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¹³C-detected HN(CA)C and HMCMC experiments using a single methyl-reprotonated sample for unambiguous methyl resonance assignment

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Abstract

Methyl groups provide an important source of structural and dynamic information in NMR studies of proteins and their complexes. For this purpose sequence-specific assignments of methyl ¹H and ¹³C resonances are required. In this paper we propose the use of ¹³C-detected 3D HN(CA)C and HMCMC experiments for assignment of methyl ¹H and ¹³C resonances using a single selectively methyl protonated, perdeuterated and ¹³C/¹⁵N-labeled sample. The high resolution afforded in the ¹³C directly-detected dimension allows one to rapidly and unambiguously establish correlations between backbone H_N strips from the 3D HN(CA)C spectrum and methyl group H_mC_m strips from the HMCMC spectrum by aligning all possible side-chain carbon chemical shifts and their multiplet splitting patterns. The applicability of these experiments for the assignment of methyl ¹H and ¹³C resonances is demonstrated using the 18.6 kDa B domain of the *Escherichia coli* mannose transporter (IIB^{Mannose}).

Introduction

Resonance assignments are central to NMR studies of protein structure and dynamics (Wuthrich, 1986; Clore and Gronenborn, 1991, 1998; Mittermaier and Kay, 2006). Methyl groups are important reporters of dynamics in relaxation studies (Kay, 2005) and NOEs involving methyl groups yield invaluable information for structure determination of proteins (Kay, 2005) and their complexes (Williams et al., 2005; Suh et al., 2006). Sequence-specific assignments of methyl ¹H and ¹³C resonances are therefore an essential

prerequisite for many NMR studies. Previously, 3D HCCH-COSY (Bax et al., 1990a; Kay et al., 1990) and HCCH-TOCSY (Bax et al., 1990b; Fesik et al., 1990; Olejniczak et al., 1992) experiments via isotropic mixing of ¹³C magnetization have been widely used for side-chain ¹H and ¹³C resonance assignment in protonated proteins. Conventional ¹H detected COSY-based NMR experiments have also been developed for the assignment of methyl ¹H and ¹³C chemical shifts of Ile, Leu and Val residues in a large perdeuterated protein, malate synthase G, in which the Ile, Leu and Val residues were ¹³CH₃-labeled at only a single methyl position, with the other methyl group ¹²CD₃-labeled (Tugarinov et al., 2003). This special methyl group labeling scheme is required to “linearize” the spin systems for efficient coherence transfer. In the

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experiments described by Tugarinov et al. (2003), selective pulses are repeatedly applied and their phases have to be adjusted. The “out-and-back” approach also requires many INEPT elements, leading to additional signal loss. A constant-time MQ-(H)CC_mH_m-TOCSY experiment has been proposed for sequence-specific assignments of methyl groups using a large uniformly ¹³C-labeled protein (Yang et al., 2004). However, for some Leu residues with strong scalar coupling between ¹³C^{δ1}/¹³C^{δ2} and ¹³C^γ, this experiment is less sensitive due to incomplete refocusing of the ¹³C magnetization during the constant time period.

The first 2D ¹³C-detected spectrum for protein sequence-specific assignment was reported by Markley and coworkers (Oh et al., 1988). Recently, ¹³C-detected NMR spectroscopy has been proposed as an attractive alternative for studying large macromolecules (Bermel et al., 2003, 2006; Eletsky et al., 2003; Hu et al., 2003, 2005; Bertini et al., 2004; Kovacs et al., 2005). In this paper, we propose the use of ¹³C-detected 3D HN(CA)C and HMCMC TOCSY-type experiments for assignment of methyl ¹H and ¹³C resonances using a single methyl protonated, highly deuterated, ¹³C/¹⁵N-labeled protein sample. These ¹³C-detected spectra offer high resolution and the observation of all side-chain carbon chemical shifts in the direct ¹³C dimension. Correlation between methyl groups and their backbone H_N groups is established via alignment of all observed side-chain carbon chemical shifts and their multiplet splitting pattern in the ¹H_N-¹³C strips from the 3D HN(CA)C spectrum and in the ¹H_m-¹³C strips from the 3D HMCMC spectrum (Figure 1), which affords unambiguous assignment of methyl resonances. These ¹H-start, ¹³C-detect experiments make use of magnetization originating from ¹H spins (H_N of amide groups and H_m of methyl groups) and can therefore be expected to have higher sensitivity compared to 2D ¹³C-start, ¹³C-observe NMR experiments (Eletsky et al., 2003). Using methyl re-protonated, perdeuterated protein samples offers three times the initial magnetization while relaxation of deuterated carbons is minimized. The experiments are demonstrated using the 18.6 kDa ¹⁵N/¹³C/²H/[Leu,Val]-methyl-protonated B domain (IIB^{Man}) of the *Escherichia coli* mannose transporter. The ¹³C-detected “out-and-stay” 3D HN(CA)C and HMCMC experiments proposed here offer a general route for the

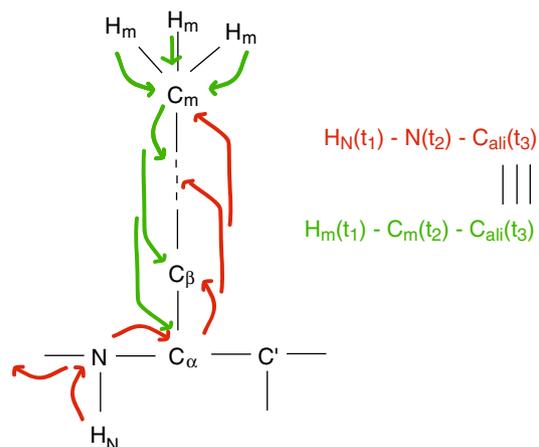


Figure 1. Schematic representation of the coherence transfer pathways observed in the 3D ¹³C-directly detected HMCMC-TOCSY (green) and HN(CA)C-TOCSY (red) experiments. Correlation between methyl groups and their backbone H_N groups is established via alignment of all side-chain carbon chemical shifts and their multiplet splitting patterns.

unambiguous assignment of methyl ¹³C and ¹H chemical shifts of high molecular weight ¹⁵N/¹³C/²H-labeled, methyl selectively protonated proteins.

Material and methods

NMR sample preparation

The B domain (IIB^{Man}) of the *E. coli* mannose transporter, residues 156–322, was cloned into a modified pET-32a vector (Legler et al., 2004) to form a thioredoxin fusion protein with a His₆-tag. After transformation, *E. coli* strain BL21(DE3) (Novagen) was cultured in Luria Bertini medium, then adapted and grown in minimal medium in ²H₂O using 1 g/l of ¹⁵NH₄Cl and 2 g/l of ²H₇/¹³C₆-glucose (Cambridge Isotope Laboratories) as the sole nitrogen and carbon sources, respectively. One hour prior to induction (at A₆₀₀ ~ 0.6–0.7) with 1 mM isopropyl-D-thiogalactopyranoside, 50 mg 3-²H, ¹³C₅-α-ketoisovaleric acid (Cambridge Isotope Laboratories) was added as precursor for Leu and Val re-protonation at their methyl groups. The cells were grown overnight and harvested. The cell pellet was resuspended in 150 ml (per liter of culture) of 20 mM Tris, pH 8.0, 150 mM NaCl. The suspension was lysed using a microfluidizer and centrifuged at 10,000 × g for

20 min. The supernatant was loaded onto a nickel-Sephacryl column (Amersham Biosciences), and the fusion protein was eluted with a 150 ml gradient of imidazole (25–500 mM). The fusion protein was then digested with thrombin (100 units) and dialyzed in 20 mM Tris, pH 8.0, 200 mM NaCl. After addition of 1 mM phenylmethylsulfonyl fluoride, the cleaved His₆-thioredoxin was removed by loading the digested proteins over a nickel-Sephacryl column. The IIB^{Man} fractions were collected and further purified by Sephadex-75 gel filtration column (Amersham Biosciences) equilibrated with 20 mM Tris, pH 7.4, 1 mM EDTA. The final ¹⁵N/¹³C/²H/[Leu,Val]-methyl-protonated IIB^{Man} NMR sample was concentrated to 0.7 mM in 20 mM phosphate buffer, pH 6.5 containing 0.01% (w/v) sodium azide and 8% ²H₂O.

NMR experiments

NMR experiments were performed at 308 K on a Bruker AVANCE 800 MHz spectrometer equipped with a cryogenic z-gradient DUL ¹³C{¹H} probe. NMR data were processed with the program NMRPipe (Delaglio et al., 1995).

Results and discussion

NMR pulse sequences

Figure 2 shows the pulse schemes for the 3D HMCNC and HN(CA)C TOCSY-type experiments.

The HMCNC-TOCSY experiment (Figure 2A) is a ¹H^{methyl}-resolved [¹³C^{methyl}, ¹³C^{ali}]-TOCSY experiment, which is a modified version of the original 3D HCC-TOCSY (Hu et al., 2005) aimed at optimal performance for deuterated, methyl-reprotonated samples. While preparing this paper, a similar pulse sequence was published (Jordan et al., 2006). The initial ¹H^{methyl} polarization is excited and evolved to anti-phase coherence under scalar coupling with ¹³C^{methyl} during a semi-constant time INEPT step while chemical-shift labelling ¹H^{methyl} (*t*₁). Subsequent 90° pulses on ¹H^{methyl} and ¹³C^{methyl} create transverse anti-phase ¹³C^{methyl} magnetization, followed by a second semi-constant time element with chemical-shift labelling of ¹³C^{methyl} (*t*₂). The second semi-constant time aims to refocus

¹³C^{methyl} magnetization into the in-phase form with respect to all three attached methyl protons. The optimal effective evolution time under three *J*_{HC}-couplings is approximately 1/(5*J*_{HC}) (Cavanagh et al., 1996). Subsequent transfer of the in-phase ¹³C^{methyl} magnetization to all aliphatic ¹³C spins is achieved via an isotropic FLOPSY-16 sequence (Kadkhodaie et al., 1991; Eletsky et al., 2003). The 90° cleaning pulse on ¹H after the TOCSY mixing period converts residual anti-phase ¹³C magnetization into unobservable multiple quantum coherences. ¹H and ²H decoupling is applied during acquisition. ¹⁵N decoupling is not applied because a substantial amount of signal is lost due to heteronuclear interference between ²H, ¹⁵N and ¹³C frequencies (Vogeli et al., 2005).

In the 3D HN(CA)C TOCSY experiment (Figure 2B), the ¹H_{N(i)} polarization is excited and followed by a semi-constant time INEPT step while chemical-shift labelling ¹H_N (*t*₁). Subsequent 90° pulses on ¹H^N and ¹⁵N transfer magnetization to ¹⁵N(i). The ¹⁵N (*t*₂) chemical shift is labeled in a constant time element while in-phase magnetization on ¹⁵N(i) is generated via its scalar coupling to ¹H_N and by refocusing of ¹³C^{ali} and ¹³C' spins. The total duration of this element is determined by the desired resolution. In-phase to in-phase magnetization transfer from ¹⁵N(i) to ¹³C^α(i) and ¹³C^α(i-1) is achieved via heteronuclear cross polarization (HCP) for a duration of 56 ms (Richardson et al., 1993). Transfer efficiency by HCP is comparable to in-phase to in-phase transfer using two consecutive INEPT steps. Relaxation depends on spin-system specific parameters, but is expected to be about equal (Ernst et al., 1991). However, during the application of the DIPSI-2 pulse train (Shaka et al., 1988), HCP already initiates in-phase to in-phase transfer from ¹³C^α to neighbouring ¹³C spins. Therefore, the subsequent FLOPSY-16 mixing time can be substantially shortened. Here, we decrease the mixing time from 15.9 ms to 10.6 ms. ¹H and ²H decoupling is applied during acquisition.

Resonance assignment using 3D HN(CA)C and HMCNC experiments

To illustrate the application of these experiments for methyl ¹H and ¹³C chemical shift assignments, Figure 3 shows data from the ¹³C-detected 3D HN(CA)C and HMCNC spectra recorded on the

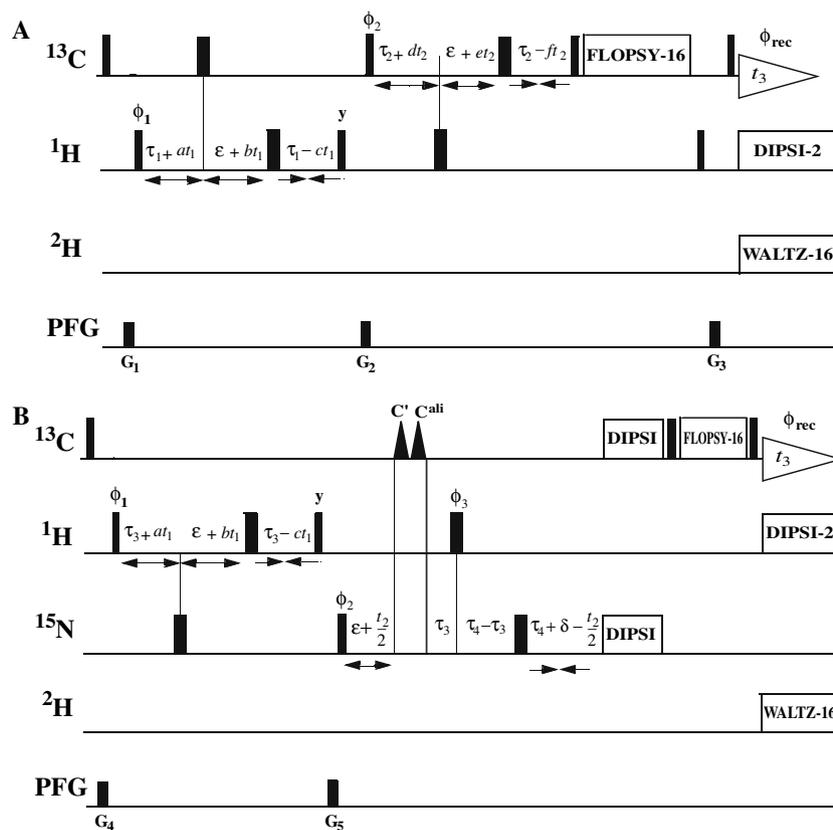


Figure 2. (A) 3D HMC-MC-TOCSY and (B) 3D HN(CA)C-TOCSY pulse sequences for methyl group side-chain assignment. The carrier frequencies for ^{13}C , ^1H and ^2H pulses are set to 23 ppm (last ^{13}C -pulse and FLOPSY at 37 ppm), 0.8 ppm and 3.0 ppm, respectively, in (A) and to 58 ppm (last ^{13}C -pulse and FLOPSY at 40 ppm), 4.7 ppm and 3.0 ppm, respectively, in (B). The carrier frequency for the ^{15}N pulses is set to 118 ppm. Narrow and wide bars indicate non-selective 90° and 180° pulses, respectively, and triangles represent selective 180° rectangular pulses. The last ^1H pulse in (A) converts unwanted magnetization into unobservable multiple-quantum coherences. ^1H - and ^2H -decoupling is achieved with DIPSI-2 and WALTZ-16 pulse trains using field strengths γB_1 of 1.25 kHz and 0.625 kHz, respectively. DIPSI ($\gamma B_1 = 2.083$ kHz) and FLOPSY ($\gamma B_1 = 8.333$ kHz) mixing are applied for 56 ms and 15.9 ms (A)/10.6 ms (B), respectively. The delays are as follows: $\tau_1 = 1/(4J_{\text{HC}}) = 1.85$ ms, $\tau_2 = 1/(10J_{\text{HC}}) = 0.72$ ms, $\tau_3 = 1/(4J_{\text{HN}}) = 2.65$ ms, $\tau_4 > t_{\text{max}}(^{15}\text{N})/2$, $\epsilon = 2$ μs and $\delta = p_{\text{H}} + 2p_{\text{C}} + 2$ μs , where p_{H} and p_{C} are the pulse lengths of the 180° ^1H and selective ^{13}C pulses, respectively. Pulsed field gradients indicated on the line marked PFG are applied along the z-axis with durations and strengths as follows: G_1 , 300 μs and 30 G/cm; G_2 , 300 μs and 30 G/cm; G_3 , 300 μs and 30 G/cm; G_4 , 600 μs and 30 G/cm; and G_5 , 600 μs and 30 G/cm. Unless indicated otherwise, all radio-frequency pulses are applied with phase x . The phase cycles are as follows: in (A) $\phi_1 = \{x, -x\}$, $\phi_2 = \{x, x, -x, -x\}$, and $\phi_{\text{rec}} = \{x, -x, -x, x\}$; in (B) $\phi_1 = \{x, -x\}$, $\phi_2 = \{y, y, -y, -y\}$, $\phi_3 = \{x, x, x, x, -x, -x, -x, -x, y, y, y, y, -y, -y, -y, -y\}$, and $\phi_{\text{rec}} = \{x, -x, -x, x, x, -x, -x, x, -x, x, x, x, -x, -x, x, x, -x\}$. Incrementations follow the rules: $b = a - c$ and $\epsilon = d - f$. The ^1H and ^{13}C chemical shifts evolve effectively during $2at_1$ and $2dt_2$. Quadrature detection in the $^1\text{H}(t_1)$, $^{13}\text{C}(t_2)$ (in A) and $^{15}\text{N}(t_2)$ (in B) dimensions is achieved using States-TPPI (Marion et al., 1989) applied to the phases ϕ_1 and ϕ_2 , respectively, together with ϕ_{rec} .

18.6 kDa $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ /[Leu, Val]-methyl-protonated IIB^{Man}.

Figure 3A show $^1\text{H}_{\text{m}}-^{13}\text{C}$ strips for $^{13}\text{C}^{\gamma 1}$ and $^{13}\text{C}^{\gamma 2}$ (middle and right panels) from the 3D ^{13}C -detected HMC-MC spectrum and a $^1\text{H}_{\text{N}}-^{13}\text{C}$ strip (left panel) from the ^{13}C -detected 3D HN(CA)C spectrum for Val-133. The alignment of the direct dimension $^{13}\text{C}^{\beta}$ and two $^{13}\text{C}^{\text{methyl}}$ chemical shifts in these strips relates the methyl groups to their backbone H_{N} group. Figure 3B

and D show the detailed peak pattern matching in the expanded regions corresponding to the $^{13}\text{C}^{\beta}$ and $^{13}\text{C}^{\text{m}}$ chemical shifts of Val-133. In Figure 3C and E, 1D slices are extracted to align their multiplet splitting patterns and the corresponding side-chain carbon assignments are indicated. The expanded regions and 1D slices from the HN(CA)C spectrum in Figure 3F and G show the correlation to intra- and inter-residue $^{13}\text{C}^{\alpha}$ chemical shifts and to the $^{13}\text{C}^{\beta}$

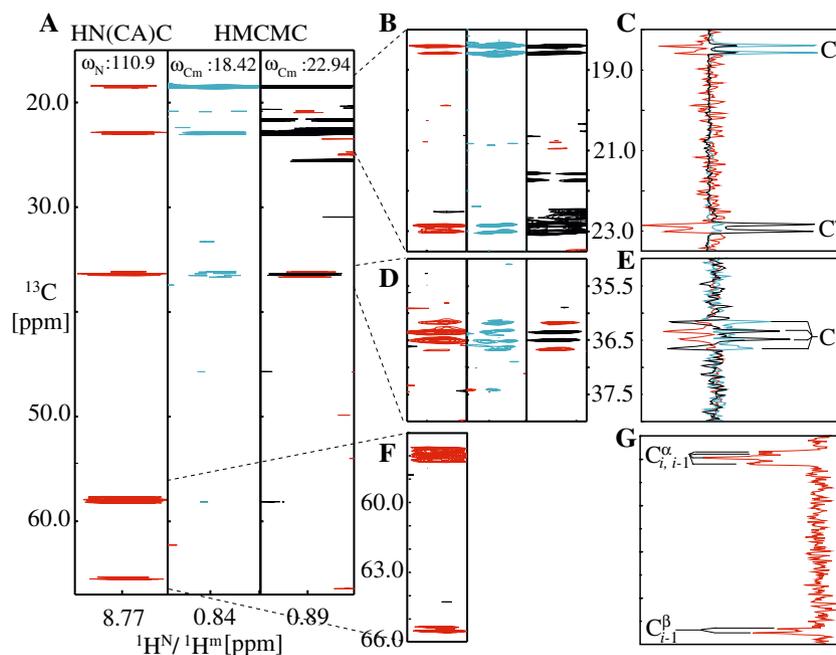


Figure 3. (A) H_N strip taken from the 3D ^{13}C -detected HN(CA)C-TOCSY spectrum and H_mC_m strips taken from the 3D HmCmC spectrum for Val-133 of $^{15}\text{N}/^{13}\text{C}, ^2\text{H}/[\text{Leu,Val}]$ -methyl-protonated IIB^{Man}. The corresponding indirect $^{15}\text{N}/^{13}\text{C}$ chemical shifts are indicated at the top of each strip. Chemical shifts of the $^{13}\text{C}^\beta$ and two $^{13}\text{C}^\gamma$ resonances are aligned. (B) and (D) show the multiplet pattern for these carbons. (F) Expanded region shows the multiplet pattern of the intrareidue $^{13}\text{C}^\alpha$ correlation and the interresidue $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ correlations to the preceding residue (Ser-132). (C), (E) and (G) Slices taken along the direct ^{13}C dimension are expanded to show the resolved multiplets due to J_{CC} couplings. Alignment of the splitting pattern resolved at the high resolution obtained in the directly detected ^{13}C dimension helps to confirm the assignment of the methyl resonances. The assigned carbons are labeled. The experiments were recorded at 800 MHz. For the HmCmC experiment, $35(t_1) \times 33(t_2) \times 2048(t_3)$ complex points were accumulated, with $t_{1\text{max}}(^1\text{H}_m) = 54.68$ ms, $t_{2\text{max}}(\text{indirect } ^{13}\text{C}_m) = 16.4$ ms and $t_{3\text{max}}(\text{direct } ^{13}\text{C}) = 168.7$ ms with 64 scans per increment. For the HN(CA)C experiment, $28(t_1) \times 31(t_2) \times 2048(t_3)$ complex points were accumulated, with $t_{1\text{max}}(^1\text{H}_N) = 10.94$ ms, $t_{2\text{max}}(^{15}\text{N}) = 15.29$ ms and $t_{3\text{max}}(\text{direct } ^{13}\text{C}) = 168.7$ ms with 112 scans per increment. Both experiments were run with an interscan delay of 0.9 s resulting in total experiment times of 92 h and 120 h for the 3D HmCmC-TOCSY and HN(CA)C-TOCSY experiments, respectively.

chemical shift of the preceding residue (Ser-132). The disappearance of the $^{13}\text{C}^\alpha$ peak in the two H_mC_m strips from the 3D ^{13}C -detected HmCmC spectrum is probably due to the absence of ^{15}N decoupling and the scalar couplings between $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$, $^{13}\text{C}^\gamma$. The observed splitting pattern in the directly detected ^{13}C dimension helps identification of residue type and assignment of side-chain carbon resonances. For example, the $^{13}\text{C}^\beta$ and $^{13}\text{C}^{\gamma 1/\gamma 2}$ resonances of Val appear as a quadruplet and doublet, respectively (Figure 3); while the $^{13}\text{C}^\beta$, $^{13}\text{C}^\gamma$ and $^{13}\text{C}^{\delta 1/\delta 2}$ resonances of Leu appear as a triplet, quadruplet and doublet, respectively (Figure 4). Alignment of these splitting patterns resolved at the high resolution obtained in the directly detected ^{13}C dimension helps to confirm the assignment of the methyl resonances.

To further demonstrate the application of these experiments for unambiguous methyl resonance assignment, an expanded crowded region of a ^1H -detected constant time (CT) ^{13}C -HMQC spectrum recorded on the same sample is shown in Figure 4A. For comparison, the overlaid cross-section of the 3D ^{13}C -detected HmCmC spectrum for the corresponding region is shown in Figure 4B, and clearly exhibits much higher resolution. To illustrate the assignment strategy employed for these methyl resonances, strips corresponding to Leu-57 and Leu-23 are taken as examples (Figure 4C and E), as indicated by the open dashed arrows. These strips can be almost perfectly aligned with the other methyl $^1\text{H}_m$ - ^{13}C strip from the same residues at the two $^{13}\text{C}^{\delta 1}/^{13}\text{C}^{\delta 2}$ chemical shifts and also at their $^{13}\text{C}^\gamma$ chemical shift. The horizontal dashed lines in Figure 4C and

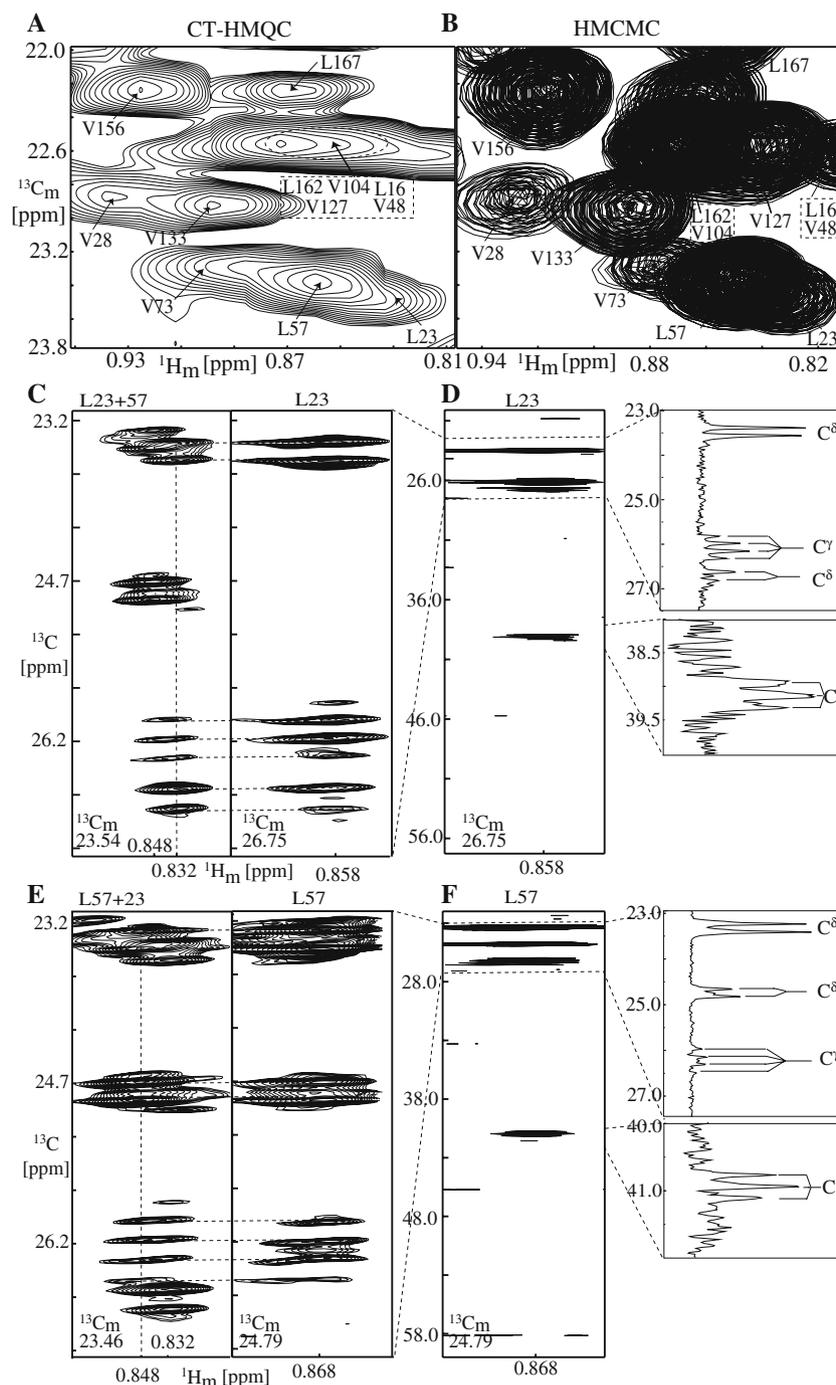


Figure 4. (A) An expanded region from the 2D ^{13}C CT-HMQC spectrum of $^{15}\text{N}/^{13}\text{C}/^2\text{H}/[\text{Leu, Val}]$ -methyl-protonated IIB^{Man}. (B) Overlaid cross-sections of the H_mC_m planes of the 3D ^{13}C -detected HMCMTOCOSY spectrum showing the corresponding region for comparison. (C) and (E) H_mC_m strips taken from the 3D HMCMTOCOSY spectrum corresponding to Leu-57 and Leu-23. The horizontal dashed lines indicate the alignment of the multiplet pattern of these carbons. (D) and (F) Zoom-out of (C) and (E) showing the full range of ^{13}C chemical shifts in the ^{13}C directly-detected dimension, including two $^{13}\text{C}^{\delta 1}/^{13}\text{C}^{\delta 2}$ resonances and $^{13}\text{C}^{\gamma}$ and $^{13}\text{C}^{\beta}$ resonances. 1D slices (the right-hand panels in D and F) taken at the position of the methyl resonances of Leu-57 and Leu-23 show clear peak splitting patterns at these carbons.

E indicate the alignment of the multiplet pattern for these peaks. The zoom-out shown in Figure 4D and F reveals the full range ^{13}C chemical shifts in the ^{13}C directly-detected dimension, including two $^{13}\text{C}^{\delta 1}/^{13}\text{C}^{\delta 2}$ chemical shifts and $^{13}\text{C}^{\gamma}$, $^{13}\text{C}^{\beta}$ chemical shifts. These chemical shifts allow unambiguous identification of these methyl groups. Further, 1D slices (the right panels in Figure 4D and F) taken at the position of these methyl resonances show a clear peak splitting pattern at these carbons. The splitting pattern can be used to further confirm the assignments. This strategy clearly demonstrates how one can obtain unambiguous assignment for two methyl resonances located in a very crowded region of the 2D ^{13}C CT-HMQC spectrum, which have only slight chemical shift differences in both dimensions, 0.848 ppm versus 0.832 ppm in the H_m dimension, and 23.46 ppm versus 23.54 ppm in C_m dimension. Following the same approach, the crowded cluster of methyl resonances for Leu-162, Val-104, Leu-16, Val-127 and Val-48 can also be unambiguously assigned (Figure 4B).

Concluding remarks

In this paper we have presented a robust approach for the direct assignment of methyl group resonances by linking them to backbone amide groups using a single sample and two 3D ^{13}C -detected experiments, namely the HMCMC-TOCSY and HN(CA)C TOCSY experiments. Alignment of all side-chain carbon chemical shifts together with their multiplet patterns can lead to unambiguous identification of methyl resonances. For high molecular weight proteins and protein complexes for which high deuteration levels are required, the new methods described could find wide application. The HMCMC pulse sequence can be readily incorporated into relaxation experiments probing methyl group dynamics. In addition, the measurement of C–C residual dipolar couplings in the highly resolved ^{13}C -directly detected dimension may yield further insights into side-chain structure and dynamics (Vogeli et al., 2004).

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References

- Bax, A., Clore, G.M., Driscoll, P.C., Gronenborn, A.M., Ikura, M. and Kay, L.E. (1990a) *J. Magn. Reson.*, **87**, 620–628.
- Bax, A., Clore, G.M. and Gronenborn, A.M. (1990b) *J. Magn. Reson.*, **88**, 425–431.
- Bermel, W., Bertini, I., Felli, I.C., Kummerle, R. and Pierattelli, R. (2003) *J. Am. Chem. Soc.*, **125**, 16423–16429.
- Bermel, W., Bertini, I., Felli, I.C., Piccioli, M. and Pierattelli, R. (2006) *Prog. Nucl. Magn. Reson. Spect.*, **48**, 25–45.
- Bertini, I., Felli, I.C., Kummerle, R., Moskau, D. and Pierattelli, R. (2004) *J. Am. Chem. Soc.*, **126**, 464–465.
- Cavanagh, J., Fairbrother, W.J., Palmer, A.G. and Skelton, N.J. (1996) *Protein NMR Spectroscopy: Principles and Practice*, Academic Press, New York.
- Clore, G.M. and Gronenborn, A.M. (1991) *Science*, **252**, 1390–1399.
- Clore, G.M. and Gronenborn, A.M. (1998) *Trends Biotech.*, **16**, 22–34.
- Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.
- Eletsky, A., Moreira, O., Kovacs, H. and Pervushin, K. (2003) *J. Biomol. NMR*, **26**, 167–179.
- Ernst, M., Griesinger, C., Ernst, R.R. and Bermel, W. (1991) *Mol. Phys.*, **74**, 219–252.
- Fesik, S.W., Eaton, H.L., Olejniczak, E.T., Zuiderweg, E.R.P., McIntosh, L.P. and Dahlquist, F.W. (1990) *J. Am. Chem. Soc.*, **112**, 886–888.
- Hu, K.F., Eletsky, A. and Pervushin, K. (2003) *J. Biomol. NMR*, **26**, 69–77.
- Hu, K.F., Vogeli, B. and Pervushin, K. (2005) *J. Magn. Reson.*, **174**, 200–208.
- Jordan, J.B., Kovacs, H., Wang, Y., Mobli, M., Luo, R., Anklin, C., Hoch, J.C. and Kriwacki, R.W. (2006) *J. Am. Chem. Soc.*, **128**, 9119–9128.
- Kadkhodaie, M., Rivas, O., Tan, M., Mohebbi, A. and Shaka, A.J. (1991) *J. Magn. Reson.*, **91**, 437–443.
- Kay, L.E. (2005) *J. Magn. Reson.*, **173**, 193–207.
- Kay, L.E., Ikura, M. and Bax, A. (1990) *J. Am. Chem. Soc.*, **112**, 888–889.
- Kovacs, H., Moskau, D. and Spraul, M. (2005) *Prog. Nucl. Magn. Reson. Spect.*, **46**, 131–155.
- Legler, P.M., Cai, M.L., Peterkofsky, A. and Clore, G.M. (2004) *J. Biol. Chem.*, **279**, 39115–39121.
- Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.
- Mittermaier, A. and Kay, L.E. (2006) *Science*, **312**, 224–228.
- Oh, B.H., Westler, W.M., Darba, P. and Markley, J.L. (1988) *Science*, **240**, 908–911.
- Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1992) *J. Biomol. NMR*, **2**, 655–659.
- Richardson, J.M., Clowes, R.T., Boucher, W., Domaille, P.J., Hardman, C.H., Keeler, J. and Laue, E.D. (1993) *J. Magn. Reson. Series B*, **101**, 223–227.
- Rosen, M.K., Gardner, K.H., Willis, R.C., Parris, W.E., Pawson, T. and Kay, L.E. (1996) *J. Mol. Biol.*, **263**, 627–636.

- Schwieters, C.D., Kuszewski, J.J. and Clore, G.M. (2006) *Prog. Nucl. Magn. Reson. Spect.*, **48**, 47–62.
- Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.
- Suh, J.Y., Cai, M.L., Williams, D.C. and Clore, G.M. (2006) *J. Biol. Chem.*, **281**, 8939–8949.
- Tugarinov, V. and Kay, L.E. (2003) *J. Am. Chem. Soc.*, **125**, 13868–13878.
- Vogeli, B., Kovacs, H. and Pervushin, K. (2004) *J. Am. Chem. Soc.*, **126**, 2414–2420.
- Vogeli, B., Kovacs, H. and Pervushin, K. (2005) *J. Biomol. NMR.*, **31**, 1–9.
- Williams, D.C., Cao, M.L., Suh, J.Y., Peterkofsky, A. and Clore, G.M. (2005) *J. Biol. Chem.*, **280**, 20775–20784.
- Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
- Yang, D.W., Zheng, Y., Liu, D.J. and Wyss, D.F. (2004) *J. Am. Chem. Soc.*, **126**, 3710–3711.