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Comprehensive and Cost-Effective NMR Spectroscopy of Methyl Groups in Large Proteins

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Abstract: An NMR approach is described which yields the methyl resonance assignments of alanine, threonine, valine, leucine, and isoleucine residues in proteins with high sensitivity and excellent resolution. The method relies on protein samples produced by bacterial expression using [^{1}H,^{13}C]-D-glucose and ~100% D_2O, which is cost-effective and ensures the isotopic enrichment of all possible methyl groups. Magnetization transfer throughout the methyl-containing side chains is possible with this labeling scheme due to the high level of deuteration along the amino acid side chain, coupled with the selection of the favorable CHD_2 methyl isotopomer for detection. In an application to the 34 kDa periplasmic binding protein FepB 164 out of 195 methyl groups (85%) were assigned sequence-specifically and stereospecifically. This percentage increases to 91% when taking into account that not all backbone assignments are available for this system. The remaining unassigned methyl groups belong to six leucine residues, caused by low cross-peak intensities, and four alanine residues due to degeneracy of the $^{13}C^{\alpha}/^{13}C^{\beta}$ frequencies. Our results demonstrate that NMR spectroscopic investigations of protein structure, dynamics, and interactions can be extended to include all methyl-containing amino acids also for larger proteins.

Introduction

Over the past decade the potential of NMR spectroscopy to study the structure and dynamics of higher molecular weight protein systems has been greatly enhanced by taking advantage of NMR hardware developments (e.g., cryogenic probes) and combining advances in sample preparation and NMR methodology. In particular, methyl groups have been targeted since they often occur in the hydrophobic core of a protein and at the interface of complexes.[1] Hence, methyl groups are considered important reporters of structure[2,3] and dynamics[3−7] in proteins and are suitable to use in ligand-binding or protein−protein interaction studies.[8−10] Furthermore, methyl groups are particularly beneficial for NMR spectroscopic studies of high molecular weight systems, as methyl ^{1}H/^{13}C NMR lines are narrow due to rapid 3-fold jumps about the methyl symmetry axis[11] and the TROSY principle[12] can be employed.[13] Approaches using only deuteration or a combination of deuteration and methyl spectroscopy[14−16] have enabled NMR studies of protein structure and dynamics for several high molecular weight systems.[7,17−23]

Deuteration, in particular, has the potential to improve both the sensitivity and spectral resolution of NMR spectra. Although early applications focused on simplifying proton spectra through isotopic substitution,[24,25] the dilution of the highly polarized ^{1}H spins by deuteration greatly improved the relaxation proper-

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ties of the remaining proton nuclei in perdeuterated samples.[15,16,26] As a result, even for systems with higher molecular weight, perdeuterated samples give spectra with narrow lines. However, the concomitant reduction in the number of available NOE-based distance restraints severely impedes the determination of a high-resolution structure.[2] Therefore, when working with high molecular weight systems, one would prefer protein samples with specific protonation at some desired positions (e.g., methyl groups) while at the same time retaining high levels of deuteration at all other sites. In such a situation NMR spectra would demonstrate good sensitivity and resolution due to the high level of deuteration, while the spectroscopically interesting atoms (e.g., methyl groups) would be present in protonated form.

Different approaches to achieve this goal have been published, either by providing chemically synthesized amino acids or precursors or by making clever use of the amino acid biosynthetic pathways of *Escherichia coli*. An example of the first approach is the so-called *stereoarray isotope-labeling* (SAIL) strategy developed by Kainosho and co-workers,[27] in which specifically labeled amino acids are incorporated using a cell-free protein expression system. While the resulting protein molecules are ideal for studying them by NMR, this technique is not in general use, mainly due to the high costs or lack of availability of the required isotopically labeled amino acids. Another rather obvious biosynthetic approach is to employ random fractional deuteration, as explored by the groups of Laue,[28] LeMaster,[15] and Fesik.[29] It was shown that, depending on the protein size, a deuteration level between 50% and 75% presents a good compromise between spectral quality and the number of measurable distance restraints. However, a disadvantage of random fractional deuteration is that it leads to spectral deterioration due to isotope effects. Obtaining a more defined fractional labeling is possible by performing the bacterial growth in fully deuterated medium with a protonated carbon source (i.e., acetate,[30] pyruvate,[31,32] or glucose[31,33]). Protein samples prepared in such a way are highly deuterated at the $^{13}C^{\alpha}$ position and contain different isotopomer compositions for methylene and methyl groups. Although many labeling schemes based on random fractional deuteration have been proposed, the tailoring of NMR experiments to the obtained labeling has lagged behind. Finally, the approach pioneered by Kay and co-workers, in which suitably labeled compounds are added to the growth medium to obtain protonated methyl groups, has received the most attention. Selective methyl protonation strategies in an otherwise deuterated background have been described for Ile-$\delta1$[34,35] and [Val, Leu][35] using [^{2}H,^{13}C]-D-glucose and α-ketobutyrate or α-ketoisovalerate, respectively, as precursors and more recently for Met[36] and Ala.[37] A number of specifically labeled α-ketoacid precursors are commercially available and have been used for different applications, reviewed recently by Tugarinov and Kay.[38]

Despite the success of the above-mentioned labeling strategies, they suffer from one or a combination of the following drawbacks: (i) the labeled precursors are rather expensive, (ii) bacterial growth on carbon sources other than glucose proceeds more slowly and to smaller cell density, (iii) protein production levels are lower, and, most importantly, (iv) only a subset of methyl groups in the target protein is labeled. Ideally, one would prefer to apply a protocol using [^{1}H,^{13}C]-D-glucose as the precursor and grow in high levels of D_2O, because this is cost-effective and ensures enrichment of all available methyl groups.[31,33] In practice, such a sample may already be available since it is commonly used for obtaining the backbone resonance assignment. This procedure does have its drawbacks as it yields varying levels of ^{2}H enrichment throughout the amino acid side chains, giving rise to peak doubling or unresolved line broadening due to isotope shifts.[31,33] Fortunately, most positions in the side chains of methyl-containing amino acids become highly deuterated using this procedure while favorable isotopomer distributions are found for the methyl groups.[31,33]

We hereby propose to fully exploit the aforementioned strategy to label all methyl groups and present a novel experiment for their sequence-specific assignment, tailored to the given labeling pattern. In our approach, a 3D doubly sensitivity-enhanced C-TOCSY-CHD_2 experiment correlates the ^{13}C chemical shifts of the entire side chain with the ^{13}C/^{1}H frequencies of each methyl group. Because of the high deuteration level in the side chains, except for the methyl groups, the magnetization originates on ^{13}C and can be transferred efficiently by ^{13}C homonuclear mixing with little loss due to relaxation. The magnetization that has been transferred to the methyl group is subsequently frequency-labeled with the methyl ^{13}C chemical shift in a constant-time fashion, and only the CHD_2 isotopomer is detected to obtain a high-resolution spectrum.

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In an application to the 34 kDa bacterial periplasmic binding protein FepB[39] we were able to sequence-specifically and stereospecifically assign 110 out of the 121 methyl-containing amino acids (i.e., 91%) for which the backbone assignments were available. To mimic a larger system, the experiment was also performed at 5 °C, where the tumbling time of the protein is ~38 ns, and the sensitivity was found to be only about 30% lower. Although the use of specific precursors might be necessary for really large single-chain proteins, we anticipate that our approach is a viable alternative for many interesting protein systems. For single-chain proteins of ~30−40 kDa an assignment level of more than 80% is feasible, and oligomeric proteins, complexes, or solubilized membrane proteins with a correlation time of ~40 ns are also within reach.

Materials and Methods

Sample Preparation. Mature *fepB*[40] with the signal sequence removed was cloned into pET-19b (Novagen) to generate plasmid pFepB. *E. coli* BL21(DE3) was used to express recombinant FepB with an N-terminal 10xHis tag. For the induction of protein synthesis, cells were grown at 37 °C in M9 minimal medium with 100 μg/mL ampicillin to an $OD_{600} \approx 0.8$ and incubated for 3 h at the same temperature in the presence of 0.5 mM isopropyl β-D-thiogalactoside (IPTG). To purify FepB, cell pellets were resuspended in a buffer containing 20 mM Tris, pH 8.0, and 500 mM NaCl with 10 μg/mL DNase I and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) added. After French press (SLM-Aminco/Spectronic), the cell lysate was loaded onto a nickel Sepharose column (GE Healthcare) pre-equilibrated with 20 mM Tris, pH 8.0, and 500 mM NaCl. After extensive washing, FepB was eluted with 20 mM Tris, pH 8.0, 500 mM NaCl, and 200 mM imidazole, and homogeneity was confirmed by 15% SDS−PAGE.

A U-[^{13}C,^{15}N]-^{1}H/^{2}H sample of FepB was obtained using the general isotope-labeling strategy outlined by Tugarinov et al.[38] Briefly, pFepB−*E. coli* BL21(DE3) was grown in 1 L of D_2O M9 medium containing 3 g/L U-[^{1}H,^{13}C]-D-glucose (Cambridge Isotope Laboratories) as the sole carbon source and 1 g/L $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) as the nitrogen source. M9 medium initially contained 90 nM $FeCl_2$, and additional supplementation with $FeCl_2$ (300 nM final concentration) was required to suppress native enterobactin production. A second NMR sample for the stereospecific assignment of methyl groups was prepared using 10% U-[^{1}H,^{13}C]-D-glucose and 90% unlabeled glucose as described earlier by Neri,[41] but growth was performed in 100% D_2O.

A third sample, [U-^{13}C,^{15}N]-^{1}H/^{2}H-FepB loaded with Ga^{3+}−enterobactin, was prepared using iron-free enterobactin obtained from Professor G. Winkelmann (University of Tübingen). A 10 mM Ga^{3+}−enterobactin stock solution (1 mg of iron-free enterobactin dissolved in 10 mM Ga_3NO_4, 20 mM Tris, pH 8, and 33% v/v methanol) was prepared and titrated into the protein solution until the [^{1}H−^{15}N]-HSQC spectrum did not change anymore. Complete removal of the methanol by extensive dilution−concentration steps and freeze-drying of the sample was confirmed by 1D ^{1}H NMR (data not shown).

All NMR samples contained ~1 mM FepB and 50 mM sodium phosphate, pH 6.4 (93% H_2O and 7% D_2O).

NMR Spectroscopy. NMR experiments were performed at 25 °C on a Varian Unity Inova 600 MHz four-channel spectrometer, equipped with a triple-resonance room temperature probehead (^{1}H S/N on 0.1% ethylbenzene = ~600:1) with pulsed field gradient capabilities.

Conventional and CHD_2-detected 2D constant-time (CT) [^{1}H−^{13}C]-HSQC experiments were recorded with the constant time period set to 28 ms, recording 111 (^{13}C) × 512 (^{1}H) complex points, with maximum evolution times equal to 27.75 (^{13}C) × 64 (^{1}H) ms. An interscan delay of 1.0 s was used along with 128 or 32 scans per transient, giving rise to a net acquisition time of 8.5 and 2 h for the conventional and CHD_2-detected CT-[^{1}H−^{13}C]-HSQC experiment, respectively. The CHD_2-detected 2D CT-[^{1}H−^{13}C]-HSQC experiment of holo-FepB was recorded on a Bruker Avance 700 MHz spectrometer, equipped with a cryogenic probe.

Stereospecific assignments for Leu and Val residues were obtained from the CHD_2-detected CT-[^{1}H−^{13}C]-HSQC spectrum acquired on the 10% ^{13}C-labeled sample (measured in ~16 h) on the basis of the sign of the cross-peaks relative to the ε-methyl group of Met.

The 3D C-TOCSY-CHD_2 experiment was acquired with 75 (^{13}C) × 111 ($^{13}C^m$) × 512 ($^1H^m$) complex points, with maximum evolution times equal to 6.25 (^{13}C) × 27.75 ($^{13}C^m$) × 64 ($^1H^m$) ms. An interscan delay of 3.0 s was used with two scans per transient, giving rise to a net acquisition time of 58 h. Homonuclear carbon mixing was achieved using four cycles of the DIPSI-3 mixing scheme, with a total mixing time of 24.2 ms.

Deuterium T_1 and T_2 measurements on the CH_2D isotopomer were performed using the experimental schemes by Muhandiram et al.[42] T_1 values were measured from eight 2D spectra recorded with T delays of 0.1, 7.0, 15.0, 23.5, 32.9, 43.1, 54.5, and 70.0 ms. T_2 values were measured from nine 2D spectra recorded with T delays of 0.05, 2.0, 3.5, 6.5, 10.0, 14.5, 20.0, 25.0, and 29.0 ms using a 739 Hz spin-lock field. All data sets were recorded with 85 ($^{13}C^m$) × 768 ($^1H^m$) complex points, with maximum evolution times equal to 28.3 × 85.3 ms, and the experimental time was approximately 80 min for each 2D plane.

Carbon $T_{1\rho}$ measurements on the CHD_2 isotopomer were performed using the pulse scheme described by Brath et al.[6] T_1 relaxation times were determined from 10 data points, including two duplicates, covering $T = 0{-}1.4$ s. $T_{1\rho}$ relaxation times were measured using 10 points per decay curve, covering relaxation delays of $T = 0{-}240$ ms, including two duplicates ($T = 40$ ms and $T = 100$ ms). Six spin-lock field strengths were used, covering the range $\omega_1/2\pi = 699.1{-}3932.4$ Hz. The different spin-lock field strengths were combined with offsets ranging from 3.5 kHz upfield to 3.5 kHz downfield of the center of the methyl region, resulting in 21 data points per dispersion curve, including the T_1 data point. Each data set comprised 105 ($^{13}C^m$) × 512 ($^1H^m$) complex points, with maximum evolution times equal to 26.2 ($^{13}C^m$) × 64 ($^1H^m$) ms. The total acquisition time for each spectrum was approximately 25 min.

Pulse Sequence. The experimental scheme for the doubly sensitivity-enhanced C-TOCSY-CHD_2 experiment is shown in Figure 1. The experiment starts with (^{1}H−^{13}C)-NOE enhancement to increase the steady-state magnetization of carbon atoms that have protons attached. The proton pulse train is at the same time used for presaturation of the water signal, and the increased steady-state magnetization of the methyl groups is obtained for free. The carbon chemical shifts of the aliphatic carbons in the side chain are measured during a short (~6 ms) evolution period to minimize losses due to relaxation and carbon−carbon coupling evolution. Subsequently, ^{13}C−^{13}C homonuclear TOCSY mixing is performed using an isotropic DIPSI-3 sequence,[43] and therefore, sensitivity enhancement can be employed in the first indirect domain.[44] The magnetization is then frequency labeled in a constant-time manner during CT ≈ $1/J_{cc}$. In the period κ evolution under the ^{1}H−^{13}C coupling takes place, such that, just before the pulse with phase ϕ_3, the desired anti-phase magnetization is obtained for CHD_2

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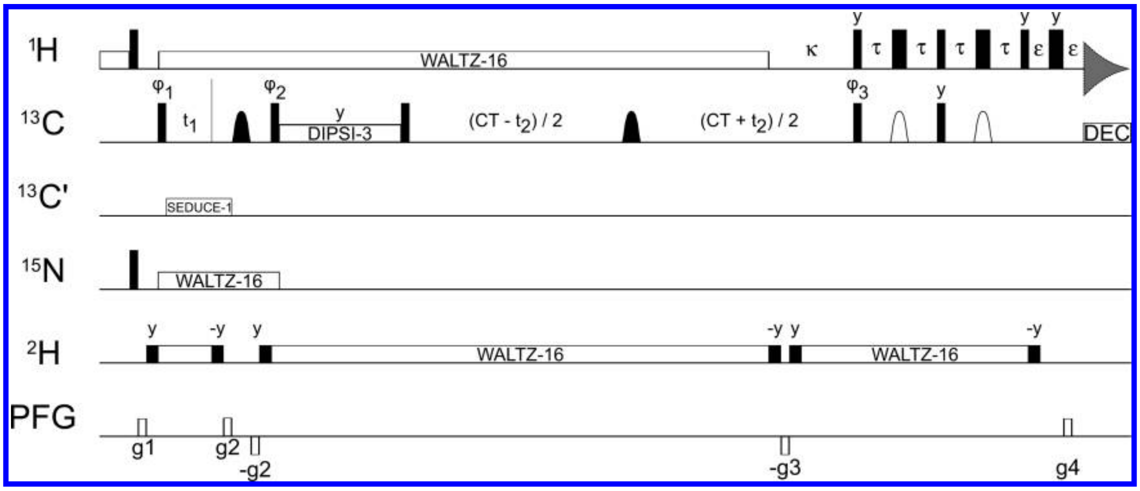

Figure 1. Pulse sequence for the C-TOCSY-CHD_2 experiment.

groups ($C_x \rightarrow C_yH_z$), whereas double anti-phase terms develop for CH_2D isotopomers ($C_x \rightarrow -C_xH_{1z}H_{2z}$). The remaining part of the sequence consists of a conventional gradient sensitivity enhancement detection scheme,[45] for which the signals originating from the CH_2D isotopomer do not lead to observable magnetization. Since gradient coherence selection is used in both indirect domains, the experiment can be performed with a phase cycle of only two scans per transient.

Narrow (wide) filled bars indicate 90° (180°) rf pulses applied along the x axis, unless otherwise indicated. The ^{1}H carrier is centered at the water resonance (4.75 ppm), and proton pulses are applied with a field strength of $\omega_1/2\pi = 36.8$ kHz. During the last second of the recycle delay a 120° pulse train ($\omega_1/2\pi = 27.6$ kHz, separated by 5 ms) on the proton channel was used to saturate the proton transitions. Alternatively, weak ($\omega_1/2\pi = 30$ Hz) continuous-wave (cw) irradiation can be used to saturate the solvent water resonance.

Rectangular ^{13}C pulses are centered at 40 ppm and applied with $\omega_1/2\pi = 21.4$ kHz. The first two shaped carbon pulses (filled domes) have a REBURP profile[46] applied on resonance, with a peak rf field strength of $\omega_1/2\pi = 19.0$ kHz and a pulse length of 350 μs, so as to cover all aliphatic carbon spins (bandwidth ~67 ppm). The two other shaped carbon pulses (open domes) have a REBURP profile ($\omega_1/2\pi = 6.6$ kHz, 1.0 ms duration) and are frequency shifted to 20 ppm and thereby refocus only the methyl resonances (bandwidth ~24 ppm). Carbon homonuclear TOCSY mixing is achieved using the DIPSI-3 scheme[43] applied along the y axis, with $\omega_1/2\pi = 9.0$ kHz. Decoupling during acquisition is done using GARP-1,[47] with $\omega_1/2\pi = 2.5$ kHz. Carbonyl decoupling is performed using an amplitude-modulated SEDUCE-1[48] decoupling scheme with $\omega_1/2\pi = 3.5$ kHz, applied at 176 ppm. Proton, nitrogen, and deuterium decoupling are achieved by WALTZ-16,[49] using $\omega_1/2\pi = 7.0$, 1.0, and 0.7 kHz and the carrier positions at 4.75, 117.0, and 4.75 ppm, respectively. Flanking 90° deuterium pulses are given at the same field strength and frequency. Values of the delays are $\tau = 1.98$ ms, CT = 28.0 ms, $\kappa = 3.96$ ms, $\varepsilon = 0.75$ ms, and $d_1 = 3.0$ s. The gradient strengths in G/cm (length in ms) are $g_1 = 5.0$ (1.0), $g_2 = 20.0$ (0.5), $g_3 = 25.0$ (0.5), and $g_4 = 16.3$ (0.5). All gradients are applied along the z axis. The phase cycling is $\phi_1 = [x, -x]$, $\phi_2 = [x]$, $\phi_3 = [y]$, and $\phi_{\mathrm{rec}} = [x, -x]$. Quadrature detection in F_1 and F_2 is obtained using the gradient sensitivity enhancement scheme:[45] the echo and antiecho signals are collected separately by inverting the sign of g_2 together with inversion of ϕ_2 and inverting the sign of g_3 together with inversion of ϕ_2 and ϕ_3, respectively. The pulse sequence and parameter file can be obtained from the authors upon request.

Data Processing and Analysis. The data sets were processed with the NMRPipe/NMRDraw software package[50] and analyzed using the program Sparky.[51] Briefly, mirror image linear prediction[52] was used to extend the $^{13}C^{m}$ domain of the 2D and 3D data sets, while forward−backward linear prediction[53] was employed to double the other indirect ^{13}C domain. A cosine-squared window function was employed in all domains.

Results and Discussion

Labeling Protocol and Residual Protonation. Using a labeling protocol based on [^{1}H,^{13}C]-D-glucose in ~100% D_2O,[33] we prepared an NMR sample of the periplasmic binding protein FepB. A nearly complete backbone assignment was obtained using TROSY variants of the standard backbone experiments[54] on this sample.

A CT-[^{1}H−^{13}C]-HSQC spectrum of FepB is shown in Figure 2 (left panel). It can be observed that for most amino acids CHD_2 presents the major isotopomer, followed by CH_2D, and that CH_3 isotopomers are hardly present. To assess the isotopomer incorporation and residual protonation along the side chain of methyl-containing amino acids in a more quantitative manner, we compared [^{1}H−^{13}C]-HSQC spectra for the small (8.5 kDa) protein calbindin D_{9k} using samples grown on H_2O and D_2O. To compensate for differential ^{13}C relaxation effects, the experiments were recorded with real-time carbon evolution and the signal intensities were normalized for differences in the sample concentrations. The average percentages mentioned in Table 1 were obtained by dividing the integral of the various isotopomer signals and their corresponding protonated peak.

Our results agree well with previous observations using the REDPRO procedure[33] and findings by Rosen et al.[31] The $^{13}C^{\alpha}$ position is highly deuterated (~95%) for all amino acids, and

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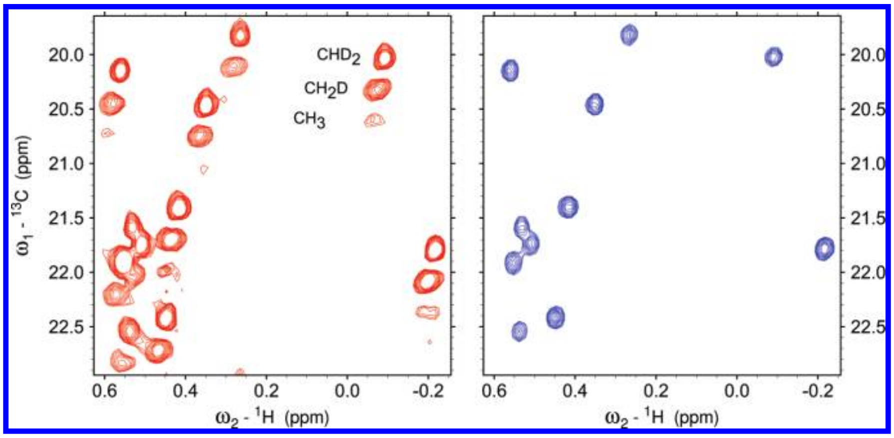

Figure 2. Part of the methyl region in the 2D CT-[^{1}H−^{13}C]-HSQC spectrum recorded with either the conventional (left) or CHD$_2$-detected (right) variant of the experiment.

Table 1. Isotopomer Incorporation and Residual Protonation for Protein Samples Grown on U-[^{1}H,^{13}C]-D-Glucose in ~100% D$_2$O

(A) Average Incorporation (%) of Isotopomers in Methyl Groups[a]

	Ala-β	Thr-γ2	Val-γ1	Val-γ2	Ile-δ1	Ile-γ2	Leu-δ1	Leu-δ2	Met-ε
CH$_3$	2	1	5	5	1	5	8	5	3
CH$_2$D	33	13	40	44	12	49	45	37	25
CHD$_2$	46	45	55	49	41	49	47	56	30
CD$_3$	19	41	0	2	46	~0	0	2	42

(B) Average Isotopomer Incorporation (%) in the Side Chain of Methyl-Containing Amino Acids[a,b]

	^{13}Cα		^{13}Cβ			^{13}Cγ		
Ala	CH: 6	CD: 94						
Thr	CH: 5	CD: 95	CH: 19	CD: 81				
Leu	CH: 5	CD: 95	CH$_2$: 0	CHD: 4	CD$_2$: 96	CH: 8	CD: 92	
Val	CH: 4	CD: 96	CH: 11	CD: 89				
Ile	CH: 5	CD: 95	CH: 8	CD: 92		CH$_2$: 0	CHD: 34	CD$_2$: 66
Met	CH: 8	CD: 92	CH$_2$: 5	CHD: 12	CD$_2$: 83	CH$_2$: 5	CHD: 41	CD$_2$: 54

[a] The uncertainty in the incorporation levels is on the order of 3%. [b] For methylene groups CHD represents the average of CHD and CDH.

also the ^{13}C$^\beta$ positions are very highly deuterated, although Thr and Val residues display detectable protonation (19% and 11%, respectively). Finally, the ^{13}C$^\gamma$ atom of Leu is highly deuterated (92%), whereas there is a significant fraction of CHD/CDH for Met and Ile at this position. The methyl groups show a clear pattern in the incorporation of ^{1}H/^{2}H: in most cases, the CHD$_2$ isotopomer represents the major isotopomer and CH$_3$ groups are hardly present. Furthermore, from Table 1 it is clear that for the Val, Ile-γ2, and Leu residues there is no significant fraction of CD$_3$, while the other methyl groups do show CD$_3$ incorporation ranging from 19% for Ala to 40−46% for Thr, Ile-δ1, and Met.

The ^{1}H/^{2}H incorporation levels in the methyl groups can be rationalized from the biosynthetic pathways of *E. coli*[55] as follows. Glucose is converted into two pyruvate molecules, and therefore, the CH$_3$ group in pyruvate will originate from either a CH or a CH$_2$ moiety of glucose. For amino acids synthesized directly from pyruvate (Val, Ala) or indirectly via precursors derived from pyruvate (Leu, Ile-γ2) it is, therefore, observed that the incorporation of CH$_2$D and CHD$_2$ isotopomers, originating from the CH or CH$_2$ moiety of glucose, respectively, are similar. The Thr-γ2, Ile-δ1, and Met-ε methyl groups display higher levels of deuteration, as they do not originate directly from pyruvate, but follow a different pathway in which the intermediates become more highly deuterated (Table 1).

NMR Spectroscopy and Assignments. Selective detection of an isotopomer with an odd/even number of protons can be easily achieved in an NMR experiment using a refocused INEPT transfer based on the difference in evolution under the J_{CH} scalar coupling. We therefore developed a CT-[^{1}H−^{13}C]-HSQC pulse sequence (see the Supporting Information) to detect only the CHD$_2$ isotopomer based on the conventional CT-[^{1}H−^{13}C]-HSQC scheme.[56] The part for the selection of the CHD$_2$ isotopomer was taken from Brath et al.,[6] and further improvements include the addition of continuous proton decoupling during the CT period[57] and detection using a gradient sensitivity enhancement scheme.[45]

Due to the difference in the relaxation properties of the individual lines of the carbon quartet of a CH$_3$ moiety,[11,58] it is difficult to suppress CH$_3$ signals completely[59] if one wants to detect the CHD$_2$ isotopomer. However, with protonated glucose as the precursor and bacterial cultures grown in ~100% D$_2$O, the CH$_3$ incorporation is extremely low, and therefore, a clean CHD$_2$ detection can readily be achieved (Figure 2, right panel). Finally, in the currently prepared sample the CHD$_2$ isotopomer exhibits the smallest proton line width, which, together with the high incorporation and convenient selection, makes it the most suitable NMR probe for assignment purposes.

From the high-resolution CHD$_2$-detected CT-[^{1}H−^{13}C]-HSQC spectrum (Figure 3) it is clear that the proposed isotope-labeling scheme ensures the enrichment of methyl groups for all methyl-containing amino acids. Moreover, excellent spectra in terms of resolution and signal-to-noise ratio were obtained. The fact that a good-quality spectrum can be recorded for all methyl groups is promising in itself, but to obtain structural or dynamical information of a protein system the residue-specific assignment of these cross-peaks is crucial. NMR assignments of methyl groups can be obtained from (TROSY[12]) experiments based on Hartmann−Hahn polarization transfer (TOCSY)

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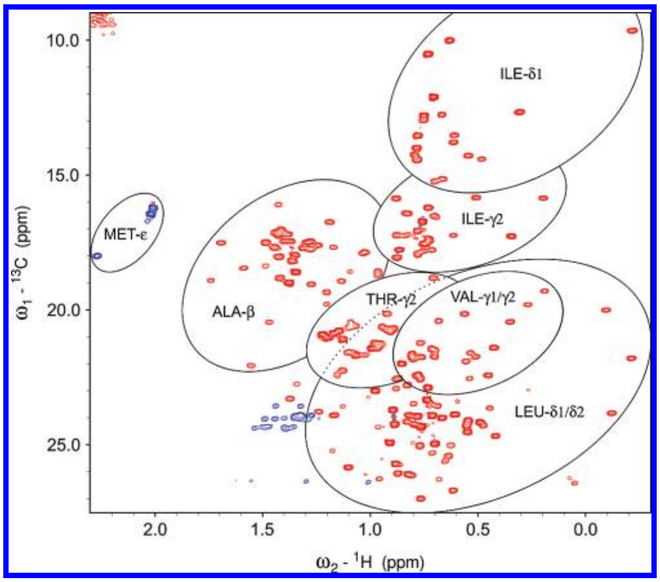

Figure 3. CHD_2-detected CT-[^{1}H−^{13}C]-HSQC spectrum of FepB containing cross-peaks for all the methyl-containing amino acids. Areas indicate where most assignments are found for the different amino acids. Negative (blue) signals between 1.2 and 1.5 ppm originate from CHD methylene groups.

schemes detected on the backbone NH.[60] The sensitivity of these experiments is, however, rather low in the case of larger systems due to the long transfer times from ^{13}C to ^{15}N, and attention has shifted more toward COSY-type experiments in combination with specific labeling of I(δ1)LV[61−63] or AI(γ2).[64] As an alternative, Yang and co-workers devised a strategy for the assignment of side chains and protein structure determination of large proteins without deuteration.[65] A key component in this procedure is a multiple-quantum version of the (H)CCH-TOCSY experiment[66] as an improvement of existing CH-detected experiments[57,67] to assign the side chain resonances. Although their ingenious approach can produce very complete methyl group assignments for large proteins, this procedure is quite a lot more time-consuming and labor-intensive.

Our approach is different from the above strategies in that we try to obtain all methyl assignments from one protein sample using a single experiment based on prior backbone assignments. Figure 1 illustrates the 3D doubly sensitivity-enhanced C-TOCSY-CHD_2 experiment that has been developed to correlate the ^{13}C chemical shifts of the entire side chain with the ^{1}H/^{13}C frequencies of each methyl group. The magnetization transfer of the experiment is schematically as follows:

$$^{13}\mathrm{C}\ (t_1) \xrightarrow{\text{TOCSY, SE}} {}^{13}\mathrm{C}^{\mathrm{m}}\ (\mathrm{CT},\ t_2) \xrightarrow{\text{SE scheme}} {}^{1}\mathrm{H}^{\mathrm{m}}\ (t_3)$$

In our experiment the magnetization originates from all side chain carbon nuclei rather than methyl protons. This may seem to produce an insensitive experiment, but avoids nonproductive magnetization transfer at the branching point of Ile, Leu, and Val residues, which otherwise reduces the sensitivity of COSY- or TOCSY-based experiments that start on methyl protons.[62,63]

The C-TOCSY-CHD_2 experiment was applied to the 34 kDa protein FepB, which has an overall rotational correlation time of 19 ns at 25 °C (as determined from ^{15}N relaxation data). The quality of the experimental data can be appreciated from Figure 4, where representative strip plots are shown for various methyl-containing amino acids. The spectral pattern (number of cross-peaks and frequencies) in a strip immediately reveals the amino acid type. In the case of Ile, Leu, and Val two strips can be observed, originating from each of the methyl groups, with the same spectral pattern. If the ^{13}C$^\alpha$ or ^{13}C$^\beta$ signal is weak in one of the strips, it might be more intense in the other, so that combining the information from both strips will give a more complete assignment of the side chain. Provided that the ^{13}C$^\alpha$ and ^{13}C$^\beta$ frequencies of the methyl-containing amino acids are (i) assigned from the backbone experiments and (ii) not collectively degenerate within a residue type, the residue-specific assignment of the methyl resonances can be obtained. For FepB we thus established the unique identity of 164 methyl groups out of 195 present in the protein. The stereospecific assignments of the *pro-R*- and *pro-S*-methyl groups for Leu and Val were subsequently obtained from a CHD_2-detected CT-[^{1}H−^{13}C]-HSQC spectrum (see Figure 5), recorded on a sample prepared using 10% U-[^{1}H,^{13}C]-D-glucose and 90% unlabeled glucose as precursors.[41]

Thus, a highly complete sequence-specific and stereospecific assignment was obtained for the methyl groups in FepB. This result is summarized in Table 2, and a complete table with side chain ^{13}C and ^{1}H assignments for the methyl-containing amino acids Ala, Thr, Val, Ile, and Leu of FepB can be found in the Supporting Information. It should be noted that the numbers of assigned methyl groups in row 4 of Table 2 are lower limits because not all methyl-containing amino acids were observed in the backbone experiments. When taking this into account (Table 2, row 5), the assignment percentages increase significantly. For example, in the case of Ile, all signals are observed in our TOCSY experiment and the methyl group assignments will be 100% complete once these residues can be assigned from the backbone experiments. For both Val and Thr, one residue could not be observed in the backbone experiments. The missing Val and Thr residues could be assigned from the remaining correlations in the methyl TOCSY experiment after assignment of the other methyl groups. For four Ala residues the ^{13}C$^\alpha$/^{13}C$^\beta$ combinations are degenerate, thereby precluding the residue-specific assignment of the methyl groups. This degeneracy may be lifted by recording additional experiments (see below).

Although almost all of the Leu residues show cross-peaks in a CHD_2-detected CT-[^{1}H−^{13}C]-HSQC spectrum, we found that some of the methyl groups were difficult or impossible to assign due to low sensitivity or lack of ^{13}C$^\alpha$/^{13}C$^\beta$ cross-peaks. This is reflected in the lowest assignment percentage obtained for this residue type (Table 2). Part of the explanation lies in the fact that, for Leu residues, the difference between the resonance frequencies of the ^{13}C$^\delta$ and ^{13}C$^\gamma$ spins is comparable to the *J* coupling (strong coupling regime), which will partly convert

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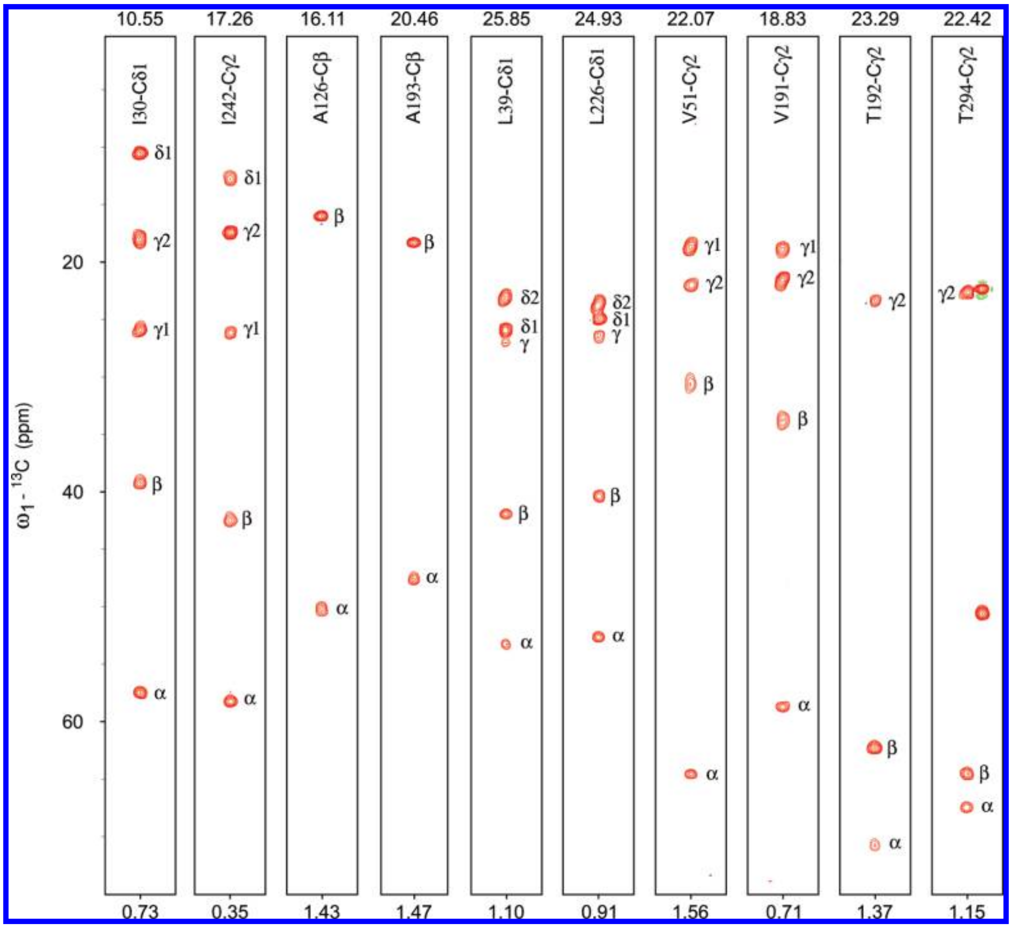

Figure 4. Representative strips from the 3D C-TOCSY-CHD$_2$ spectrum for Ile, Val, Leu, Val, and Thr residues. In each F_1(^{13}C)−F_3(^{1}H^m) slice the assignment of the methyl-containing residue is shown. The frequencies (ppm) for F_2(^{13}C^m) and F_3(^{1}H^m) are shown at the top and bottom of each slice, respectively.

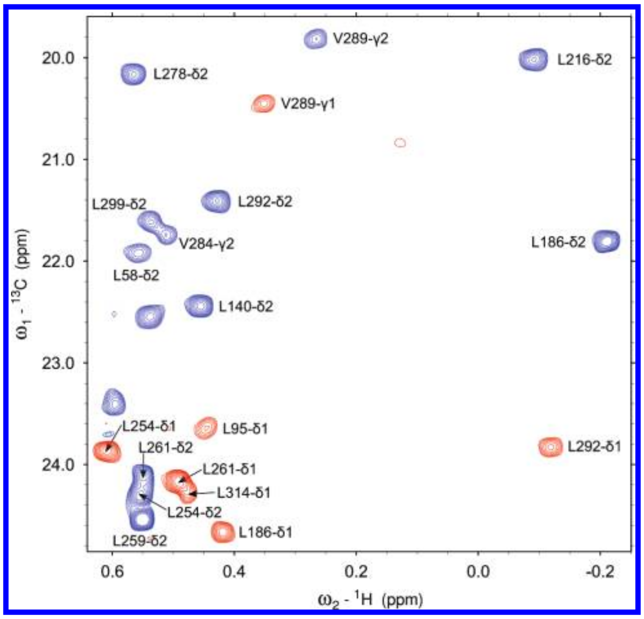

Figure 5. Region of the CHD$_2$-detected CT-[^{1}H−^{13}C]-HSQC spectrum containing Leu and Val cross-peaks showing some of the stereospecific assignments. *pro-R*-methyl groups (Leu-δ2 and Val-γ2) are shown in blue and have the same sign as the ε-methyl group of Met.

Table 2. Overview of the Assignments for Methyl Groups in the 34 kDa Protein FepB[a]

	Ala	Thr	Leu	Val	Ile	Met	total
occurrence	41	19	37	11	18	3	129
C$^\alpha$/C$^\beta$ assigned in backbone	41	18	34	10	15	3	121
C^m assigned in TOCSY	37	19	28[b, c]	11[b, c]	15[b]	–[d]	110
assigned percentage	90	100	76	100	83	–	85
assigned percentage[e]	90	100	82	100	100	–	91

[a] Assignments were obtained from data recorded at 25 °C using a 600 MHz spectrometer on a sample grown on ^{15}NH$_4$Cl and U-[^{1}H,^{13}C]-D-glucose in ∼100% D$_2$O. [b] Both methyl groups are assigned. [c] Methyl groups are stereospecifically assigned. [d] For the ε-methyl group the diagonal peak is observed, but no cross-peaks. [e] Percentage of possible assignments (also assigned in backbone experiments).

the initial magnetization into unobservable terms during the CT period, thereby decreasing the signal intensity. Recording the experiment at higher $\mathbf{B}_0$ field and/or the use of a cryogenic probe might alleviate this problem by increasing the overall sensitivity as well as slightly lifting the strong coupling regime.

Degeneracy in the ^{13}C$^\alpha$/^{13}C$^\beta$ frequencies will become a limiting factor for the number of assignments possible from TOCSY-based experiments for larger single-chain proteins (>400 residues), in particular for Ala and Leu, as was shown by Yang et al.[66] On the basis of the statistics they presented, we calculated how many methyl groups can be assigned using glucose labeling in D$_2$O, and this analysis can be found in the Supporting Information. In short, assignment levels of more than 80% of the methyl groups are expected for proteins of 30−40 kDa, whereas about 60% of the methyl groups have a unique combination of ^{13}C$^\alpha$/^{13}C$^\beta$ frequencies in an 80 kDa protein. Of note, ILV-labeling generates proteins in which about 60% of the methyl groups are isotopically enriched. The additional

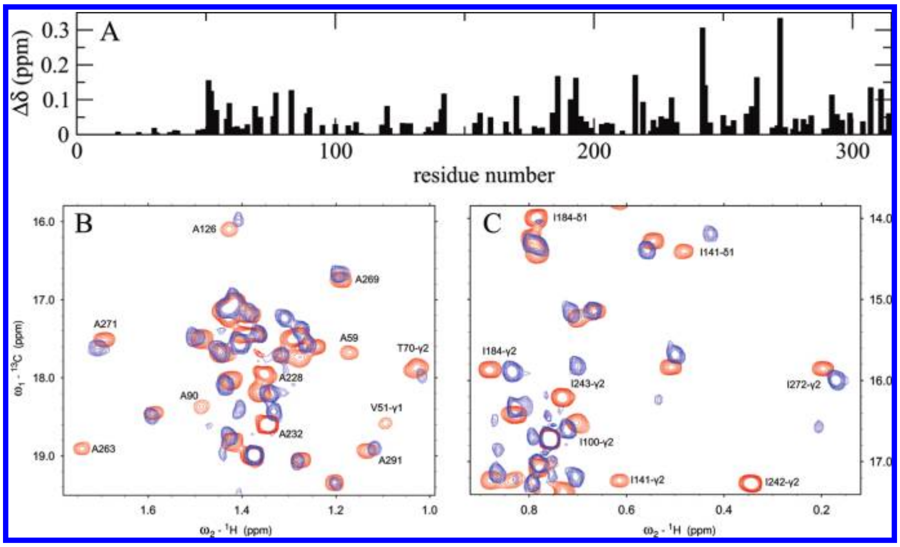

Figure 6. Chemical shift mapping of the ligand binding site of *E. coli* FepB. Panel A shows the chemical shift changes (minimal distance approach) upon ligand binding, and panels B and C show insets of the CHD_2-detected CT-[$^1H-^{13}C$]-HSQC spectra of apo-FepB (red) and holo-FepB (blue).

incorporation of Ile-$\gamma 2$, Thr, and Ala methyl groups using protonated glucose compensates for the lower assignment success for large proteins. A larger number of assignments may still be possible, however, by correlating the methyl and $^{13}C'$ frequencies as well.[68] In that case, additional experiments will need to be performed, since measurement of the $^{13}C'$ chemical shift is not included in our TOCSY experiment. To test whether our approach is applicable to higher molecular weight systems, we repeated the experiment at 5 °C to mimic a system with a correlation time of ~40 ns, and under these conditions the sensitivity decreased by only ~30%. This suggests that the sensitivity of the experiment is adequate for larger proteins as well, but that the completeness of the assignment will depend on the complexity of the system.

Applications Using the Assignment of the Methyl Groups. Sequence-specific assignments are a prerequisite for protein NMR spectroscopy. Having established the identity of most methyl ^{13}C and ^{1}H resonance frequencies, several experiments can be performed. We include here two applications to the protein FepB, a periplasmic binding protein involved in the ferric iron uptake of Gram-negative bacteria.[69] We investigated the effect of siderophore binding to the apoprotein by methyl group chemical shift mapping, recording a high-resolution CHD_2-detected CT-[$^1H-^{13}C$]-HSQC spectrum in the absence and presence of ligand (Figure 6).

The chemical shift difference for every methyl group was calculated according to the formula $\Delta\delta$ (ppm) = $[\Delta\delta_H{}^2 + (\Delta\delta_C/R_{scale})^2]^{1/2}$, where $R_{scale} = 5.4$ was determined according to Mulder et al.[70] Since methyl group assignments in the ligand-bound form of the protein are not yet available, we have used a "minimal distance approach", where peaks for holo-FepB were provisionally assigned the identity of the nearest correlation for apo-FepB. From Figure 6 it is clear that the binding of enterobactin significantly affects FepB. This conclusion is supported by extensive backbone amide chemical shift changes observed in the [$^1H-^{15}N$]-TROSY-HSQC spectrum (data not shown). The first ~50 residues seem to be unaffected by ligand binding, and whereas the region 50−90 shows the strongest perturbation for the N-terminal domain, most changes occur in the C-terminal part of the protein. A detailed analysis of the ligand-binding interface and structural differences between apo- and holo-FepB is currently pursued in our laboratories.

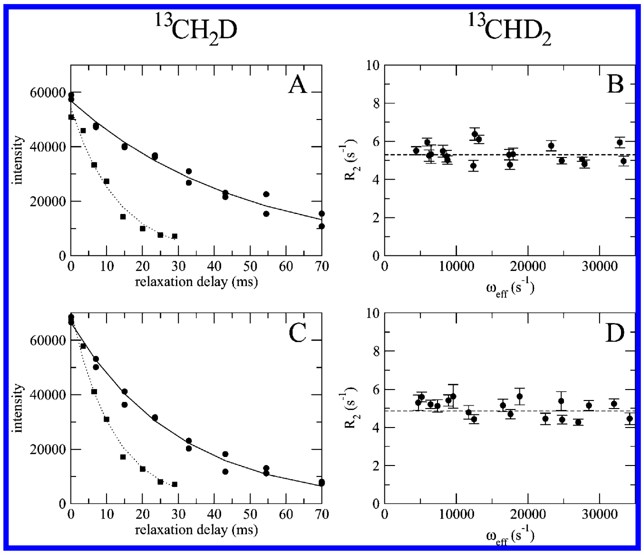

Figure 7. Representative curves for deuterium (circles, T_1; squares, T_2) and carbon ($T_{1\rho}$) relaxation of methyl groups A232-C^β (A, B) and I242-$C^{\gamma 2}$ (C, D) of *E. coli* apo-FepB.

As a second example, we describe the application of nuclear spin relaxation experiments to study methyl group dynamics. Samples produced on protonated U-[^1H,^{13}C]-D-glucose in ~100% D_2O are ideally suited for this purpose, since CHD_2 and CH_2D isotopomers are present in significant proportions within the same sample (see Table 1). For example, carbon relaxation studies are preferably performed on the CHD_2 isotopomer,[6,59] while the CH_2D isotopomer is favorable for measuring ^2H relaxation.[42] The results of deuterium and carbon relaxation experiments for FepB are illustrated in Figure 7. In panels A and C the fast (picosecond to nanosecond) side chain dynamics are characterized by deuterium relaxation on CH_2D, for A232-C^β and I242-$C^{\gamma 2}$, respectively. The relaxation param-

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eters can be extracted by fitting a monoexponential decay curve to the data points, and the T_1 and T_2 values were determined to be 47.9 and 13.1 ms (A232-C$^{\beta}$) and 30.0 and 11.9 ms (I242-C$^{\gamma 2}$). Furthermore, the presence of slow side chain dynamics for the same residues was assessed by ^{13}C $T_{1\rho}$ relaxation, measured on the CHD$_2$ isotopomer, as shown in panels B and D. The flat dispersion profiles, R_2 versus ω_{eff}, indicate that no dynamical processes can be detected for these residues on the microsecond to millisecond time scale. Although the above examples substantiate the usefulness of these samples and the obtained assignments, a detailed analysis of the methyl group dynamics in this biological system is outside the scope of this paper, especially since no three-dimensional structure is currently available.

Finally, it is worth noting that $^3J_{\text{CC}}$ coupling constants[71] and methyl RDCs[72] can be recorded on partially deuterated samples to obtain angular restraints for the side chains in large molecular weight systems, where other structural information is scarce or absent.

In summary, we have described how a suitable isotope-labeling strategy, together with a new 3D doubly sensitivity-enhanced C-TOCSY-CHD$_2$ experiment, can be used to obtain highly complete assignments for methyl-containing amino acids in large proteins. Our results show that very nearly complete methyl group assignments can be obtained for single-chain proteins consisting of ~300 residues. It is anticipated that the proposed methodology can be extended to bigger systems to facilitate cost-effective and comprehensive studies of protein structure, dynamics, and interactions for many large, single-chain proteins, oligomeric proteins, protein complexes, and solubilized membrane proteins.

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Supporting Information Available: One figure showing the pulse scheme for the CHD$_2$-detected CT-[^{1}H$-$^{13}C]-HSQC, one table listing the assignments of the methyl-containing amino acids for apo-FepB, and a paragraph with the analysis of assignment ambiguity for large proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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