# Measurement of Three-Bond Nitrogen–Carbon $J$ Couplings in Proteins Uniformly Enriched in $^{15}N$ and $^{13}C$

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The utility of three-bond $J$ couplings for determining backbone and side-chain conformation in peptides and proteins has long been established.[1] Historically, the focus has been primarily on three-bond $^1H$–$^1H$ couplings to obtain information on the dihedral backbone angle $\phi$ and the side-chain angle $\chi_1$, but measurement of heteronuclear $^3J_{CH}$ and $^3J_{NH}$ couplings is becoming increasingly popular.[2-10] Recently, measurement of $^3J_{CC}$ to methyl carbons in proteins was also demonstrated to be feasible,[11] facilitating the stereospecific assignment of the methyl groups of valines and leucines and providing information about the side-chain $\chi_1$ (Val, Ile, Thr) and $\chi_2$ (Leu) angles.[12] Here, an experiment is described that provides quantitative information on the $J$ coupling between methyl carbons and backbone amide $^{15}N$ nuclei. Together with the measurement of $^3J_{CC}$, this allows one to determine the $\chi_1$ angle in valine, isoleucine, and threonine residues.

Most methods available to date for measurement of unresolved $J$ couplings in proteins rely on the E.COSY principle,[13] in which the $J$ coupling is measured from the frequency difference between two resonances in a 2D or 3D spectrum. More recently, a different approach for measurement of unresolved $J$ couplings was proposed which relies on quantitating coherence transfer[11] or measuring the magnetization loss due to dephasing caused by the unresolved $J$ coupling.[14] As will be demonstrated below, this approach is also very well suited for measurement of small unresolvable $^3J_{CN}$ couplings in proteins.

The method described here is essentially a 2D difference experiment, and the pulse scheme is sketched in Figure 1. When the $^{15}N$ 180° pulse is applied in position $a$, the scheme is identical to the constant-time $^1H$–$^{13}C$ correlation experiment (CT-HSQC) described elsewhere.[15-17] The effect of one-bond $^{13}C$–$^{13}C$ $J$ couplings during the constant-time evolution period is suppressed by adjusting the duration ($2T$) of the constant-time evolution

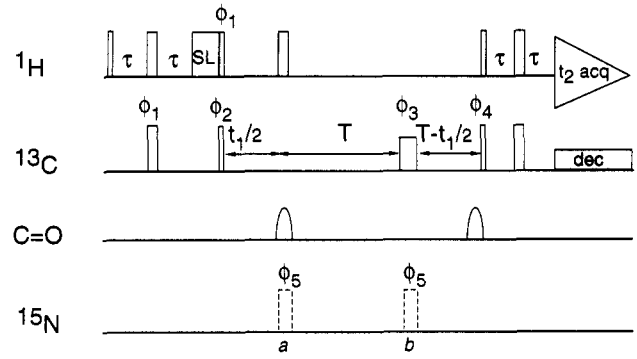


**Figure 1.** Pulse scheme for the {$^{15}N$} spin–echo difference CT-HSQC experiment. Narrow and wide pulses denote 90° and 180° flip angles, respectively. The power of the 180°$_{\phi 3}$ pulse is adjusted to avoid excitation of the carbonyl resonances. Further details are described elsewhere.[17] The reference CT-HSQC experiment is recorded using the $^{15}N$ pulse labeled $a$ and omitting the pulse labeled $b$, whereas the attenuated CT-HSQC experiment is recorded using pulse $b$ and omitting pulse $a$. Unless indicated otherwise, all pulses are applied along the $x$-axis. The phase cycle is as follows: $\phi_1 = y,-y$; $\phi_2 = x$; $\phi_3 = 2(x),2(-x),2(y),2(-y)$; $\phi_4 = 8(x),8(-x)$; $\phi_5 = 16(x),16(-x)$; receiver $= 2(x,-x),4(-x,x),2(x,-x)$. Quadrature detection in $t_1$ is obtained by the States-TPPI technique, incrementing $\phi_2$. The delays $\tau$ and $T$ were set to 1.7 and 28.6 ms, respectively.

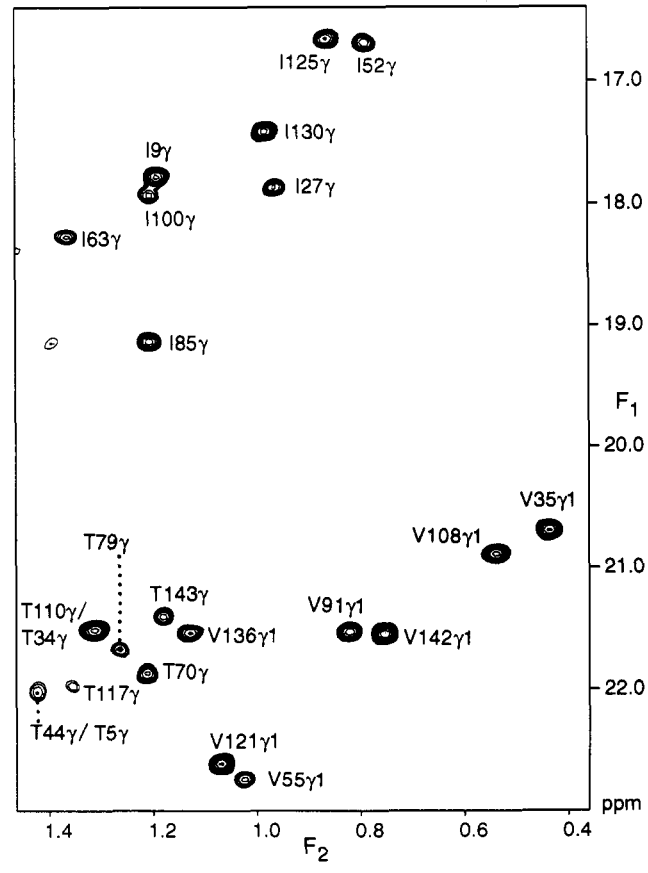


**Figure 2.** Methyl region of the {$^{15}N$} spin–echo difference CT-HSQC spectrum, recorded at 600 MHz $^1H$ frequency. The two spectra were recorded in an interleaved manner with acquisition times of 51 ($t_1$) and 53 ms ($t_2$) and spectral widths of 33 ($F_1$,$^{13}C$) and 8 ppm ($F_2$,$^1H$). Total measuring time was 17 h.

period to $2/^1J_{CC}$ (58 ms). The effect of $^{13}C$–$^{15}N$ $J$ coupling is eliminated by the 180° pulse applied at position $a$. In the second experiment, the 180° $^{15}N$ pulse is shifted to position $b$, causing the $J_{CN}$ coupling to be active during the entire $2T$ period. In the latter case, the $^{13}C$ magnetization is attenuated by $\cos(2\pi J_{NC}T)$

(1) Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 41–81.
(2) Montelione, G. T.; Winkler, M. E.; Rauenbühler, P.; Wagner, G. *J. Magn. Reson.* **1989**, *82*, 198–204.
(3) Wider, G.; Neri, D.; Otting, G.; Wüthrich, K. *J. Magn. Reson.* **1989**, *85*, 426–431.
(4) Kurz, M.; Schmieder, P.; Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1329–1331.
(5) Edison, A. S.; Westler, W. M.; Markley, J. L. *J. Magn. Reson.* **1991**, *92*, 434–438.
(6) Xu, R. X.; Olejniczak, E. T.; Fesik, S. W. *FEBS Lett.* **1992**, *305*, 137–143.
(7) Sattler, M.; Schwalbe, H.; Griesinger, C. *J. Am. Chem. Soc.* **1992**, *114*, 1126–1127.
(8) Edison, A. S.; Westler, W. M.; Markley, J. L. *J. Magn. Reson.* **1991**, *92*, 434–438.
(9) Zuiderweg, E. R. P.; Fesik, S. W. *J. Magn. Reson.* **1991**, *93*, 653–658.
(10) Eggenberger, U.; Karimi-Nejad, Y.; Thüring, H.; Rüterjans, H.; Griesinger, C. *J. Biomol. NMR* **1992**, *2*, 583–590.
(11) Bax, A.; Max, D.; Zax, D. *J. Am. Chem. Soc.* **1992**, *114*, 6924–6925.
(12) Powers, R.; Garrett, D. S.; March, C. J.; Frieden, E. A.; Gronenborn, A. M.; Clore, G. M. *Biochemistry*, in press.
(13) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Chem. Phys.* **1986**, *85*, 6837–6852.
(14) Blake, P. R.; Summers, M. F.; Adams, M. W. W.; Park, J.-B.; Zhou, Z. H.; Bax, A. *J. Biomol. NMR* **1992**, *2*, 527–533.
(15) Santoro, J.; King, G. C. *J. Magn. Reson.* **1992**, *97*, 202–207.
(16) van de Ven, F. J. M.; Philippens, M. E. P. *J. Magn. Reson.* **1992**, *97*, 637–644.
(17) Vuister, G. W; Bax, A. *J. Magn. Reson.* **1992**, *98*, 428–435.

**Table I.** $^3J_{C\gamma N}$, $^3J_{C\gamma CO}$ Values and Stereospecific Assignments of the Valine Residues in the Calmodulin–Peptide Complex

| | $C^{\gamma 1}$ [c] | | | $C^{\gamma 2}$ [c] | | | |
|---|---|---|---|---|---|---|---|
| | $\delta C^{\gamma 1}$ | $^3J_{C\gamma 1N}$ | $^3J_{C\gamma 1CO}$ | $\delta C^{\gamma 2}$ | $^3J_{C\gamma 2N}$ | $^3J_{C\gamma 2CO}$ | $\chi_1$ |
| Val35 | 20.6 | 2.26 ± 0.07 | <0.8[b] | 23.2 | <0.5[b] | 3.0 | 180° |
| Val55 | 22.6 | 1.22 ± 0.07 | 1.7 | 21.4 | <1.1[a,b] | 2.4[a] | *d* |
| Val91 | 21.4 | 2.24 ± 0.08 | <0.8[b] | 22.9 | <0.6[b] | 3.0 | 180° |
| Val108 | 20.8 | 1.90 ± 0.05 | <0.8[b] | 23.6 | 0.59 ± 0.2 | ~3[a] | 180° |
| Val121 | 22.5 | 2.20 ± 0.05 | <0.9[b] | 23.5 | 0.81 ± 0.15 | ~3[a] | 180° |
| Val136 | 21.4 | ~2[a] | <1.4[b] | 22.9 | <0.8[a,b] | ~3[a] | 180° |
| Val142 | 21.4 | 1.93 ± 0.04 | 1.0 | 23.2 | <0.6[b] | 2.4 | 180° |

[a] Value could not be measured precisely due to partial overlap. [b] Upper limit, based on $(S_a - X_b + D)/S_a$. [c] Chemical shifts are in ppm relative to TSP, J couplings are in hertz. [d] Rotamer averaging.

relative to the scheme with the 180° pulse in position *a*. The two experiments are executed in an interleaved manner with the two signals, $S_a$ and $S_b$, stored separately. $(S_a - S_b)/S_a$ gives a relative intensity of $1 - \cos(2\pi J_{NC}T) = 2\sin^2(\pi J_{NC}T)$, allowing $J_{NC}$ to be calculated straightforwardly.

The method is demonstrated for uniformly $^{13}C/^{15}N$-enriched (>95%) calmodulin (1.5 mM) complexed with a 26-residue synthetic peptide[18] and 4 molar equivalents of $Ca^{2+}$. Spectra were recorded at 35 °C on a Bruker AMX600 spectrometer.

The methyl region of the $\{^{15}N\}$ spin–echo difference CT-HSQC spectrum is shown in Figure 2. As can be seen, all eight Ile $C^{\gamma 2}$ methyl carbons are present in the difference spectrum. From the intensities in the difference spectrum, the corresponding couplings are calculated to fall in the 1.9 (Ile130) to 2.2 Hz (Ile27) range, indicating that all Ile residues in the complex have $\chi_1$ angles of –60°. This is confirmed by small couplings between $C^{\gamma 2}$ and the carbonyl carbon for all of these residues.[11] For each of the seven valines, only one of the two diastereotopic methyl carbons is observed in the difference spectrum. Values for the corresponding *J* couplings, plus upper limits for *J* couplings not observed in the difference spectrum, are presented in Table I. For six valine residues, the $^3J_{C\gamma N}$ couplings together with $^3J_{C\gamma CO}$ values indicate a $\chi_1$ angle of 180°. For Val55, however, $^3J_{C\gamma 1N}$ is smaller than for the other residues. Moreover, $C_{\gamma 1}$ also shows a 1.7-Hz coupling to the carbonyl, suggesting rotamer averaging.

The error in the measured *J* value is determined primarily by the precision with which the difference in resonance intensity, $S_a - S_b$, can be determined. This precision is estimated from the root-mean-square (rms) value, *D*, of the difference in intensities $(S_a - S_b)$ observed for 27 well-resolved Leu- and Ile-$C^{\delta}$ and Met-$C^{\epsilon}$ resonances. As no *J* coupling to $^{15}N$ is expected for these resonances, *D* may be used as a measure for the precision of the difference intensity for the $^{15}N$-coupled resonances. It was found that *D* is 14% higher than the rms thermal noise level in the difference spectrum. Error estimates for the derived *J* value follow from $2\sin^2(\pi J_{NC}T) = (S_a - S_b \pm D)/S_a$ and are reported in Table I. The measured *J* value is also affected by systematic errors,[14] including the effect of (a) faster relaxation of $^{13}C$ magnetization which is antiphase with respect to $^{15}N$ compared to in-phase magnetization,[19,20] (b) the level of $^{15}N$ enrichment, and (c) the fraction of $^{15}N$ nuclei that are not inverted by the $^{15}N$ 180° pulse (primarily due to radio frequency inhomogeneity). These latter three factors reduce the measured *J* coupling by 3% (assuming a $^{15}N$ $T_1$ value of 500 ms at 600 MHz), 1.5% (for 97% $^{15}N$ labeling), and 3% (for a 180° $^{15}N$ inversion of 94%), respectively. The *J* values reported in Table I have been scaled by 1.075 to account for these systematic errors.

If a methyl group were coupled to two $^{15}N$ nuclei, N1 and N2, one would obtain $(S_a - S_b)/S_a = 2[\sin^2(\pi J_{N1C}T) + \sin^2(\pi J_{N2C}T)]$. For large values of $J_{N1C}$ (~2 Hz), the presence of an additional four-bond $J_{N2C}$ coupling of, for example, 0.2 Hz would cause a negligible increase (0.01 Hz) in the value measured for $J_{N1C}$. This effect, therefore, may be safely ignored.

The simple 2D experiment presented here, together with the analogous 2D or 3D experiment for measurement of long range $^{13}C$–$^{13}C$ couplings,[11] provides stereospecific assignments for valines and $\chi_1$ angles for valine, isoleucine, and threonine residues in a very straightforward manner. The high precision with which the small coupling constants can be measured also allows identification of residues that are subject to $\chi_1$ rotamer averaging. In addition to yielding the magnitude of the $^3J_{CN}$ coupling constants, the experiment described here is also useful for identification of Pro-$C^{\delta}H_2$, Lys-$C^{\epsilon}H_2$, Arg-$C^{\delta}H_2$, Gln-$C^{\gamma}H_2$, and Asn-$C^{\beta}H_2$ resonances, which are of high intensity in the 2D difference spectrum because of the substantial $^1J_{CN}$ and $^2J_{CN}$ coupling to the adjacent nitrogen.

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(18) Ikura, M.; Clore, G. M.; Gronenborn, A. M.; Zhu, G.; Klee, C. B.; Bax, A. *Science* **1992**, *256*, 632–638.

(19) Peng, J.; Thanabal, V.; Wagner, G. *J. Magn. Reson.* **1991**, *95*, 421–427.

(20) Vuister, G. W.; Bax, A. *J. Am. Chem. Soc.*, submitted for publication.