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COMMUNICATION

Selective ^{1}H-^{13}C NMR spectroscopy of methyl groups in residually protonated samples of large proteins

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Abstract Methyl $^{13}CHD_2$ isotopomers of all methyl-containing amino-acids can be observed in residually protonated samples of large proteins obtained from [U-^{13}C,^{1}H]-glucose/D_2O-based bacterial media, with sensitivity sufficient for a number of NMR applications. Selective detection of some subsets of methyl groups (Ala$^{\beta}$, Thr$^{\gamma 2}$) is possible using simple 'out-and-back' NMR methodology. Such selective methyl-detected 'out-and-back' NMR experiments allow complete assignments of threonine $\gamma 2$ methyls in residually protonated, [U-^{13}C,^{1}H]-glucose/D_2O-derived samples of an 82-kDa enzyme Malate Synthase G. [U-^{13}C,^{1}H]-glucose/D_2O-derived protein samples are relatively inexpensive and are usually available at very early stages of any NMR study of high-molecular-weight systems.

Keywords Isotope labeling · [U-^{13}C,^{1}H]-glucose · $^{13}CHD_2$ methyl group · MSG

Abbreviations

MSG	Malate Synthase G
HSQC	Heteronuclear single-quantum coherence spectroscopy
CT-HSQC	Constant-time HSQC

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Current state-of-the-art NMR methodology for the studies of large proteins and macromolecular assemblies relies upon selective protonation of Ile$^{\delta 1}$, Leu$^{\delta}$ and Val$^{\gamma}$ (ILV) positions of large proteins on a deuterated background (Goto et al. 1999; Tugarinov et al. 2006). The ILV labeling achieved via the use of biosynthetic keto-acid precursors has facilitated NMR studies of structure and dynamics in very large protein systems (Sprangers and Kay 2007; Sprangers et al. 2007; Tugarinov et al. 2005a; Velyvis et al. 2007; Sheppard et al. 2009c). However, the availability of only these three methyl probes limits the scope of information that can be obtained by NMR of large proteins. Significant efforts have been dedicated recently to extension of the ILV labeling methodology to other methyl sites (Ayala et al. 2009; Isaacson et al. 2007; Sheppard et al. 2009a, b). Here we report that residual protonation in protein samples obtained using [U-^{13}C,^{1}H]-glucose in 99% D_2O-based media, is sufficient for sensitive observation of all methyl groups in large proteins. This observation is not new in itself—earlier, Rosen et al. have thoroughly investigated the protonation patterns of all residues in [U-^{13}C,^{1}H]-glucose/D_2O-derived proteins (Rosen et al. 1996). Such residually protonated protein samples have been used previously for structure determination of smaller systems (Shekhtman et al. 2002); however, no NMR studies have focused so far on residually protonated methyl sites of large proteins in the 50-100 kDa molecular weight range.

Figure 1 shows the methyl region of the $^{13}CHD_2$-filtered (Brath et al. 2006; Ishima et al. 1999) ^{1}H-^{13}C CT-HSQC (Vuister and Bax 1992) correlation map of a 723-residue monomeric enzyme Malate Synthase G (MSG) obtained using U-[^{13}C,^{1}H]-glucose as the sole carbon source in 99% D_2O-based bacterial medium. In agreement with predictions based on analysis of biosynthetic pathways and

previous observations (Rosen et al. 1996; Shekhtman et al. 2002), $^{13}CHD_2$ isotopomers are dominant among residually protonated methyl groups. Earlier, Cowburn and co-workers (Shekhtman et al. 2002) reported that among 'NMR-observable' methyl isotopomers (excluding $^{13}CD_3$ methyls), $^{13}CHD_2$ and $^{13}CH_2D$ isotopomers in U-[^{13}C,^{1}H]-glucose/D_2O-derived ubiquitin are present at the levels of 73 and 22%, respectively, with the remaining 5% of $^{13}CH_3$ variety. In MSG, the only other non-$^{13}CHD_2$ methyls detected are of a $^{13}CH_2D$ type. Very different relaxation properties of $^{13}CHD_2$ and $^{13}CH_2D$ methyls in large proteins (Ollerenshaw et al. 2005; Tugarinov et al. 2005b) (vide infra) preclude direct quantification of isotopomer ratios from CT-HSQC-type spectra. However, using the $^{13}CH_2D$-TROSY experiment (Ollerenshaw et al. 2005; Tugarinov et al. 2005b) and taking into account the effects of $^{13}CH_2D$-TROSY acquisition and processing schemes on signal-to-noise ratios (Ollerenshaw et al. 2005; Tugarinov et al. 2005b), we have been able to estimate the average approximate $^{13}CHD_2$:$^{13}CH_2D$ ratio in U-[^{13}C,^{1}H]-glucose/D_2O-derived MSG as ~3.5:1—in good agreement with the measurements of (Shekhtman et al. 2002). We have also estimated that the detection of $^{13}CHD_2$ methyls in U-[^{13}C,^{1}H]-glucose/D_2O-derived samples of MSG is approximately 5-to-6 times less sensitive than Methyl-TROSY (Tugarinov et al. 2003) detection of (close to 100% abundant) $^{13}CH_3$ methyl groups.

Clearly, the degree of resonance overlap between different regions of methyl resonances among the total pool of 446 methyls in MSG (Fig. 1) warrants the development of NMR methods allowing, where possible, selective detection of methyls of individual amino-acid groups. Figure 2a shows a pulse-scheme that selects for methyls of Ala or Thr depending on the excitation/refocusing bandwidth of the selective $^{13}C_{\phi 3}$ pulse. The selective Ala$^{\beta}$(Thr$^{\gamma 2}$) methyl correlation maps are shown in Fig. 2b, c). The experiment of Fig. 2a 'filters' the magnetization through ^{13}C nuclei directly attached to methyl carbons (Thr $^{13}C^{\beta}$ and Ala $^{13}C^{\alpha}$), and takes advantage of the uniqueness of Ala $^{13}C^{\alpha}$ and Thr $^{13}C^{\alpha}$/$^{13}C^{\beta}$ chemical shifts in proteins. The inclusion of the dashed element in Fig. 2a ensures active suppression of signals originating from $^{13}CH_2D$ isotopomers. It has not been used in MSG because of very unfavorable relaxation properties of $^{13}CH_2D$ methyls in HSQC-type magnetization transfer schemes in large proteins (Ollerenshaw et al. 2005; Tugarinov et al. 2005b), but may be necessary for smaller systems. Using the same concept of selective refocusing, it is also possible to restrict detection to all methyls *except* Ala$^{\beta}$ and Thr$^{\gamma 2}$ sites. Figure 3a shows such a correlation map obtained using a selective RE-BURP pulse (Geen and Freeman 1991) whose excitation/refocusing profile is illustrated in Fig. 3b. In a similar manner, if needed, Met $^{13}C^{\varepsilon}$ methyls can be targeted for

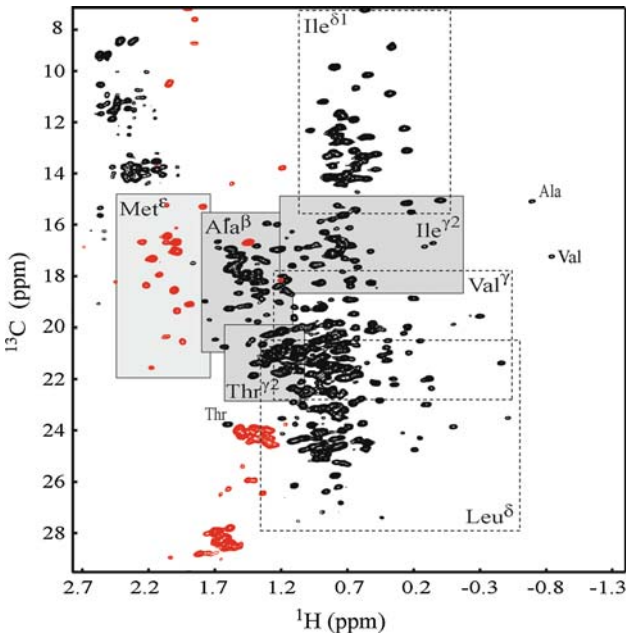

Fig. 1 The methyl region of the ^{1}H-^{13}C CT-HSQC (Vuister and Bax 1992) correlation map recorded on a 0.5 mM [U-^{13}C]-glucose/D_2O-derived sample of MSG (pH = 7.1; 99.9% D_2O; room temperature probe operating at 37°C; 600 MHz; 1 h. acquisition time) and actively 'filtered' for $^{13}CHD_2$ (against $^{13}CH_2D$) isotopomers (Brath et al. 2006; Ishima et al. 1999). Approximate regions of ILV methyl resonances are enclosed in *dashed rectangles*, whereas the regions corresponding to non-ILV methyl groups are enclosed in *shaded solid rectangles*. *Negative peaks* are shown with *red* contours. In methyl-containing side chains, substantial residual protonation can be observed at Val $^{13}C^{\beta}$ and Ile $^{13}C^{\gamma 1}$ positions

selective observation based on the absence of one-bond ^{13}C-^{13}C couplings at Met methyl positions. Of note, the pulse scheme of Fig. 2a as well as all the experiments described below can be gradient sensitivity-enhanced (Kay et al. 1992; Schleucher et al. 1993) in a straightforward manner. For $^{13}CHD_2$ methyls of MSG, however, the sensitivity enhancement with gradient coherence selection provides only ~15% gain in signal-to-noise ratio on average. This gain is likely to be even lower in larger systems. Therefore, for the sake of simplicity, we present the amplitude-modulated versions of the pulse-schemes.

In this work, we have chosen to concentrate primarily on methyl groups of threonines because these are the only methyl sites remaining unassigned in MSG (besides Met$^{\varepsilon}$ positions). Thr methyls are not easily amenable to selective protonation on a deuterated background possible for other methyl-bearing side-chains. For example, $^{13}CH_3$ Thr$^{\gamma 2}$ methyls are absent from the spectra of proteins derived from {U-^{13}C}-pyruvate/D_2O bacterial media (Gardner and Kay 1998; Gardner et al. 1997; Rosen et al. 1996; Sheppard et al. 2009b), while $^{13}CHD_2$ Thr$^{\gamma 2}$ methyl signals appear only as very weak peaks (Gardner and Kay 1998; Rosen et al. 1996). Figure 4a shows the methyl-detected

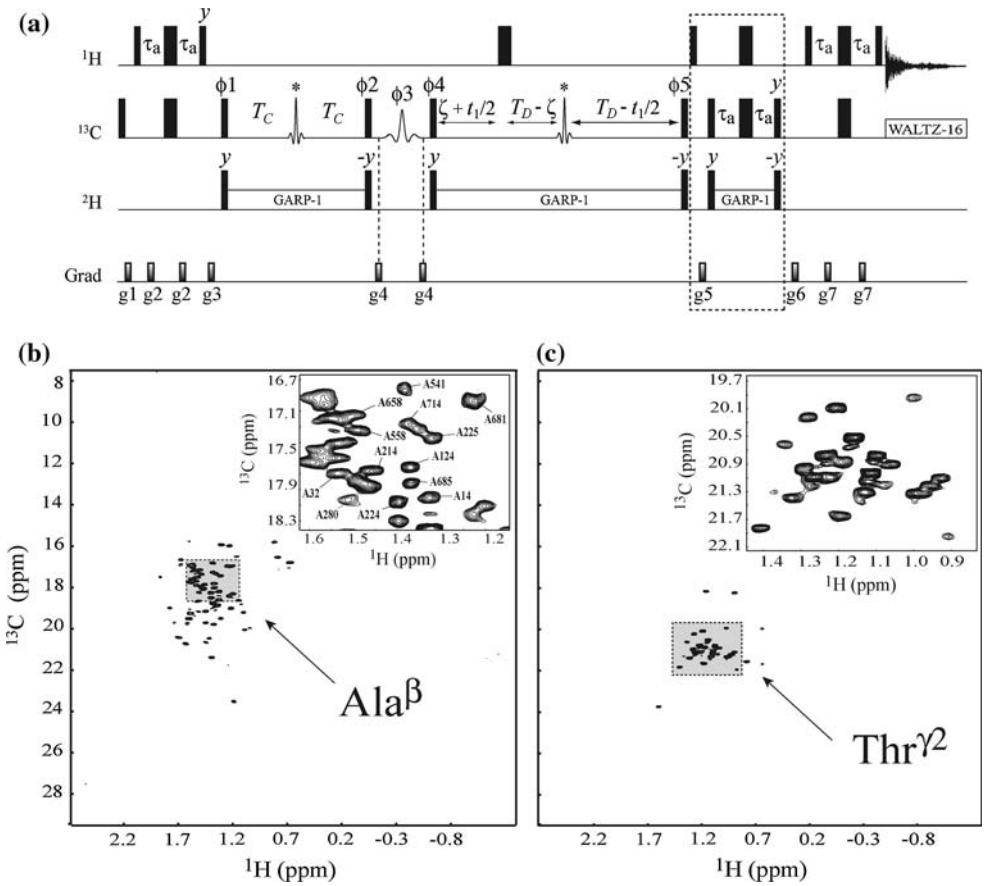

Fig. 2 **a** Methyl-detected 'out-and-back' 2D pulse-scheme designed for selective detection of Ala$^{\beta}$ and Thr$^{\gamma 2}$ methyl groups in large proteins. All the pulses are applied along the *x*-axis unless indicated otherwise. All pulses shown with *black rectangles* are applied with the highest possible power. ^{13}C WALTZ-16 (Shaka et al. 1983) decoupling during acquisition is achieved using a 2.5 kHz field, while ^{2}H GARP-1 (Shaka et al. 1985) decoupling uses a 0.8 kHz field. The ^{1}H(^{2}H) carrier is positioned at 4.7(2.5) ppm. ^{13}C carrier is placed at 18(21) ppm for Ala$^{\beta}$(Thr$^{\gamma 2}$) filtering, switched to 36(45) ppm before the ^{13}C pulse with phase $\phi 1$, and returned to 18(21) ppm after the ^{13}C pulse with phase $\phi 5$ or after the element enclosed in the dashed rectangle. The ^{13}C shaped pulses marked with asterisks are high-power 350 μs (600 MHz) RE-BURP pulses (Geen and Freeman 1991) applied on-resonance. The ^{13}C shaped pulse applied with phase $\phi 3$ is a 3.0 ms-long RE-BURP pulse (600 MHz; refocusing bandwidth of ±600 Hz) applied at 53(71) ppm by phase-modulation of the carrier (Boyd and Soffe 1989; Patt 1992) for selective refocusing of Ala$^{\alpha}$(Thr$^{\beta}$) regions. Delays are: $\tau_a = 2.0$ ms; $T_C = 7$ ms; $T_D = 7$ ms. To increase resolution in the F_1(^{13}C) dimension delay T_D can be extended to 21 ms. The element of the scheme enclosed in the *dashed rectangle* filters the methyl signals for ^{13}CHD$_2$ (against ^{13}CH$_2$D) isotopomers. If it is included the delay ζ should be set to 2.0 ms; otherwise ζ should be set to zero. The phase-cycle is: $\phi 1 = x,-x$; $\phi 2 = 2(y),2(-y)$; $\phi 3 = x,-x,y,-y$; $\phi 4 = 4(y),4(-y)$; $\phi 5 = x$ if $\zeta = 0$, and $\phi 5 = y$ if $\zeta = 2.0$ ms; rec. $= x,-x,-x,x$. Quadrature in F_1 is achieved via States (States et al. 1982) incrementation of $\phi 5$. Durations and strengths of the gradients in units of (ms;G/cm): G1 = (1.0;15), G2 = (0.3;8), G3 = (1.5;25), G4 = (0.25;30), G5 = (0.8;15), G6 = (0.5;12), G7 = (0.4;10); **b–c** 2D ^{1}H-^{13}C correlation maps with selectively detected Ala$^{\beta}$ (**b**) and Thr$^{\gamma 2}$ (**c**) methyl resonances recorded using the pulse-scheme shown in (**a**) with $T_D = 21$ ms on the same sample as in Fig. 1 (total acquisition time ~1.5 h.). The shaded regions are zoomed in the insets of *panels b* and *c*. No signals from other methyl-containing amino-acids have been observed in either Ala$^{\beta}$- or Thr$^{\gamma 2}$-selective experiments

'out-and-back' (Sheppard et al. 2009b; Tugarinov and Kay 2003) experiment developed for assignments of Thr$^{\gamma 2}$ methyls. The selectivity of the experiment is ensured by the application of Thr-^{13}C$^{\alpha,\beta}$-selective refocusing pulse, ^{13}C$_{\phi 4}$, applied after the t_1 evolution period. About 90% of Thr$^{\gamma 2}$ methyl assignments in MSG can be obtained from this single short 3D data set (~24 h. net acquisition time on a room-temperature probe; 600 MHz) by matching the observed pairs of ^{13}C$^{\alpha}$/^{13}C$^{\beta}$ shifts (Fig. 4b) to those available from the previous studies (Tugarinov et al. 2002). Notably, since the ^{13}C$_{\phi 4}$ pulse refocuses most of Ala ^{13}C$^{\alpha}$ resonances, such Thr-selective spectra are 'contaminated' by Ala ^{13}C$^{\alpha}$ correlations. However, in contrast to similar experiments developed earlier for assignments of ILV sites (Tugarinov and Kay 2003), Thr ^{13}C$^{\gamma 2}$ methyl cross-peaks and multiple-quantum peaks are not observed in the F_1 dimension of the Thr-selective spectra as they are not refocused by the ^{13}C$_{\phi 4}$ pulse.

The ambiguities arising from chemical shift degeneracy in Thr ^{13}C$^{\alpha}$-^{13}C$^{\beta}$ pairs have been resolved with the help of

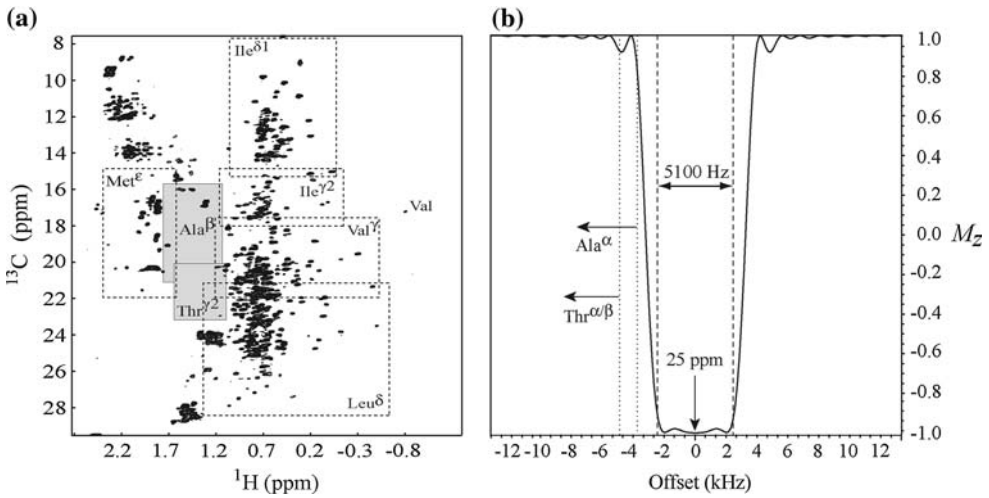

Fig. 3 **a** 2D ^{1}H-^{13}C methyl correlation map of a 0.5 mM [U-^{13}C,^{1}H]-glucose/D_2O-derived sample of MSG (pH = 7.1; 37°C; 99.9% D_2O; 600 MHz; ~1.5 h. acquisition time) 'filtered' for all $^{13}CHD_2$-type methyl groups *except* those of Ala$^{\beta}$ and Thr$^{\gamma 2}$. The spectrum has been acquired using the pulse-scheme of Fig. 2a (T_D = 21 ms) with the $^{13}C_{\phi 3}$ pulse implemented as an 800 μs RE-BURP pulse (Geen and Freeman 1991) centered at 25 ppm by phase modulation of the carrier (Boyd and Soffe 1989; Patt 1992) (see **b** for details). Approximate regions of all observable methyl types are enclosed in *dashed rectangles*, while the regions of (unobservable) Ala$^{\beta}$ and Thr$^{\gamma 2}$ methyls are enclosed in *solid rectangles* and *shaded*. The only signals appearing in the Ala$^{\beta}$ region belong to Met$^{\varepsilon}$ and Ile$^{\gamma 2}$ methyl groups, while those appearing in the Thr$^{\gamma 2}$ region are Val$^{\gamma}$ methyls. **b** The inversion profile (M_z magnetization, *y*-axis, vs. resonance offset in kHz, *x*-axis) of an 800 μs RE-BURP 180° pulse employed in the experiment of Fig. 2a for 'filtering-out' of Ala$^{\beta}$ and Thr$^{\gamma 2}$ peaks. Approximate limits of chemical shift ranges of Ala$^{\alpha}$ and Thr$^{\alpha,\beta}$ ^{13}C nuclei (600 MHz spectrometer, ^{1}H frequency) are shown with dotted vertical lines, while the inversion/refocusing bandwidth of the pulse spans the offset range enclosed in *dashed vertical lines* (5,100 Hz, 34 ppm at a 600 MHz spectrometer field). The 800 μs RE-BURP pulse thus refocuses the chemical shift evolution of Ile$^{\gamma 1}$, Ile$^{\beta}$, Leu$^{\gamma}$, Val$^{\beta}$ and Met$^{\varepsilon}$ positions (8–42 ppm), and leaves Ala$^{\alpha}$ and Thr$^{\alpha/\beta}$ positions (downfield of 50 ppm) unrefocused

a Thr$^{\gamma 2}$-selective 3D HMCM(CBCA)CO experiment (see Figure S1 in the Supporting Information for details). Practically complete assignments of all 33 Thr$^{\gamma 2}$ positions in MSG have been obtained using the [U-^{13}C]-glucose/D_2O-derived sample (Fig. 4c). Interestingly, Thr^{99} methyl has been observed in at least two distinct conformations (labeled 'T99^{a}' and 'T99^{b}' in Fig. 4c). This is the only Thr methyl whose coordinates are not available in the x-ray structure of MSG (Howard et al. 2000). It is located in a flexible loop connecting two domains of the enzyme and is likely to be conformationally disordered.

Although Ala$^{\beta}$ and Ile$^{\gamma 2}$ methyls have been assigned in MSG earlier (Ayala et al. 2009; Sheppard et al. 2009a; Sheppard et al. 2009b), it is of interest to note that NMR experiments can be designed for simultaneous assignments of all non-ILV sites in [U-^{13}C]-glucose/D_2O-derived large proteins except Met$^{\varepsilon}$ (*i.e.* Ala$^{\beta}$, Thr$^{\gamma 2}$, Ile$^{\gamma 2}$). These methyl-detected pulse-schemes are very similar to those developed by us earlier (Sheppard et al. 2009b), and are presented in the Supporting Information. Although ~75% of Ile$^{\delta 1}$ and ~50% of Val$^{\gamma}$ methyls can be assigned using the methyl-detected 'out-and-back' methodology (Tugarinov and Kay 2003) modified for the branched ^{13}C spin-systems of Val and Leu side-chains, the sensitivity of these experiments performed on residually protonated [U-^{13}C]-glucose/D_2O-derived samples proves insufficient for complete assignments of ILV methyls in MSG. Thus, the selective isotope labeling of ILV side chains via biosynthetic keto-acid precursors (Tugarinov and Kay 2003) remains the methodology of choice for assignments of ILV methyl groups in large proteins.

Analysis of amino-acid biosynthetic pathways shows that the single proton position of $^{13}CHD_2$ methyls targeted in this work derives predominantly from the proton attached to ^{13}C-1' position of U-[^{13}C,^{1}H]-glucose used as a carbon source in 99% D_2O-based bacterial medium (Lundström et al. 2007; Malaisse et al. 1994a; Malaisse et al. 1994b), whereas $^{13}CH_2D$ methyls originate from the protons attached to ^{13}C-6' positions of glucose. This ensures the paucity of $^{13}CH_3$ methyl isotopomers in U-[^{13}C,^{1}H]-glucose/D_2O-derived protein samples, and shifts the isotopomer distribution toward methyls of the $^{13}CHD_2$ variety (vide supra). Any partial protonation of the solvent used in the medium—via the use of D_2O/H_2O mixtures—would lead to the distribution of additional (solvent) protons among the isotopomers of all types including those of $^{13}CH_3$ variety. As a result, the addition of substantial amounts of H_2O into the medium for the purpose of increasing the concentration of $^{13}CHD_2$ methyls would be very inefficient. This would also degrade the quality of the $^{13}CHD_2$-directed NMR spectra since methyls of the $^{13}CH_3$ type can not be 'filtered out' as easily as $^{13}CH_2D$ isotopomers because of fast intra-methyl ^{1}H-^{1}H cross-correlated relaxation (Brath et al. 2006; Ishima et al. 1999).

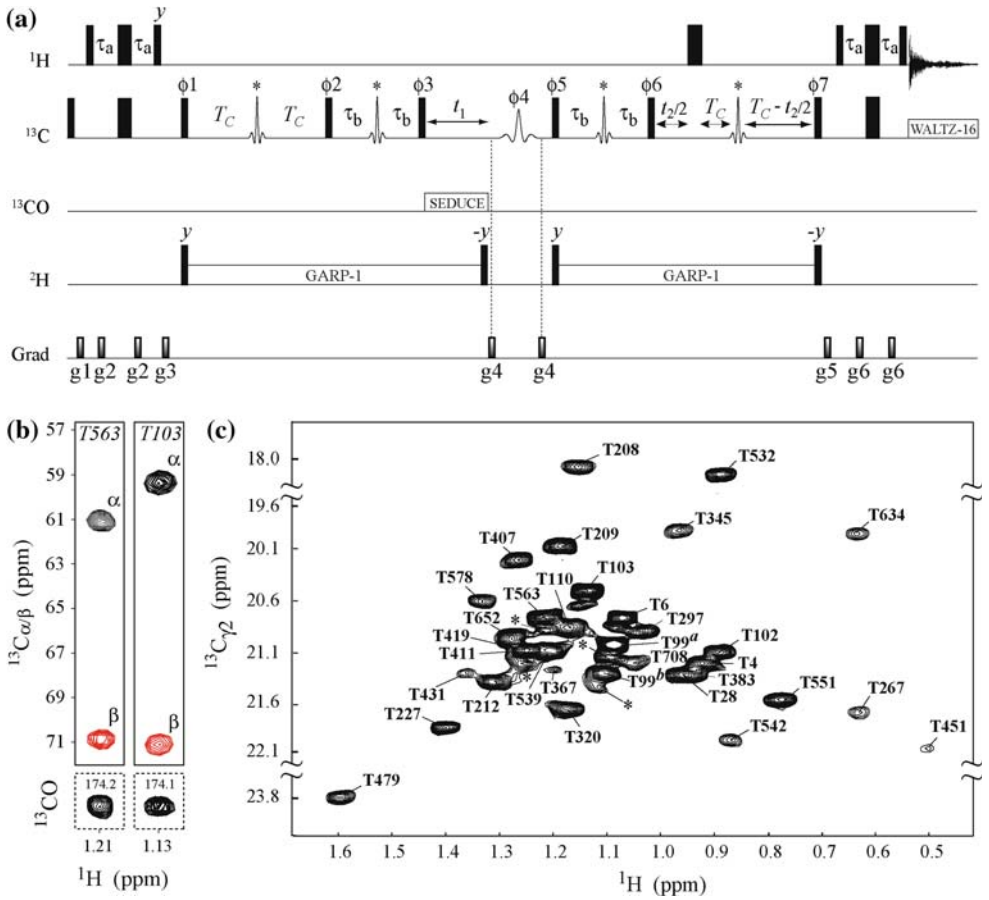

Fig. 4 Methyl-detected 'out-and-back' 3D HMCMCBCA pulse-scheme designed for assignment of Thr$^{\gamma 2}$ methyl groups in large proteins. Many details of the pulse-scheme are as described in the legend to Fig. 2a. The ^{13}C carrier is placed at 21 ppm, switched to 45 ppm before the ^{13}C pulse with phase $\phi 1$, and returned to 21 ppm after the ^{13}C pulse with phase $\phi 7$. SEDUCE (McCoy and Mueller 1992) ^{13}CO decoupling is implemented with 300 μs seduce-shaped pulses, with a 132 ppm cosine modulation of the waveform. The ^{13}C shaped pulse applied with phase $\phi 4$ is a 1.5 ms RE-BURP pulse (Geen and Freeman 1991) (refocusing bandwidth of ±1,200 Hz) that selectively refocuses Thr$^{\alpha,\beta}$ positions and is centered at 66.5 ppm by phase-modulation of the carrier (Boyd and Soffe 1989; Patt 1992). Delay τ_b is set to 3.5 ms. The phase-cycle is: $\phi 1 = x,-x$; $\phi 2 = \phi 3 = 2(y),2(-y)$; $\phi 4 = x,-x,y,-y$; $\phi 5 = \phi 6 = 4(y),4(-y)$; $\phi 7 = x$; rec. $= x,-x,-x,x$. Quadrature in F_1 is achieved via States-TPPI (Marion et al. 1989) of phases $\phi 1$, $\phi 2$, and $\phi 3$, while the quadrature in F_2 is achieved via States incrementation of $\phi 7$. Durations and strengths of the gradients are the same as listed in Fig. 2a except for G6 that is set to (0.3; 10) in units of (ms;G/cm). See Supporting Information for experimental details. **b** Selected 2D ^{1}H$^{\gamma 2}$-^{13}C$^{\alpha/\beta}$ and ^{1}H$^{\gamma 2}$-^{13}CO strips from the Thr$^{\gamma 2}$-selective 3D HMCMCBCA and HMCM(CBCA)CO data sets drawn at ^{13}C$^{\gamma 2}$ chemical shifts of Thr^{563} and Thr^{103}. *Negative peaks* are shown with *red* contours. **c** A region of Thr$^{\gamma 2}$-selective ^{1}H-^{13}C correlation map recorded using the pulse-scheme of Fig. 2a ($T_D = 21$ ms). Assignments of Thr methyls are indicated with residue numbers. The peaks marked with *asterisks* arise from isotopic ^{13}C shifts due to small (~12%) protonation of Thr ^{13}C$^{\beta}$ positions. The assignments of Thr^{431} and Thr^{451} are tentative because the ^{13}C$^{\alpha}$,^{13}C$^{\beta}$ chemical shifts remain unassigned (Tugarinov et al. 2002)

Furthermore, partial protonation of the solvent would entail partial protonation of ^{13}C$^{\alpha}$ and additional protonation of ^{13}C$^{\beta}$ aliphatic sites seriously compromising the sensitivity of the 'out-and-back' methyl assignment experiments. Therefore, for the purposes of this work, it seems unadvisable to increase protonation levels of the proteins the size of MSG and larger beyond those achieved using of U-[^{13}C,^{1}H]-glucose in a fully deuterated solvent.

In summary, we have shown that NMR detection of *all* methyl sites in large proteins is possible in residually protonated uniformly ^{13}C-enriched samples obtained using [U-^{13}C,^{1}H] glucose as a carbon source in D_2O-based media. Resonance assignments of all non-ILV methyl sites except Met$^{\varepsilon}$ are possible in such samples of proteins within ~100 kDa molecular weight range. [U-^{13}C]-glucose is an inexpensive carbon source, and [U-^{13}C]-glucose/D_2O-derived protein samples are usually available at very early stages of any NMR study of a large protein. Although the sensitivity of the $^{13}CHD_2$ methyl detection in residually protonated samples is clearly insufficient for more demanding experiments, for the simplest of NMR applications (such as, for example, resonance assignments, ligand binding studies etc.), selective detection of Ala$^{\beta}$ and Thr$^{\gamma 2}$ $^{13}CHD_2$ methyls in residually protonated uniformly

^{13}C-enriched samples obviates the need for costly selective isotope labeling techniques.

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