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Measurement of two- and three-bond ^{13}C–^{1}H J couplings to the C_δ carbons of leucine residues in staphylococcal nuclease

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SUMMARY

A new ^{1}H-detected 3D NMR experiment is described that permits quantitative measurement of two- and three-bond ^{13}C–^{1}H couplings in proteins with selectively ^{13}C-enriched methyl sites. The method is demonstrated for staphylococcal nuclease selectively [5,5 ^{13}C]-labeled in all 11 leucine positions and ligated with thymidine 3',5'-biphosphate and Ca^{2+}. Two- and three-bond ^{13}C methyl–proton couplings are reported and, together with the measured three-bond $J_{C\sigma C\delta}$ in uniformly ^{13}C-enriched staphylococcal nuclease, the χ_2-angles and the stereospecific assignments of the C_δ methyl group with respect to the prochiral β-protons were determined. The same residues that were previously found to have high degrees of internal mobility on the basis of ^{13}C relaxation times have measured coupling constants that are indicative of motional averaging.

INTRODUCTION

Heteronuclear coupling constants have long been known to contain a wealth of conformational information (Bystrov, 1976). However, it is difficult to measure such couplings in proteins because of the low sensitivity of natural abundance studies and because of the small values of these couplings relative to typical protein resonance linewidths. With recent advances in molecular biology, many proteins of interest can be overexpressed in microorganisms, allowing both uniform and residue-specific isotopic enrichment with ^{13}C and ^{15}N. This development opens the way to explore fully the information contained in one-, two-, and three-bond J couplings. Three-bond J_{CH} couplings are of particular interest because of their well-established Karplus-like dependence upon the intervening torsion angle (Bystrov, 1976).

Many of the recently proposed experiments for measuring heteronuclear J couplings rely on the

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so-called E.COSY principle (Griesinger et al., 1986) in which the coupling of interest to spin I is measured from the relative displacement of cross peaks between two other spins, both coupled to I (Montelione et al., 1989; Wider et al., 1989; Edison et al., 1991; Emerson and Montelione, 1992; Griesinger and Eggenberger, 1992; Sattler et al., 1992; Vuister and Bax, 1992; Xu et al., 1992). Alternatively, 3D spectra employing COSY transfer via long-range couplings have been used to obtain qualitative information about the size of these couplings and to make stereospecific assignments of non-equivalent β-methylene protons (Archer et al., 1991; Chary et al., 1991; Grzesiek et al., 1992). As was recently shown for the measurements of ^{13}C–^{13}C long-range couplings (Bax et al., 1992) and for the measurement of very small ^{1}H–^{113}Cd and ^{1}H–^{199}Hg couplings (Blake et al., 1992), these COSY-based experiments can also be conducted in a quantitative manner, and offer a convenient method for the precise measurement of long-range heteronuclear J couplings. The present paper describes the use of this COSY-based approach for the measurement of two- and three-bond ^{1}H–^{13}C J couplings involving the C_δ methyl carbons of the 11 selectively [5,5 ^{13}C]-labeled leucine residues in staphylococcal nuclease (SNase) ligated with thymidine 3',5'-bisphosphate (pdTp) and Ca^{2+}. In combination with the measurement of the $^{13}C_\delta$–$^{13}C_\alpha$ J couplings in uniformly ^{13}C-enriched SNase, these couplings can be used to determine the leucine side-chain conformations and to aid the stereospecific assignment of the C_β methylene protons and the C_δ methyl groups.

Generally, the ^{13}C methyl resonances in proteins have smaller linewidths than most other ^{13}C spins, due to the rapid rotation about the methyl 3-fold symmetry axis. In addition, because of cross-correlation effects, the decay of ^{13}C methyl transverse magnetization is strongly non-exponential, giving rise to the superposition of lines with different widths (Müller et al., 1987; Kay et al., 1992a). The narrowest of these components typically has a width of only a few Hz in proteins of ~20 kDa. Since many of the long-range J couplings are larger than this width, they can be measured in a long-range ^{13}C–^{1}H correlation experiment as described below.

DESCRIPTION OF THE PULSE SCHEME

The pulse scheme for the ^{1}H-detected [^{13}C–^{1}H] long-range correlation experiment (LRCH) is sketched in Fig. 1, and its mechanism will briefly be described below. The sequence starts with presaturation of the proton spins to obtain NOE-enhanced ^{13}C methyl magnetization. In practice, an enhancement of about 2.5 is obtained (Nicholson et al., 1992). After the $90°_{\phi 1}$ ^{13}C pulse, the C_δ magnetization is frequency labeled during the constant-time evolution period of total duration 2T. Because the $180°_{\phi 2}$ ^{13}C pulse and 180° ^{1}H pulse are applied simultaneously, dephasing of the ^{13}C methyl magnetization due to heteronuclear ^{1}H–^{13}C J couplings during the time 2T is independent of t_1. The net effect of dephasing caused by $^{1}J_{CH}$ (~127 Hz) is minimized by adjusting 2T to $k/^{1}J_{CH}$ (k=1,2,3,...). Just before the ^{1}H $90°_{\phi 3}$ pulse, the ^{13}C magnetization is described by a sum of terms given by:

$$\Pi_i \cos(2\pi J_{CH_i} T)\, S_y - 2\Sigma_i[\sin(2\pi J_{CH_i} T)\Pi_{j\neq i} \cos(2\pi J_{CH_j} T) S_x I_{iz}] + \ldots \quad (1)$$

where S is the spin operator for the ^{13}C, and I_i is the operator for proton i. The dots in expression (1) denote terms that contain products of I_z operators. Of these terms, those products containing an even number of I_z operators are removed by the ^{1}H phase cycling, and terms containing

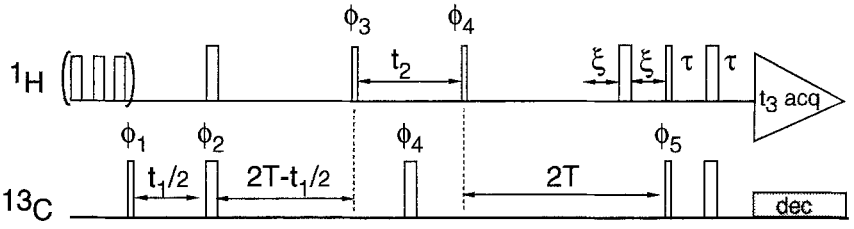


Fig. 1. Pulse sequence of the 3D ^{1}H-detected [^{13}C–^{1}H] long-range correlation experiment. Narrow and wide pulses denote 90° and 180° flip angles, respectively. The pulse train on the ^{1}H channel during the relaxation delay consists of low-power 135° pulses spaced by 10-ms delays and serves to build up the heteronuclear NOE. The carrier for the carbon 90° and 180° pulses was positioned at 25 ppm. Unless indicated otherwise, all pulses are applied along the x-axis. The phase cycle is as follows: ϕ_1 = x; ϕ_2 = 4(x),4(y).4(−x),4(−y); ϕ_3 = x,−x; ϕ_4 = 2(x),2(−x); ϕ_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 the following receiver phase cycle: 4(x),4(−x),4(x),8(−x),4(x),4(−x).4(x). Quadrature detection in t_1 and t_2 is obtained by the States-TPPI method (Marion et al., 1989) by incrementing phases ϕ_1 and ϕ_3, respectively. Delay durations: τ = 1.7 ms, 2T = 15.8, 23.7, or 31.6 ms, and ξ=1 ms.

products of three or more I_z operators are negligibly small because they contain the product of three or more small $\sin(2\pi J_{CH_j}T)$ coefficients. The terms containing the S_xI_z operator products in expression 1 are the ones of interest. As a result of the 90°(^{1}H) − t_2/2 − 180°(^{13}C) − t_2/2 − 90°(^{1}H) sequence, such terms are modulated by $\cos(\omega_1 t_2)$ in a regular HMQC manner. After the 90°$_{\phi 4}$ ^{1}H pulse, the antiphase S_xI_z terms refocus for a total time of 2T−2ξ prior to INEPT transfer to the observed protons. Rephasing during this 2T−2ξ delay gives rise to trigonometric terms that are analogous to the ones in expression 1. The 180° ^{1}H pulse, applied at time ξ before the end of the second 2T interval, is needed to bring the methyl ^{13}C magnetization in antiphase with respect to its directly attached protons, a requirement for the subsequent reverse INEPT transfer.

J values from integrated intensity ratios

As will be discussed below, the value of the long-range J_{CH} coupling can be calculated in a straightforward manner from the ratio of the integrated intensity observed for the long-range correlation in the 3D spectrum, to the integral of a reference peak in a 2D spectrum. Comparison of integrated intensities in spectra of different dimensionality is most easily analyzed by considering that the integral over the frequency domain spectrum equals the first point of the time domain. This forms the basis for the well-known fact that the volume integral is independent of the decay rate of the time domain signal. When considering a discrete Fourier transform with the time domain starting at time zero, the first data point, s(0), needs to be multiplied by 0.5 to obtain zero baseline (Otting et al., 1986) and the integral of the spectrum becomes s(0)/2. Also, the spectrum resulting from most discrete Fourier transform routines is not scaled for the number of time domain data points. If the total length of the time domain, including an arbitrary amount of zero-filling, is N complex data points, the integral then becomes $N \times s(0)/2$. Similarly, a time domain signal $s(t_1,t_2)$ after Fourier transformation has an integral equal to $N_1 \times N_2 \times s(0,0)/4$, where N_1 and N_2 are the number of complex data points used in the t_1 and t_2 domains. The integral for a 3D signal $s(t_1,t_2,t_3)$ becomes $N_1 \times N_2 \times N_3 \times s(0,0,0)/8$. Integrated peak intensities are therefore related in a very straightforward manner to the signal intensity of the corresponding

component at time zero. Below, these simple scaling factors will not be considered as they may vary depending on the Fourier-transform algorithm used. Which scaling factor is needed with a given software package is determined most easily by using a simple simulated signal with a single resonance of unit intensity, at zero frequency in all dimensions.

The 3D time domain signal at $t_1,t_2,t_3 = 0$, and thus its integrated intensity in the 3D spectrum of the long-range ^1H–^{13}C correlation signal between a given carbon and a proton i, V_{CH_i}, then depends on the J values according to:

$$V_{CH_i} = -A \times \{\sin(2\pi J_{CH_i}T)\sin(2\pi J_{CH_i}[T - \xi])\Pi_{j\neq i}\cos(2\pi J_{CH_j}T)\cos(2\pi J_{CH_j}[T - \xi])\} \quad (2a)$$

where A is a constant which depends on a large number of factors that need not be discussed here. The pulse scheme of Fig. 1 can also be used to obtain a direct one-bond $^1J_{CH}$ type 2D spectrum by keeping $t_2 = 0$ and by changing the phase cycling as indicated in the legend to Fig. 1. The integrated intensity of the one-bond correlation, V_{CH}, is again equal to the time domain signal at $t_1,t_3 = 0$ and to a good approximation given by:

$$V_{CH} = A \times \Pi_i\cos(2\pi J_{CH_i}T)\cos[2\pi J_{CH_i}(T - \xi)] \quad (2b)$$

where the constant A has the same value as in Eq. 2a, and the product extends over all protons i that have a J interaction with the ^{13}C spin considered. For $2J_{CH_i}T < 0.5$, the size of the long-range couplings can therefore be calculated from the ratio of the peak volumes measured in the 3D (V_{CH_i}) and 2D spectra (V_{CH}):

$$-V_{CH_i}/V_{CH} = \tan(2\pi J_{CH_i}T)\tan[2\pi J_{CH_i}(T - \xi)] \quad (3)$$

The product of the tangent terms of Eq. 3 can be approximated for small ξ/T ratios by $\tan^2(\pi J_{CH_i}\{2T - \xi\})$, yielding a simple expression for J_{CH_i}:

$$J_{CH_i} = \tan^{-1}\sqrt{(-V_{CH_i}/V_{CH}}\,/\,\pi(2T-\xi) \quad (4)$$

EXPERIMENTAL

The 3D ^1H-detected long-range ^1H–^{13}C correlation experiment is demonstrated on a sample of SNase, selectively [5,5 ^{13}C]-Leu labeled, 1.5 mM in D_2O, p^2H 7.0, and ligated with pdTp (5 mM) and Ca^{2+} (10 mM). Three LRCH experiments were recorded at 35 °C on a Bruker AMX600 spectrometer with the pulse scheme of Fig. 1, using dephasing periods, 2T, of 15.8, 23.7, and 31.6 ms corresponding to $2T = 2/^1J_{CH}$, $3/^1J_{CH}$, and $4/^1J_{CH}$, respectively. For the experiment recorded with 2T = 31.6 ms it was found that no correlations involving $C_{\delta 1,2}$ of Leu^{37}, $C_{\delta 1}$ of Leu^{38} and $C_{\delta 2}$ of Leu^{89} were observed. It was determined that hydrolyzation of a significant fraction of the pdTp resulted in exchange broadening for these resonances, which are known to be significantly perturbed by ligand binding (Nicholson et al., 1992). For the subsequent experiments with 2T durations of 15.8 and 23.7 ms, pdTp and hydrolyzed pdTp were removed by dialysis and replaced by fresh pdTp.

The LRCH spectra result from 32* (t_1) × 32*(t_2) × 384*(t_3) data matrices where n* denotes n

complex points. Data were acquired with acquisition times of 26.5 (t_1), 11.9 (t_2), and 53 (t_3) ms, using 64 scans per hypercomplex t_1/t_2 increment, which resulted in a total measuring time of 36 h per 3D spectrum. All data were processed identically. A 72°-shifted squared sine-bell window was used in t_3 prior to zero-filling to 1024* and Fourier transformation. After Fourier transformation in the t_2 domain, the length of the time domain in the t_1 dimension was first doubled by mirror-image linear prediction (Zhu and Bax, 1990), then filtered with a squared cosine-bell window, zero-filled to 128*, and Fourier transformed. Inverse Fourier transformation, followed by backward-forward linear prediction (Zhu and Bax, 1992) doubled the data to 64* in the t_2 domain. The t_2 domain was then filtered with a squared cosine-bell window, zero-filled to 256*, and Fourier transformed. 2D reference spectra were recorded in 1 h using the phase-cycle given in the legend to Fig. 1 with acquisition times of 26.5 (t_1) and 53 (t_2) ms, and 64 scans per complex t_1 increment. Identical processing parameters for the corresponding domains of the 2D and 3D spectra were used. Peak volumes were determined by integration, using integration limits derived from an iterative non-linear least-squares line fitting program (Delaglio, F., unpublished results).

The $^{13}C_\delta$–$^{13}C_\alpha$ J couplings were measured with a ^{1}H-detected long-range ^{13}C–^{13}C correlation experiment as described previously (Bax et al., 1992), using a 1.5 mM solution of uniformly ^{13}C enriched SNase in D_2O, also ligated with pdTp and Ca^{2+}. Experimental parameters used are reported by Bax et al. (1992). Long-range correlations were observed for all $^3J_{CC}$ values larger than 1.8 Hz.

RESULTS AND DISCUSSION

Figure 2 shows F_2 strips, centered at the F_1 (^{13}C) and F_3 (^{1}H) frequencies of the $C_{\delta1}$ and $C_{\delta2}$ methyl groups of Leu[137], taken from the 3D LRCH spectrum recorded with 2T = 31.6 ms. Cross peaks to $H_{\delta2}$ or $H_{\delta1}$, H_γ, $H_{\beta2}$, and $H_{\beta3}$ can be seen in both strips. The variation in their intensities reflects the differences in the magnitudes of the pertinent couplings. Small deviations from the tuned value of 127 Hz for the $^1J_{CH}$ coupling will result in spurious $C_{\delta1}$–$H_{\delta1}$ and $C_{\delta2}$–$H_{\delta2}$ correlations, but these are of negligibly low intensity for the strips shown. The size of the long-range coupling is derived from the volume ratio of the long-range correlation and the corresponding one-bond correlation observed in the reference 2D spectrum (Eq. 4) for each of the three LRCH spectra recorded. The results are presented in Table 1.

As can be seen from the data presented in Table 1, increasing 2T results in a small but significant gradual decrease in the calculated J values. This effect is caused by the fact that relaxation of the antiphase S_xI_z term can be considerably faster than that of the in-phase S_y term (Bax et al., 1990; London, 1990; Peng et al., 1991). In the slow tumbling limit, the relaxation rate of S_xI_z equals, to a first approximation, the sum of the transverse relaxation rate, $1/T_{2S}$, of S_y and the selective inversion recovery rate, $1/T_{1I}$, of spin I.

In macromolecules, the faster relaxation of the antiphase S_xI_z magnetization is dominated by ^{1}H–^{1}H spin flips (Bax et al., 1990; London, 1990; Peng et al., 1991). For SNase, the ^{1}H–^{1}H spin flip rate is ~10 s^{-1} for amide protons (Kay et al., 1992b) and rates in the 5–10 s^{-1} range have been measured for H_α protons of the alanine residues in SNase (Nicholson, L.K. and Torchia, D.A., unpublished results). In the present case, this faster relaxation can be considered as a 'leakage' process which attenuates the observed long-range correlation relative to the corresponding resonance in the 2D reference spectrum. To a first approximation, the change in the observed intensi-

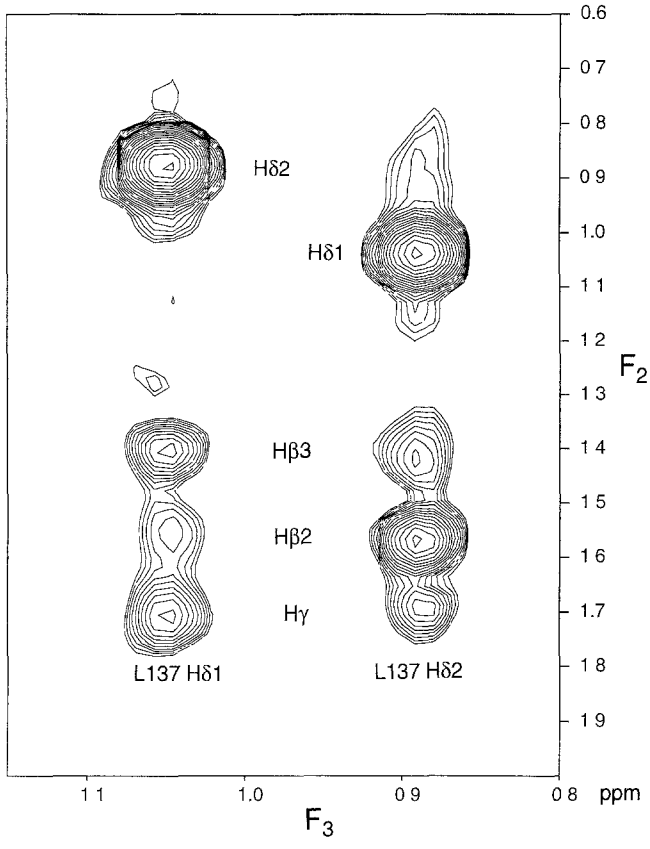


Fig. 2. Two F_2 strips from the 3D ^{1}H-detected [^{13}C–^{1}H] long-range correlation of SNase, recorded with the pulse sequence of Fig. 1, using 2T = 31.6 ms, centered at the ^{13}C (F_1) and ^{1}H (F_3) frequencies of the δ_1 and δ_2 methyl groups of Leu^{137}. The strips display correlations to all protons with a significant J coupling to $C_{\delta 1}$ (left strip) and $C_{\delta 2}$ (right strip).

ty ratio depends quadratically on 2T and $1/T_{1I}$, causing the difference between the derived and the true J coupling to increase linearly with 2T and with $1/T_{1I}$ (Vuister, G.W. and Bax, A., unpublished results). Linear extrapolation of the J values derived for the different durations of 2T to 2T = 0 then gives a reasonable estimate for the true J value. A more detailed discussion of the effect of ^{1}H–^{1}H spin flips on the measurement of H_N–H_α J couplings from quantitative J correlation will be presented elsewhere.

For coupling between leucine C_δ spins and C_β methylene protons, the situation is slightly more complex than indicated above. In this case, $H_{\beta 2}$–$H_{\beta 3}$ spin flips cause exchange between the $S_xI_z^{\beta 2}$ and the $S_xI_z^{\beta 3}$ terms. This effect results in an increase in the smaller antiphase $S_xI_z^\beta$ term at the cost of a decrease in the larger $S_xI_z^\beta$ term. From Eq. 2a it follows that the ratio of the difference and the sum of the square roots of the cross-peak volumes to the two H_β resonances, to a first approximation, depends linearly on the $H_{\beta 2}$–$H_{\beta 3}$ cross-relaxation rate, $\sigma_{\beta\beta}$, and on the duration of the de- and rephasing intervals, 2T:

$$(\sqrt{V_{CH\beta 2}} - \sqrt{V_{CH\beta 3}})/(\sqrt{V_{CH\beta 2}} + \sqrt{V_{CH\beta 3}}) \approx (J_{CH\beta 2} - J_{CH\beta 3})/(J_{CH\beta 2} + J_{CH\beta 3}) \times (1 - 2\sigma_{\beta\beta}T) \quad (5)$$

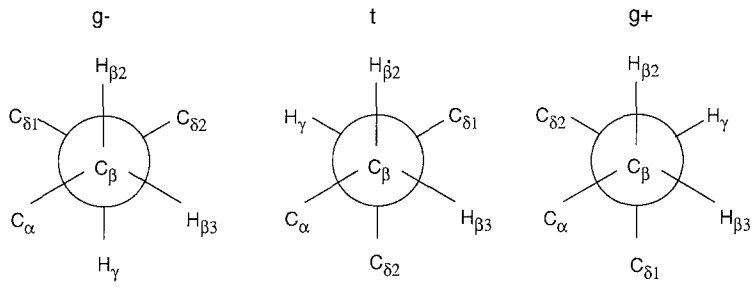
For each of the three LRCH spectra, the intensity ratios of Eq. 5 were calculated for the four residues in SNase where resolved C_δ–H_β correlations were observed for both H_β protons (Leu^{137}, Leu^{89}, Leu^{37}, and Leu^{14}). From the decrease of the ratio of Eq. 5 as a function of 2T, $\sigma_{\beta\beta}$ was estimated to be ~11 s^{-1}, in good agreement with a $\sigma_{\beta\beta}$ of 12.5 s^{-1} ($R_C = 2\sigma = 25$ s^{-1} (Ernst et al., 1987)) expected for an isolated two-spin system at 1.77 Å in a molecule tumbling isotropically with a 9-ns correlation time (Kay et al., 1989) and an assumed order parameter, S^2, of 0.8. Simulation of the buildup of the anti-phase S_xI_z terms for a methylene group in the presence of magnetization exchange between the two methylene protons, using 6 Hz and 2 Hz for the true couplings, 2T = 15.8 ms and $2\sigma_{\beta\beta} = 25$ s^{-1}, results in an apparent decrease in the large coupling to 5.4 Hz and an increase in the small coupling to 2.6 Hz. However, for the small coupling the leakage caused by cross relaxation to protons other than its geminal partner will counteract this increase. In contrast, such leakage will attenuate the larger coupling to a value smaller than 5.4 Hz. Note the similarities between this description and the initial rate analysis of NOE buildups.

From the above discussion it follows that the values obtained with 2T = 15.8 ms are closest to the true values. Simple linear extrapolation of the measured values to 2T = 0 should further increase the accuracy of the measurement. The effect of magnetization exchange between methylene protons is largest when coupling between C_δ and one of the β-protons is large and the coupling to the other β-proton is small. Even in this case, the error introduced by the ^1H–^1H spin flips does not cause any ambiguity in distinguishing the trans and gauche couplings.

TABLE 1
TWO- AND THREE-BOND J_{CH} AND J_{CC} COUPLINGS (in Hz) OF THE LEU RESIDUES IN STAPHYLOCOCCAL NUCLEASE LIGATED WITH THYMIDINE 3',5'-BIPHOSPHATE AND Ca^{2+}

Residue	$^3J_{C\delta 1H\beta 2}$	$^3J_{C\delta 1H\beta 3}$	$^2J_{C\delta 1H\gamma}$	$^3J_{C\delta 1C\alpha}$	$^3J_{C\delta 2H\beta 2}$	$^3J_{C\delta 2H\beta 3}$	$^2J_{C\delta 2H\gamma}$	$^3J_{C\delta 2C\alpha}$	Rotamer
Leu^7	a	a	4.3;3.5;2.9[a,g]	3.0	a	a	5.3;4.8;4.3[a,g]	2.0	f
Leu^{14}	3.4;3.5;3.0[b]	6.2;5.6;5.1	2.3;2.6;3.2[b]	1.2	<2.0[b]	2.5;2.3;2.1	3.9;3.3;3.0[b]	4.1	g^-
Leu^{25}	2.5;2.1;1.5	c	3.3;3.0;2.7[c,g]	3.6	6.0;5.8;5.5	c	3.1;3.1;2.9[c,g]	1.0	t
Leu^{36}	3.4;2.5;<2.0	c	5.0;4.1;4.6[c,g]	3.0	4.2;3.9;3.2	c	3.8;3.1;2.8[c,g]	2.2	f
Leu^{37}	2.3;2.4[d]	2.3;2.1[d]	h	3.5	5.8;5.4[d]	3.5;3.3[d]	3.8;2.2[d]	<1.8	t
Leu^{38}	2.6;2.1[d]	c	3.7;2.5[c,d,g]	3.5	5.4;5.1;4.6	c	3.3;2.6[c]	1.7	t
Leu^{89}	2.7;2.3;2.4	<2.0	3.4;2.8;2.8	3.7	5.9;5.5[d]	3.6;3.0[d]	3.2;2.8[d]	e	t
Leu^{103}	<2.0	c	5.0;4.5;3.8[c,g]	3.1	4.9;4.0;3.6	c	6.0;5.2;4.3[c,g]	1.2	t
Leu^{108}	b	1.8;2.0;1.8	4.3;4.1;3.3[b,g]	3.6	b	3.8;3.4;3.7	6.1;5.8;5.5[b,g]	1.0	t
Leu^{125}	3.4;4.1;2.5[b]	3.3;3.0;2.8	4.2;3.4;3.4[b]	2.7	4.3;4.1;3.2[b]	3.2;2.9;2.5	<2.0[b]	2.2	f
Leu^{137}	3.1;2.2;2.3	2.6;2.6;2.6	3.3;3.1;2.8	3.0	5.2;4.8;4.2	2.7;2.7;2.6	3.5;3.3;2.6	1.5	t

[a] $H_{\beta 2}$, $H_{\beta 3}$, and H_γ (partially) overlap.
[b] $H_{\beta 2}$ and H_γ (partially) overlap.
[c] $H_{\beta 3}$ and H_γ (partially) overlap.
[d] Correlations lost in the 2T = 31.6 ms spectrum due to exchange.
[e] Not measured due to overlap.
[f] Rotamer averaging.
[g] Upper limit.
[h] H_γ and $H_{\delta 2}$ overlap.



$^3J_{C\delta1H\beta2}$	small	small	large
$^3J_{C\delta1H\beta3}$	large	small	small
$^3J_{C\delta1C\alpha}$	small	large	small
$^3J_{C\delta2H\beta2}$	small	large	small
$^3J_{C\delta2H\beta3}$	small	small	large
$^3J_{C\delta2C\alpha}$	large	small	small

Fig. 3. Newman projections illustrating the χ_2 torsion angle in leucine. The χ_2 torsion angle and prochiral H_β and $C_\delta H_3$ have been defined in accordance with IUPAC rules (IUPAC-IUB Commission on Biochemical Nomenclature 1970).

Figure 3 shows the three rotamers g^-, t, and g^+ and the classification of the corresponding $^3J_{C\delta H\beta}$ and $^3J_{C\delta C\alpha}$ couplings. Large values of $^3J_{C\delta H\beta}$ ($\geq$5 Hz) are expected when C_δ and H_β are trans, whereas gauche values are small ($\leq$3 Hz) (Hansen, 1981). Similarly, trans $^3J_{C\delta C\alpha}$ couplings are $\geq$3 Hz and gauche $^3J_{C\delta C\alpha}$ values are $\leq$1 Hz. If each of the C_δ spins shows a large coupling to one of the H_β protons and a weak or absent coupling to the other H_β this defines χ_2 to be g^+. In this case, neither of the C_δ carbons has a significant J coupling to C_α. In proteins, however, a g^+ χ_2 rotamer for leucine residues is extremely rare (James and Sielecki, 1983). For the most common χ_2 state, t, one of the C_δ carbons ($C_{\delta2}$) has a large coupling to $H_{\beta2}$, and $C_{\delta1}$ has small couplings to both H_β protons. For the less common case of a g^- χ_2 state, one of the C_δ carbons ($C_{\delta1}$) shows a large coupling to $H_{\beta3}$ whereas $C_{\delta2}$ has small couplings to both H_β protons. In both of the latter cases, the C_δ carbon, which does not show a large coupling to either H_β, is expected to have a large coupling to C_α.

The two- and three-bond J_{CH} couplings obtained from the 3D LRCH spectrum and three-bond J_{CC} couplings obtained from the 2D ^1H detected [^{13}C–^{13}C] long-range correlation spectrum are listed in Table 1. The stereospecific assignments of the H_β methylene protons of leucine were obtained from NOE and J coupling data and from comparison with the high-resolution crystal structure (Loll and Lattman, 1989) (see Table 2). The stereospecific assignments of the C_δ methyl groups for Leu[14], Leu[103], and Leu[137], derived from the J couplings listed in Table 1, are the reverse of the ones reported in a previous ^{13}C methyl relaxation study (Nicholson et al., 1992). These earlier C_δ stereospecific assignments were based on a comparison of the NOE spectra with a preliminary crystal structure in which the conformation of the side chains of Leu[103] and Leu[137] differed substantially from their conformation in the final refined structure. The χ_2 angles of Leu[103] and Leu[137] in this less-refined structure were incompatible with the measured J couplings reported in Table 1, whereas the χ_2 angles for these residues in the final refined structure agree with Table 1. However, the χ_2 angle for Leu[14] (−136°) in both the preliminary and the final X-ray

TABLE 2
STEREOSPECIFIC ASSIGNMENTS OF THE β-METHYLENE PROTONS AND δ METHYL GROUPS OF THE LEU RESIDUES IN SNase LIGATED WITH pdTp AND Ca^{2+}

Residue	$H_{\beta 2}$	$H_{\beta 3}$	$C_{\delta 1}$	$H_{\delta 1}$	$C_{\delta 2}$	$H_{\delta 2}$
Leu^{7}	1.77	1.77	25.0[a]	0.93[a]	22.1[a]	1.03[a]
Leu^{14}	1.15	1.94	23.3	0.76	27.0	0.91
Leu^{25}	1.78	1.44	25.4	0.02	24.8	0.75
Leu^{36}	1.51	1.65	26.2	0.78	24.4	0.76
Leu^{37}	0.74	1.06	26.6	0.38	26.6	1.18
Leu^{38}	2.29	1.92	28.3	1.13	25.1	1.19
Leu^{89}	1.72	1.14	25.2	0.67	22.2	0.68
Leu^{103}	1.02	1.72	27.0	0.80	24.0	0.90
Leu^{108}	1.22	1.43	25.8	0.48	22.7	0.74
Leu^{125}	1.92	1.45	26.5	0.95	24.1	0.78
Leu^{137}	1.53	1.37	25.5	1.02	23.0	0.86

All values are in ppm from trimethylsilylpropionic acid.
[a]Stereospecific assignment deduced from NOE and crystallographic data only.

structure of ligated SNase is not compatible with the results in Table 1. The χ_2 angle derived from the J couplings (60°) is in agreement, however, with χ_2 in the X-ray structure of unligated SNase (Hynes and Fox, 1991), and the chemical shifts and NOE patterns of these methyl groups remain unchanged upon ligation.

The $^3J_{C\delta H\beta}$ coupling constants measured in the present study, before extrapolation to 2T = 0, fall in the 1.8–6.2 Hz range. Even after extrapolation to 2T = 0, the values for most leucine residues in SNase remain considerably less extreme than the values previously reported by Sattler et al. (1992) for a leucine residue in a constrained cyclic peptide. These less extreme values are in qualitative agreement with the relatively low order parameters for the leucine methyl groups in SNase derived from ^{13}C relaxation studies (Nicholson et al., 1992). Indeed, the methyl groups with the lowest order parameters (Leu^{7}, Leu^{36}, and Leu^{125}) are the ones for which the coupling constants indicate the highest degree of rotamer averaging.

CONCLUSIONS

The ^1H-detected [^{13}C–^1H] long-range correlation experiment presents a simple way for measurement of two- and three-bond J couplings between protons and methyl carbons in selectively ^{13}C-labeled proteins. The fact that the measurement is influenced by ^1H–^1H spin flips is not unique to this method, and can also cause comparably distorted values in E.COSY-based techniques or in direct measurements of multiplet splittings (Harbison, 1993). As will be described elsewhere, a modified version of the LRCH pulse scheme, which is of lower inherent sensitivity, is also applicable to uniformly ^{13}C-enriched proteins. The ^1H-detected [^{13}C–^1H] long-range correlation experiment complements information contained in $^3J_{CC}$ couplings (Bax et al., 1992), and both types of measurements are readily applicable to proteins of up to at least 20 kDa.

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