra to the same degree and therefore do not influence the J values calculated from their intensity ratios.

If the fraction of  $^{13}\text{C}$  labeling is  $1 - L$ , a fraction  $L$  of the observed  $^{13}\text{C}$  magnetization is not subject to J modulation. This results in a difference spectrum with relative intensity  $(1 - L) \times 2\sin^2(\pi J_{CCO}T)$ . Since  $J_{CCO}T \ll 1$ , the measured J value is about a factor of  $\sqrt{1 - L}$  smaller than its true value. For CaM,  $^{13}\text{C}$  filtering experiments indicate that the level of  $^{13}\text{C}$  enrichment is higher than 97%. The upper limit for the level of  $^{13}\text{C}$  labeling is set by the enrich-

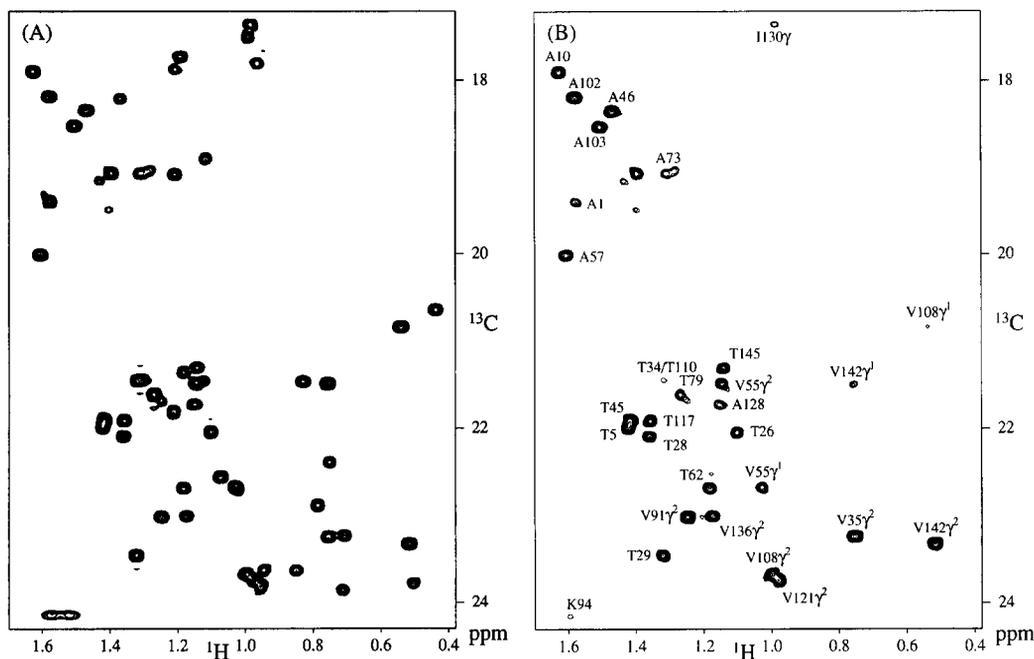


Fig. 2. Methyl region of (A) the CT-HSQC reference spectrum and (B) the  $\{^{13}\text{CO}\}$  spin-echo difference CT-HSQC spectrum of CaM-M13, recorded at a proton frequency of 600 MHz. Panel B has been plotted at a contour level 6 times lower than in (A). The reference and attenuated spectra were recorded in an interleaved manner using the pulse scheme of Fig. 1, with acquisition times of 53.2 ms ( $t_1$ ) and 80 ms ( $t_2$ ), and spectral widths of 33 ppm ( $F_1$ ,  $^{13}\text{C}$ ) and 8 ppm ( $F_2$ ,  $^1\text{H}$ ). Total measuring time was 2 h.

ment level (99%) of the  $^{13}\text{C}_6$ -glucose used during bacterial expression. Incomplete  $^{13}\text{C}$  enrichment therefore decreases the measured coupling by 0.5–1.5%.

It is well known that transverse magnetization of spin I which is antiphase with respect to spin S and denoted  $I_y S_z$ , relaxes faster than the corresponding in-phase term,  $I_x$ . To a good approximation, the difference in the relaxation rates of  $I_y S_z$  and  $I_x$  equals the longitudinal relaxation rate,  $1/T_{1S}$ , of spin S (London, 1990; Bax et al., 1990; Peng et al., 1991). For  $J_{\text{CCO}}T \ll 1$  and  $T_{1S} \gg T$ , this decreases the difference spectrum by a factor  $(1 - T/T_{1S})$  for  $t_1 = 0$  and by a factor  $(1 - 0.75T/T_{1S})$  for  $t_1 = T/2$ . The backbone carbonyl  $T_1$  values of the CaM/M13 complex fall in the 0.7–1.5 s range, and the faster antiphase relaxation decreases  $^3J_{\text{CCO}}$  between 1 and 2%.

The effect of incomplete inversion of the  $^{13}\text{CO}$  spins by the shaped  $180^\circ$  pulse cannot be corrected by phase cycling or pulsed field gradients (Freeman and Keeler, 1981). If a fraction  $K$  of the  $^{13}\text{CO}$  spins is not inverted by the shaped  $^{13}\text{CO}$  pulse, the reference signal, obtained with the  $^{13}\text{CO}$  pulse in position  $a$ , is reduced by a fraction  $K[1 - \cos(\pi J_{\text{CCO}}t_1)]$ . The attenuated signal, obtained with the  $^{13}\text{CO}$  pulse in position  $b$ , increases in amplitude by a fraction  $K[\cos(\pi J_{\text{CCO}}t_1) - \cos(2\pi J_{\text{CCO}}T)]$ . Thus, the difference spectrum is attenuated by a factor  $(1 - K)$ , independent of  $t_1$ . This results in a decrease of the measured  $J_{\text{CCO}}$  value by a factor  $1 - K/2$ . The magnitude of  $K$  has been measured experimentally for a  $180^\circ$  pulse with the shape of the center bell of a  $\sin(x)/x$  function and a duration of 300  $\mu\text{s}$ .  $K$  was found to be 2% on-resonance and 10% at an offset of 750 Hz (corresponding to 5 ppm at 150 MHz  $^{13}\text{C}$  frequency). This incomplete

TABLE 1  
 $J_{C\gamma CO}$  AND ALANINE  $J_{C\beta CO}$  VALUES (Hz) IN THE CaM-M13 COMPLEX

Residue	$J_{CCO}$	Error	Residue	$J_{CCO}$	Error
A1	1.3	0.1	T26	2.7	0.2
A10	3.1 <sup>a</sup>	b	T28	3.2	0.2
A15	3.1 <sup>a</sup>	b	T29	2.9	0.1
A46	3.0 <sup>a</sup>	b	T62	3.4	0.2
A57	2.7 <sup>a</sup>	b	T70	1.3	0.2
A88	2.8 <sup>a</sup>	b	T79	1.9	0.1
A102	3.4 <sup>a</sup>	b	T117	3.2	0.2
A103	3.0 <sup>a</sup>	b	T143	0.7	0.3
A128	1.9 <sup>a</sup>	b	T146	2.7	0.1
I9-C $\gamma^2$	1.0	0.2	V35-C $\gamma^1$	0.9	0.3
I27-C $\gamma^2$	1.1	0.4	V35-C $\gamma^2$	4.2	0.2
I52-C $\gamma^2$	1.4	0.2	V55-C $\gamma^1$	2.0	0.1
I63-C $\gamma^2$	0.7	0.4	V55-C $\gamma^2$	2.5	0.1
I85-C $\gamma^2$	1.3	0.2	V91-C $\gamma^1$	0.8	0.4
I100-C $\gamma^2$	1.2	0.4	V91-C $\gamma^2$	4.2	0.2
I125-C $\gamma^2$	1.4	0.2	V108-C $\gamma^1$	1.1	0.2
I130-C $\gamma^2$	1.3	0.2	V108-C $\gamma^2$	3.5	0.2
K13	1.9/2.3 <sup>c</sup>	0.5	V121-C $\gamma^1$	0.8	0.3
K21	4.1/4.2 <sup>c</sup>	0.7	V121-C $\gamma^2$	4.1	0.2
K30	2.1	0.3	V136-C $\gamma^2$	4.1	0.2
K75	2.7	0.2	V142-C $\gamma^1$	1.2	0.1
K94	2.0/2.0 <sup>c</sup>	0.4	V142-C $\gamma^2$	3.5	0.2
K115	2.8/2.8 <sup>c</sup>	0.2			

<sup>a</sup> Value for the interresidue  $^3J_{C\beta CO}$  coupling, assuming a 1.3-Hz value for  $^2J_{C\beta CO}$ .

<sup>b</sup> No error estimate given due to the uncertainty in  $^2J_{C\beta CO}$ .

<sup>c</sup> Values derived from the two nonequivalent C $\gamma$  H $_2$  protons.

inversion of the  $^{13}CO$  spin therefore reduces the derived  $J_{CCO}$  value by 1–5% relative to its true value.

The sum of the systematic errors listed above results in an underestimate of  $^3J_{CCO}$  by  $5.5 \pm 3\%$ . The uncertainty in the intensity difference of resonances in the reference and attenuated spectra is a second source of error. A good estimate of the uncertainty in the difference intensity can be obtained by assuming that  $J_{CC}$  couplings between the backbone carbonyl and the methionine C $^\epsilon$  resonances are zero. The root-mean-square (rms) difference,  $\epsilon$ , for these intensities was found to be virtually identical to the rms value of a noise region in the difference spectrum. The random error in the  $J_{CCO}$  value then follows from:

$$J_{CCO} = (1/\pi T) \sin^{-1}(\sqrt{(S_a - S_b \pm \epsilon)/2S_a}) \quad (2a)$$

which for  $(S_a - S_b)/2S_a \ll 1$  may be approximated by

$$J_{CCO} \approx (\pi T \sqrt{2})^{-1} \sqrt{(S_a - S_b \pm \epsilon)/S_a} \quad (2b)$$

Equation 2b indicates that the random error in the measurement of  $J$  decreases with increasing values of  $(S_a - S_b)$ , i.e. with increasing  $J_{\text{CCO}}$ . In contrast, the systematic errors mentioned above increase linearly with the size of the  $J$  coupling. The  $J$  couplings and their uncertainty, after correction for the 5.5% systematic underestimate mentioned above, are listed in Table 1.

For all valine residues, except Val<sup>55</sup>, the <sup>13</sup>CO shows a large  $J$  coupling to C<sup>γ2</sup>, whereas the  $J$  coupling to C<sup>γ1</sup> is small, indicating a  $\chi_1$  angle of 180°. The same conclusion was reached on the basis of C<sup>γ</sup>-N three-bond  $J$  couplings (Vuister et al., 1993) and C<sup>γ</sup>-H<sup>α</sup>  $J$  couplings (Vuister and Bax, 1993). For Val<sup>55</sup>, intermediate  $J$  couplings from the backbone carbonyl to both C<sup>γ</sup> carbons are observed. The C<sup>γ1</sup>-<sup>15</sup>N  $J$  coupling for this residue (1.2 Hz) was also smaller than expected for a trans configuration (2.1 Hz), but larger than for gauche (0.5 Hz), whereas <sup>3</sup>J<sub>C<sup>γ2</sup>N</sub> fell below the detection threshold. These couplings for Val<sup>55</sup> suggest rotameric averaging between  $\chi_1 = 180^\circ$  and  $\chi_1 = -60^\circ$ .

Relatively large <sup>3</sup>J<sub>C<sup>γ</sup>CO</sub> values of ~ 3 Hz are observed for Thr<sup>26</sup>, Thr<sup>28</sup>, Thr<sup>29</sup>, Thr<sup>62</sup>, Thr<sup>117</sup> and Thr<sup>146</sup>. These values suggest trans configurations, corresponding to  $\chi_1$  angles of +60°. Small values of ~ 1 Hz are observed for Thr<sup>70</sup> and Thr<sup>143</sup>, corresponding to gauche configurations. Together with relatively large  $J$  couplings to <sup>15</sup>N, this defines the  $\chi_1$  angle for the latter two residues as -60°. For Thr<sup>79</sup>, which is located in a solvent-exposed flexible loop (Ikura et al., 1992; Meador et al., 1992), rotamer averaging is observed ( $J_{\text{CCO}} = 1.9$  Hz).

For all isoleucine residues in the CaM-M13 complex, the coupling between C<sup>γ2</sup> and <sup>13</sup>CO is found to be small (~ 1 Hz), corresponding to a gauche configuration. Large <sup>3</sup>J<sub>C<sup>γ2</sup>N</sub> values (Vuister et al., 1993) indicate  $\chi_1$  angles of -60° for all these residues.

For alanine residues, both the intraresidue <sup>2</sup>J<sub>C<sup>β</sup>CO</sub> and the interresidue <sup>3</sup>J<sub>C<sup>β</sup>CO</sub> couplings cause attenuation, and to a good approximation, the  $J_{\text{CCO}}$  value derived from Eq. 1 equals  $\sqrt{(^2J_{\text{C}\beta\text{CO}})^2 + (^3J_{\text{C}\beta\text{CO}})^2}$ . Based on the LRCC experiment (Bax et al., 1992), <sup>2</sup>J<sub>C<sup>β</sup>CO</sub> is small (ca. 1.3 Hz) and rather uniform. Assuming <sup>2</sup>J<sub>C<sup>β</sup>CO</sub> equals 1.3 Hz, approximate values for <sup>3</sup>J<sub>C<sup>β</sup>CO</sub> can be extracted from the spectra in Fig. 2 (Table 1).

The CT-HSQC difference method for measuring  $J$  couplings between methyl and carbonyl carbons is simpler and more accurate than the original LRCC experiment. The CT-HSQC difference spectrum offers excellent resolution due to the long constant-time acquisition period and resonance overlap is therefore limited, even for larger proteins. The high inherent sensitivity and resolution of this type of  $J_{\text{CC}}$  measurement also makes it applicable to the measurement of CO-C<sup>γ</sup> couplings for arginine, leucine, lysine, methionine and isoleucine (C<sup>γ1</sup>) residues. For example, for Lys<sup>30</sup>, Lys<sup>75</sup>, Lys<sup>94</sup> and Lys<sup>115</sup> a <sup>3</sup>J<sub>C<sup>γ</sup>CO</sub> coupling of ~ 2 Hz is observed, suggesting  $\chi_1$  averaging. This rotameric averaging is also responsible for the relatively long C<sup>γ</sup> T<sub>2</sub> values of these residues, yielding relatively intense resonances in the CT-HSQC spectrum (outside the window shown in Fig. 2, except for Lys<sup>94</sup>). However, the much faster relaxing C<sup>γ</sup> resonance of Lys<sup>21</sup> shows a large  $J$  coupling (4.1 Hz) to <sup>13</sup>CO, indicative of a  $\chi_1$  angle of -60°. For the CaM-M13 complex, many of the C<sup>γ</sup> resonances of nonmobile arginine, leucine, lysine and methionine residues are too weak in the 2-h CT-HSQC experiment for accurate measurement of the  $J$  coupling. For the smaller protein profilin (13 kD), however, a large number of <sup>3</sup>J<sub>C<sup>γ</sup>CO</sub> couplings for these types of residues has been measured, providing important  $\chi_1$  angle constraints and providing insight into the degree of  $\chi_1$  rotameric averaging (Archer et al., unpublished results).

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