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Side Chain Assignments of Ile δ1 Methyl Groups in High Molecular Weight Proteins: an Application to a 46 ns Tumbling Molecule

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Abstract: A sensitive 3D NMR pulse scheme, (H)C(CA)NH-COSY, is presented for the assignment of $^{13}C^{\delta1}$ Ile chemical shifts in large perdeuterated, methyl-protonated proteins. The nonlinearity of branched amino acids, such as Ile, significantly degrades the quality of TOCSY schemes which transfer magnetization from methyl carbons to the backbone $^{13}C^{\alpha}$ positions, and in applications to high molecular weight proteins (correlation times on the order of 40−50 ns), this compromises the sensitivity of spectra used for methyl assignment. The experiment presented utilizes COSY-based transfer steps and refocuses undesirable $^{13}C-^{13}C$ scalar couplings that degrade the efficiency of TOCSY transfers. The (H)C(CA)NH-COSY scheme is tested on an $^{15}N,^{13}C,^{2}H$-[Leu, Val, Ile (δ1 only)]-methyl-protonated maltose binding protein (MBP)/β-cyclodextrin complex at 5 °C (molecular tumbling time 46 ± 2 ns), facilitating the assignment of $^{13}C^{\delta1}$ chemical shifts for 18 of the 19 Ile residues for which backbone assignments were previously obtained. Both sensitivity and resolution of the resulting spectra are shown to be significantly better than those for a similar TOCSY-based approach.

Introduction

The use of deuteration in concert with uniform $^{15}N,^{13}C$-labeling has proven to be critical for the chemical shift assignment of proteins and protein complexes with molecular weights larger than approximately 30 kDa.[1] Deuteration in combination with TROSY spectroscopy[2−4] has significantly increased the size of molecules amenable to detailed NMR studies.[5−7] For example, Wüthrich and co-workers have reported near complete backbone assignments of a 110 kDa homo-octameric protein, 7,8-dihydroneopterin aldolase.[8] The backbone ^{1}HN, ^{13}C, and ^{15}N and side chain $^{13}C^{\beta}$ resonances of malate synthase G from *E. coli*, a 723-residue monomeric enzyme (81.4 kDa), were recently assigned in our laboratory using four-dimensional TROSY-based spectroscopy.[9] Other applications involving membrane proteins such as OmpX,[10,11] OmpA,[12] and PagP[13] in micelles with effective molecular masses on the order of 50−60 kDa as well as studies of dimers of the human tumor suppressor protein p53 (67 kDa, 279 residues)[14] have recently appeared.

Unfortunately, deuteration reduces the number of protons that can be used to obtain NOE-based distance restraints for structure determination. The precision and accuracy of global folds determined from highly deuterated large proteins can be substantially improved through the use of deuterated, selectively methyl-protonated samples.[15−17] This labeling pattern maintains the efficiency of triple-resonance NMR experiments, while retaining a sufficient number of protons at locations where they can be used to establish NOE-based contacts between different elements of secondary structure.[18,19] A particularly useful selective protonation strategy involves the addition of precursors [3-^{2}H],^{13}C-α-ketoisovalerate and [3,3-^{2}H]-α-ketobutyrate to deuterated, $^{15}N,^{13}C$ growth media leading to the production of

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^{15}N,^{13}C,^{2}H-labeled proteins with (^{1}H-δ methyl)-Leu, (^{1}H-γ methyl)-Val, and (^{1}H-$\delta1$ methyl)-Ile.[20]

NMR strategies for the assignment of selectively protonated methyl groups are commonly based on the use of $^{13}C-^{13}C$ homonuclear Hartmann−Hahn polarization transfer (TOCSY) schemes which relay magnetization from the methyls to backbone $^{13}C^{\alpha}$ positions followed by transfer steps to the amide of the same or the following residue in the protein sequence.[21−26] These (H)C(CA)NH-TOCSY, H(CCA)NH-TOCSY, (H)C(CO)-NH-TOCSY, and H(CCO)NH-TOCSY experiments[21−26] were earlier applied successfully to obtain methyl ^{1}H and ^{13}C side chain assignments of Val, Leu, and Ile residues in a maltose-binding protein (MBP)/β-cyclodextrin complex at 37 °C.[17] Recently, side chain assignments of a membrane protein OmpX in micelles were obtained using the same methodology in combination with TROSY.[27]

The aliphatic carbon skeletons of Val, Leu, and Ile side chains are branched at β-carbon positions of Val and Ile and at the γ-carbon of Leu. The nonlinearity of these spin systems significantly compromises the efficiency of magnetization transfer schemes, such as TOCSY, from methyl carbons to the backbone $^{13}C^{\alpha}$ positions. In small proteins, the decrease in efficiency of the $^{13}C-^{13}C$ TOCSY transfer step for these residues can be tolerated due to the high intrinsic sensitivity of the experiments in the first place. In contrast, for high molecular weight systems, signal-to-noise becomes limiting and the need to develop either alternative labeling strategies and/or pulse sequence methods is apparent. Herein, we describe a sensitive 3D NMR pulse scheme, (H)C(CA)NH-COSY, for the assignment of $^{13}C^{\delta1}$ Ile methyl groups in large perdeuterated, methyl-protonated proteins. This experiment utilizes solely COSY-based transfer steps (that in contrast to the isotropic mixing schemes allow easier control over the magnetization flow) and obviates the problem of undesirable $^{13}C-^{13}C$ J couplings that degrade the efficiency of TOCSY transfers. The utility of the (H)C-(CA)NH-COSY experiment was tested on an MBP/β-cyclodextrin complex at 5 °C with an overall molecular tumbling time of 46 ± 2 ns,[7] equivalent to a molecule on the order of 100 kDa at 37 °C. The sensitivity and resolution provided by the pulse scheme are significantly better than those of (H)C(CA)NH-TOCSY experiments, allowing the assignment of 18 out of 19 $^{13}C^{\delta1}$ resonances for which backbone assignments were previously obtained.[7]

Materials and Methods

NMR spectra were recorded on a 1.4 mM sample of an ^{15}N,^{13}C,^{2}H-[Leu, Val, Ile ($\delta1$ only)]-methyl-protonated maltose binding protein (MBP)/β-cyclodextrin complex, 20 mM sodium phosphate buffer (pH 7.2), 3 mM NaN_3, 100 mM EDTA, 0.1 mg/mL Pefabloc, 1 μg/μL pepstatin, 10% D_2O, prepared as described earlier.[17,20] Experiments were recorded on a Varian Inova 800 MHz four-channel spectrometer equipped with a triple-resonance triple-axis pulsed-field gradient probe. The ^{2}H lock receiver was disabled during application of ^{2}H pulses, ^{2}H-decoupling, and pulsed field gradients.

The 3D (H)C(CA)NH-COSY experiment was acquired with 20, 32, and 768 complex points in the $^{13}C^{\delta1}$, ^{15}N, and ^{1}HN dimensions with corresponding acquisition times of 12.7 ms, 13.7 ms, and 64 ms, respectively. A relaxation delay of 1.5 s was used along with 64 scans/FID, giving rise to a net acquisition time of 76 h. Selection of the TROSY component in this experiment and in those described below was achieved passively by relying exclusively on the fast relaxation of the anti-TROSY component during the transfer delays in the pulse scheme.[6] No anti-TROSY cross-peaks were detected in spectra of the MBP/β-cyclodextrin complex at 5 °C.

2D $^{13}C^{\delta1}-^{1}HN$ (H)C(CA)NH-COSY and (H)C(CA)NH-TOCSY data sets were recorded to compare COSY versus TOCSY magnetization transfers in such a large system. The COSY and TOCSY experiments were comprised of 20 and 768 and 14 and 768 complex points with acquisition times of 12.7 and 64 ms and 8.7 and 64 ms, respectively. Unlike the COSY experiment which records the $^{13}C^{\delta1}$ chemical shift in constant-time (CT) mode (see following), the TOCSY does not. With this in mind, we adjusted the acquisition time in the TOCSY accordingly so as to minimize contributions from the one-bond $^{13}C^{\delta1} - {}^{13}C^{\gamma1}$ scalar coupling. A relaxation delay of 1.5 s was used along with 1024 (COSY) and 1472 (TOCSY) scans/FID, giving rise to equal net acquisition times of 19 h for both experiments. The (H)C(CA)NH-TOCSY experiment was derived from (H)C(CO)NH-TOCSY optimized for application to highly deuterated, methyl-protonated samples as described earlier[25] with the addition of a TROSY element at the end of the sequence using the approach of Yang and Kay.[6] The pulse scheme is similar to a recently published experiment by Hilty et al.[27] but involves further improvement through the use of selective pulses, so that delays can be optimized to account for relaxation effects without concomitant signal loss due to the evolution of passive one-bond $^{13}C-^{13}C$ scalar couplings. Simulations established that for a 9.2 kHz field (centered at 40 ppm) and a Flopsy-8 ^{13}C mixing scheme[28] a mixing time of approximately 25 ms is optimal. Ten Flopsy-8 cycles were used in the present experiment corresponding to a mixing delay of 25.4 ms.

All data sets were processed with NMRPipe/NMRDraw software,[29] and spectra were analyzed with NMRView.[30] Briefly, mirror image linear prediction[31] was used to double the ^{13}C time domain in the 2D (H)C(CA)NH-COSY, while forward−backward linear prediction[32] was employed to extend 2-fold the ^{13}C dimension of the 2D (H)C(CA)-NH-TOCSY. In the case of the 3D (H)C(CA)NH-COSY, mirror image linear prediction was employed in both the ^{13}C and ^{15}N time domains using a procedure described by Kay et al.[33] Cosine-squared window functions were used in all three dimensions.

Results and Discussion

Recently, we reported near complete ^{1}HN, ^{15}N, and ^{13}C backbone and side chain $^{13}C^{\beta}$ chemical shifts of malate synthase G (MSG, 723 residues) based on 4D TROSY-triple resonance spectroscopy.[9] Our success in obtaining backbone assignments in such a large system has encouraged us to take the next step and consider the assignment of side chain resonances. The approach that we favor for large proteins is not to attempt a complete assignment but rather to focus on those resonances that might be the easiest to identify, and we have therefore

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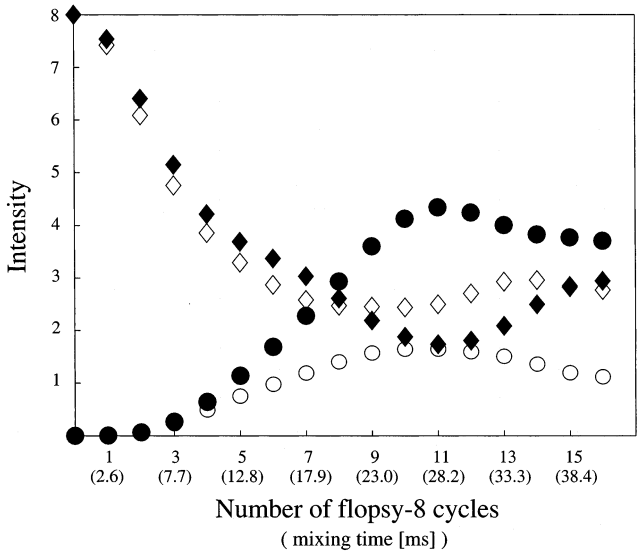

Figure 1. Numerical simulations comparing the efficiency of the Hartmann−Hahn $^{13}C^{\delta1} \rightarrow {}^{13}C^{\alpha}$ transfer in a uniformly ^{13}C-labeled Ile side chain (branched spin system) with an Ile side chain whose $\gamma2$-methyl is not ^{13}C-labeled (linear spin system). The calculated peak intensities are plotted as a function of the number of cycles (mixing time [ms]) of a 9.2 kHz Flopsy-8[28] mixing scheme centered at 40 ppm. The transfer to $^{13}C^{\alpha}$ is shown with closed and open circles for the linear and branched spin systems, respectively, while the magnetization remaining on $^{13}C^{\delta1}$ is indicated by the closed (linear) and open (branched) diamonds. The average chemical shifts of all carbon nuclei in the Ile side chain were taken from the BioMagResBank (http://www.bmrb.wisc.edu). A uniform $^{1}J_{C-C}$ coupling of 35 Hz was used in all calculations. Relaxation effects were not included in these simulations.

targeted methyl groups.[17] Biosynthetic strategies are in place for the labeling of Val, Leu, and Ile ($\delta1$) methyls with protons in a highly deuterated background.[16,17,20] Whether assignments can be obtained for even these simple targets in very high molecular weight proteins (correlation times > 35 ns) remains to be established, however.

To date, the most widely used approach for the assignment of side chains involves Hartmann−Hahn magnetization transfer from $^{13}C^{aliphatic}$ to $^{13}C^{\alpha}$ carbons.[17,21−27] Figure 1 shows the results from numerical simulations of the $^{13}C^{\delta1} \rightarrow {}^{13}C^{\alpha}$ transfer in Ile residues using the Flopsy-8 isotropic mixing scheme,[28] neglecting relaxation. By means of comparison, simulations are also included for the "hypothetical" case of a linear spin system, ^{13}C-[^{12}C-$\gamma2$-methyl]-Ile. The maximum transfer in the uniformly ^{13}C labeled side chain is only 40% of what is obtained for ^{13}C-[^{12}C-$\gamma2$-methyl]-Ile, and even in the later case, only approximately 55% of the total magnetization originating on $^{13}C^{\delta1}$ is transferred. Similar transfer profiles are obtained for the cases of Val and Leu. In principle, biosynthetic strategies in which residues are "reverse" labeled with ^{12}C at branching points so that a linear ^{13}C chain is produced would significantly improve the efficacy of TOCSY-based transfers, and such approaches are currently under development in our laboratory. However, in the case of Ile, a simpler strategy exists based on the use of COSY-type transfers with complete control of the magnetization flow at each transfer step. The undesired evolution due to the $^{13}C^{\beta} - {}^{13}C^{\gamma2}$ scalar coupling at the branch point of Ile side chains can be refocused due to the favorable distribution of ^{13}C side chain chemical shifts in this residue, resulting in reasonably efficient $^{13}C^{\delta1} \rightarrow {}^{13}C^{\alpha}$ magnetization transfer and significantly more sensitive NMR spectra than are obtained using conventional TOCSY-based approaches. In contrast, for Val(Leu), the proximity of the resonances of the methyl carbons at the branch point, $^{13}C^{\gamma}(^{13}C^{\delta})$, precludes a similar approach.

Figure 2a illustrates the (H)C(CA)NH-COSY pulse scheme that has been developed to correlate side chain $^{13}C^{\delta1}$ chemical shifts with those of the backbone amides. The pulse sequence is comprised of elements that have been described in detail previously in the literature, and therefore, we provide only a brief description here. The magnetization transfer steps in Ile can be schematically diagrammed as

$$[{}^{1}H^{\delta1}_{(i)} \xrightarrow{J_{CH}}] \, {}^{13}C^{\delta1}_{(i)} \, (CT - t_1) \xrightarrow{J_{CC}} {}^{13}C^{\gamma1}_{(i)} \xrightarrow{J_{CC}} {}^{13}C^{\beta}_{(i)} \xrightarrow{J_{CC}} {}^{13}C^{\alpha}_{(i)} \xrightarrow{J_{NC\alpha}} {}^{15}N_{(i,i+1)} \, (CT - t_2) \xrightarrow{J_{NH}} {}^{1}HN_{(i,i+1)} \, (t_3) \quad (1)$$

where J_{CH}, J_{CC}, $J_{NC\alpha}$, and J_{NH} are the scalar couplings active during each transfer step, t_i ($i = 1-3$) is an acquisition time, and an optional proton-carbon transfer at the beginning of the pulse sequence is enclosed in square brackets. Fourier transformation of the resulting data set gives rise to cross-peaks at ($\omega_{C\delta1}$, $\omega_{N(i)}$, $\omega_{HN(i)}$) and to a lesser extent at ($\omega_{C\delta1}$, $\omega_{N(i+1)}$, $\omega_{HN(i+1)}$).

The experiment starts with ($^{1}H-{}^{13}C$)-NOE-enhanced magnetization of Ile $^{13}C^{\delta1}$ selected by application of an Ile-$^{13}C^{\delta1}$-selective RE-BURP pulse[34] (labeled $\delta1$ in Figure 2a) in alternate scans along with a concomitant phase inversion of the receiver. Alternatively, magnetization can be transferred from methyl protons to the directly attached $\delta1$ carbons, as shown in Figure 2b. The $^{13}C^{\delta1}$ chemical shift is recorded in a constant-time manner for a duration of $2T_c \approx {}^{1}/_{2}J_{CC}$ with evolution of magnetization from the $^{13}C^{\delta1}-{}^{13}C^{\gamma1}$ J-coupling so that magnetization is transferred to the $^{13}C^{\gamma1}$ carbon by the pulse of phase $\phi3$. Evolution proceeds due to $^{13}C-{}^{13}C$ one-bond scalar couplings during the subsequent interval, so that at point *e* the signal of interest resides on the $^{13}C^{\beta}$ carbon. The selective $^{13}C^{\gamma2}$ inversion pulses applied during the period extending from *e* to *f* ensure complete magnetization transfer (neglecting relaxation) from $^{13}C^{\beta}$ to $^{13}C^{\alpha}$. This is critical for the success of the experiment, since in the absence of such pulses (i.e., if net evolution due to the $^{13}C^{\beta}-{}^{13}C^{\gamma2}$ coupling were to occur) the sensitivity would be reduced by approximately 2.5-fold. The $^{13}C^{\alpha}$ magnetization is subsequently refocused with respect to $^{13}C^{\beta}$ (making use of a selective $^{13}C^{\beta}$ inversion pulse) and defocused with respect to ^{15}N. Nitrogen chemical shift is recorded during the ensuing period of $2T_N$, and then magnetization transferred to ^{1}HN via a TROSY-scheme, described in detail previously.[6] For clarity, a schematic diagram of the magnetization transfer steps is included in Figure 2c along with the position of transverse magnetization during the various intervals in the pulse sequence. In addition, the expected distribution of chemical shifts for each of the carbons of the deuterated, $\delta1$-methyl-protonated Ile side chain is shown. Assignment of the $^{1}H^{\delta1}$ methyl protons can be obtained using a variation of the pulse scheme shown in Figure 2b in which the CT $^{13}C^{\delta1}$ evolution period is substituted with a semi-CT proton evolution element[35] during the first INEPT transfer.

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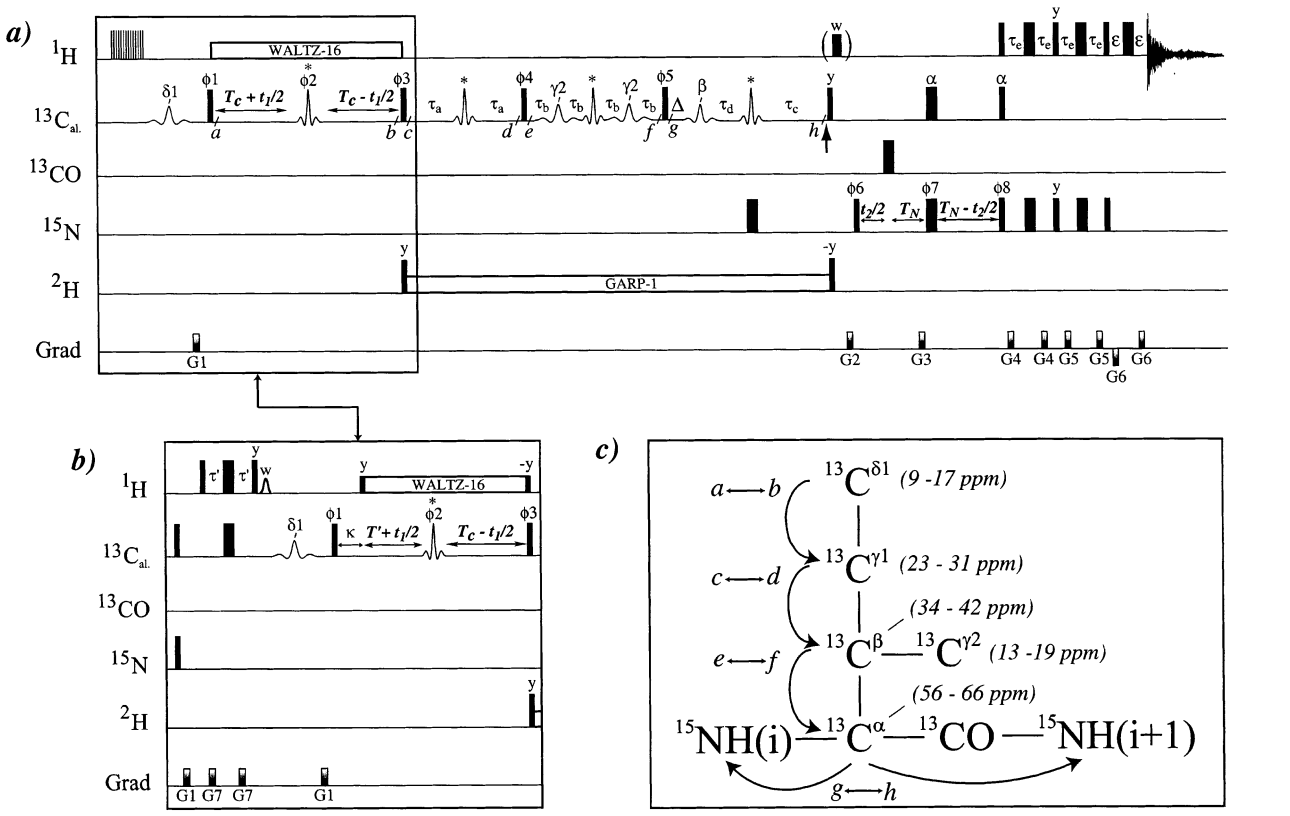

Figure 2. (a) Pulse scheme of the (H)C(CA)NH-COSY experiment with magnetization originating on ^{13}C. All narrow (wide) rectangular pulses are applied with flip angles of 90° (180°) along the *x*-axis unless indicated otherwise. The ^{1}H and ^{15}N carriers are positioned at 4.7 (water) and 119 ppm, respectively. All proton pulses are applied with a field strength of 40 kHz, with the exception of the presaturation pulse train comprised of 120° pulses (17.6 kHz field) in intervals of 5 ms and the ^{1}H WALTZ-16[36] decoupling field (and flanking pulses) which makes use of a 7.0 kHz field. The water purge labeled with "w" is comprised of 3.0 ms (phase *x*) and 1.7 ms (phase *y*) pulses (7.0 kHz) (and is only employed in the case where magnetization originates on ^{13}C). All ^{15}N(^{2}H) pulses employ a 6.9(1.9) kHz field, with ^{2}H decoupling achieved using a 0.6 kHz GARP-1[37] field. The ^{13}C carrier is set to 40 ppm and switched to 58 ppm prior to the ^{15}N 90° ϕ6 pulse. Rectangular 90° ^{13}C pulses are applied at high power, while the rectangular 180° ^{13}C pulses employ a field of $\Delta/\sqrt{3}$, where Δ is the difference (Hz) between $^{13}C^{\alpha}$ (58 ppm) and ^{13}CO (176 ppm) shifts.[38] (Pulses labeled with "α" are centered at 58 ppm.) Selective RE-BURP[34] shaped pulses are indicated by β, γ2, and δ1 to denote the carbons of the Ile side chain that are inverted. Note that other pulses such as I-BURP2[34] could also be employed equally as well in place of these RE-BURP pulses. The pulses labeled "δ1" and "γ2" have durations of 3 ms (bandwidth of ±3 kHz, 800 MHz) and are centered at 12 and 16 ppm, respectively, while the pulse labeled "β" (1.88 ms, bandwidth of ±5 ppm, 800 MHz) is centered at 38.5 ppm. RE-BURP pulses labeled with an asterisk are centered at 38 ppm (300 μs, 800 MHz). The phases of these pulses are carefully adjusted by looking for a value which nulls the signal and then adding 45°. The vertical arrow at the end of delay τ_c indicates the position of application of the "β" Bloch−Siegert shift compensation pulse.[39] If the experiment is performed at a field other than 800 MHz, the duration of all selective pulses should be scaled by 800/*y* where *y* is the spectrometer field. The pulse labeled "δ1" is applied only on every second scan to ensure the selection of Ile-$^{13}C^{\delta1}$ magnetization, with concomitant inversion of the receiver phase. The phase cycling employed is as follows: $\phi1 = x$; $\phi2 = 2(x)$, $2(y)$; $\phi3 = 4(y)$, $4(-y)$; $\phi4 = 8(y)$, $8(-y)$; $\phi5 = 16(y)$, $16(-y)$; $\phi6 = 8(y)$, $8(-y)$; $\phi7 = 2(x)$, $2(-x)$; $\phi8 = x$; rec = $2(x,-x,-x,x)$, $2(-x,x,x,-x)$. Quadrature detection in F_1 is achieved with States-TPPI[40] of phase $\phi1$, while quadrature in F_2 employs the enhanced sensitivity pulsed field gradient method[41,42] where for each value of t_2 separate data sets are recorded with (g3, $\phi8$) and (−g3, $\phi8 + 180°$). For each successive t_2 value, $\phi6$ and the phase of the receiver are incremented by 180°. The delays used are as follows: $T_C = 7$ ms, $\tau_a = 5$ ms, $\tau_b = 2.5$ ms (note that the total duration from *e* to *f* is $4\tau_b$), $\tau_c = 10$ ms (this includes the duration of the "β" Bloch−Siegert pulse), $\tau_d = 7.3$ ms, $\Delta = 2.7$ ms minus the duration of the "β" pulse. $T_N = 12.4$ ms, $\tau_e = 2.2$ ms, $\epsilon = 250$ μs. The durations and strengths of the pulsed field gradients are as follows: G1 = (1.0 ms, 4 G/cm), G2 = (0.5 ms, 10 G/cm), G3 = (1.25 ms, 30.0 G/cm), G4 = (0.2 ms, 5 G/cm), G5 = (0.25 ms, 3.5 G/cm), G6 = (62.5 μs, 28.7 G/cm). All gradients are applied along the *z*-axis. (b) Pulse scheme with magnetization originating on protons. In this case, water magnetization is preserved and placed along the +*z*-axis at the end of the sequence. All the pulses and delays are the same as in scheme a except (i) $T' = T_C - \kappa$, $\kappa = 1.52$ ms, $\tau' = 1.8$ ms, (ii) the shaped pulse labeled "w" after the INEPT scheme in panel b is a rectangular water-selective pulse of a duration of ~1.5 ms, (iii) the duration and strength of the gradient G7 is (0.2 ms, 8 G/cm), (iv) $\phi1 = y$. The parenthesized purge pulses labeled "w" in scheme a (prior to gradient G2) are not employed to avoid water saturation. (c) Schematic diagram of the magnetization transfer steps employed in the (H)C(CA)NH-COSY experiment. Each transfer step is labeled with the relevant intervals in the pulse sequence. The expected distribution of chemical shifts for each of the carbons of the deuterated, δ1-methyl-protonated Ile side chain is shown.

The (H)C(CA)NH-COSY pulse sequence was designed primarily for high molecular weight proteins and therefore avoids the use of magnetization transfer through carbonyl spins. Such transfer steps

$$^{13}C^{\alpha}_{(i)} \xrightarrow{J_{C\alpha CO}} {}^{13}CO_{(i)} \xrightarrow{J_{NCO}} {}^{15}N_{(i+1)}$$

would place transverse magnetization on the ^{13}CO spin for a period of $\sim 1/(2J_{NCO})$ which is significant relative to the transverse relaxation time of the carbonyl spin in the case of a slowly tumbling protein in a high magnetic field. Although an (H)C(CACO)NH-COSY experiment recorded at lower fields may prove to be a more sensitive alternative for methyl

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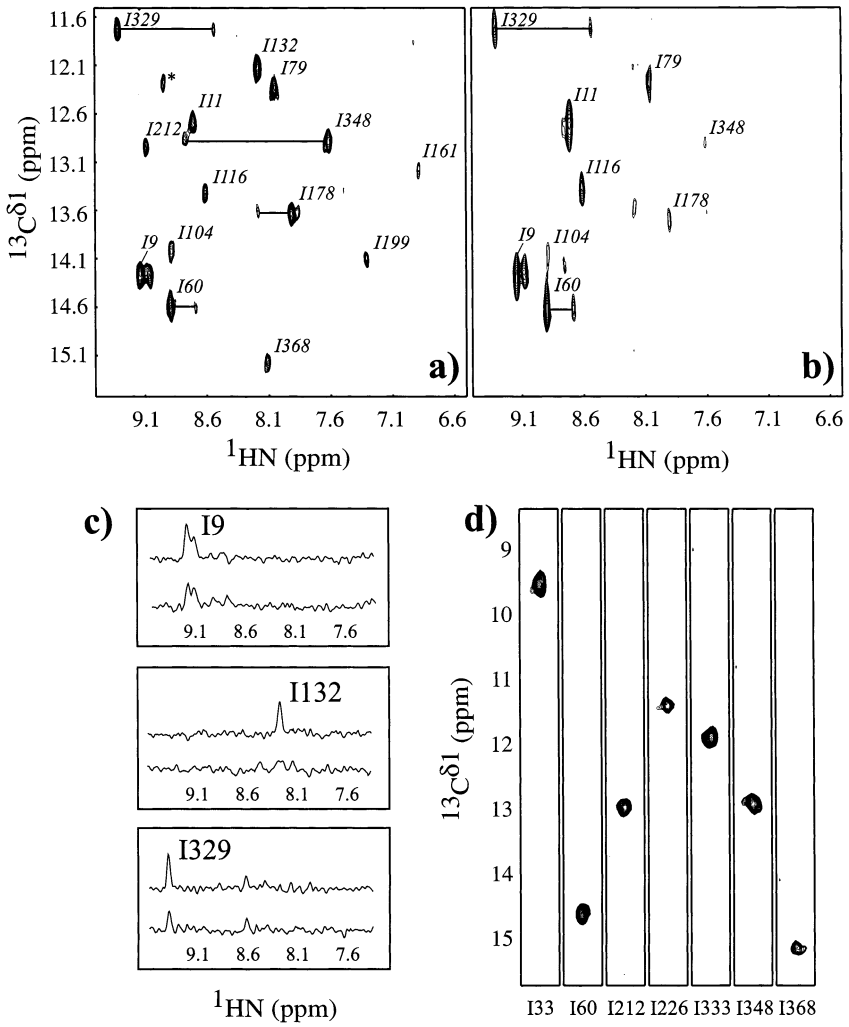

Figure 3. Sensitivity and resolution in the (H)C(CA)NH-COSY pulse scheme. Selected regions of 2D $^{13}C^{\delta 1}-^{1}HN$ planes from (a) (H)C(CA)NH-COSY and (b) (H)C(CA)NH-TOCSY data sets acquired on an [^{15}N,^{13}C,^{2}H]-(^{1}H-methyl)-MBP/β-cyclodextrin sample at 5 °C, 800 MHz, as described in Materials and Methods are shown. The intraresidue correlations are labeled with residue numbers and are connected to interresidue cross-peaks [$^{13}C^{\delta 1}_{(i)}-^{1}HN_{(i+1)}$] via horizontal lines. The peak marked with an asterisk in part a could not be identified and possibly arises due to residual amounts of apo-MBP. (c) Selected traces from the ^{1}HN dimension of 2D $^{13}C^{\delta 1}-^{1}HN$ planes of (H)C(CA)NH-COSY (upper rows) and (H)C(CA)NH-TOCSY (lower rows). Peak assignments are indicated. (d) Selected strips from the 3D (H)C(CA)NH-COSY spectrum of the [^{15}N,^{13}C,^{2}H]-(^{1}H-methyl)-MBP/β-cyclodextrin sample at 5 °C. Strips are labeled according to the identity of the methyl-containing residue.

assignments,[17,27] the resolution afforded by high magnetic fields is likely to be critical for applications to large single polypeptide chain proteins. The (H)C(CA)NH-COSY version is, therefore, the one presented here. Obviously, the relative merits of COSY- versus TOCSY-magnetization transfer schemes, described previously, are equally applicable to experiments which employ

$$^{13}C^{\alpha}_{(i)} \xrightarrow{J_{NC\alpha}} {}^{15}N_{(i)} \text{ or } {}^{13}C^{\alpha}_{(i)} \xrightarrow{J_{C\alpha CO}} {}^{13}CO_{(i)} \xrightarrow{J_{NCO}} {}^{15}N_{(i+1)}$$

transfer schemes.

The (H)C(CA)NH-COSY experiment was applied to an MBP/β-cyclodextrin complex at 5 °C for which backbone resonance assignments were obtained previously.[7] The correlation time of the complex (46 ± 2 ns)[7] is somewhat larger than that measured for MSG (37 ± 1 ns),[9] and we have therefore used the MBP sample as a rigorous test of the utility of the experiment. Parts a and b of Figure 3 illustrate a selected region from 2D $^{13}C^{\delta 1}-^{1}HN$ (H)C(CA)NH-COSY and (H)C(CA)NH-TOCSY data sets, respectively, recorded with identical total acquisition times as described in Materials and Methods. Due to the constant-time nature of the carbon (t_1) evolution period in the (H)C(CA)NH-COSY sequence, the correlations are significantly better resolved in the ^{13}C dimension relative to the TOCSY data set. This is of special importance for applications to large monomeric systems.

Figure 3c shows traces taken from the ^{1}HN dimension of 2D COSY and TOCSY $^{13}C^{\delta 1}-^{1}HN$ planes for selected residues from MBP. A significant improvement in signal-to-noise is observed in the (H)C(CA)NH-COSY data set compared to the (H)C(CA)NH-TOCSY data set. For example, out of the expected 21 Ile $^{13}C^{\delta 1}$ resonances, 16 could be detected in the 2D plane of (H)C(CA)NH-COSY as opposed to only 10 in the (H)C-(CA)NH-TOCSY experiment (cf. Figure 3a and b). Based on signal-to-noise ratios of eight common and reasonably intense peaks in the two spectra, the (H)C(CA)NH-COSY scheme is a factor of 1.5 times more sensitive. Of interest, the scheme of Figure 2a was found to be slightly more sensitive than that of Figure 2b for MBP at 5 °C (but not at 32 °C) in part due to relaxation losses associated with the first transfer step in the case where magnetization originates on protons. In a very recent application of the methodology to a sample of malate synthase

G at 37 °C, we have found that sensitivity is improved if magnetization originates on protons (Figure 2b); the choice of which sequence to use depends on the relaxation properties of the methyl groups in the protein, and we recommend comparing 2D planes using both approaches prior to recording the 3D data set. As a final note, we were able to assign 18 Ile-$^{13}C^{\delta 1}$ chemical shifts from the 3D (H)C(CA)NH-COSY data set. Figure 3d presents selected strips from the 3D (H)C(CA)NH-COSY data further demonstrating the excellent sensitivity obtained for a protein tumbling with a correlation time of 46 ns. Of note, two (Ile108, Ile235) of the three Ile that could not be assigned (Ile59, Ile108, and Ile235) were also not detected in previous backbone assignment studies of MBP loaded with β-cyclodextrin at 5 °C.[7] Interestingly, Ile235 lies in a stretch of residues extending from Pro229−Lys239 for which backbone shift assignments could not be obtained even at 37 °C, while Ile59 and 108 are close to the ligand binding interface in the protein.

In summary, we have described a sensitive 3D NMR experiment for the assignment of Ile δ1-methyl groups in deuterated and methyl-protonated protein samples. This experiment uses exclusively COSY-type transfer steps, providing a high degree of control over the magnetization flow, and thereby offering sensitivity advantages over TOCSY transfer schemes. It is anticipated that this experiment will be important for applications involving high molecular weight systems where TOCSY magnetization transfers for Ile residues are often inefficient.

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