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COMMUNICATION

Chemical shift based editing of CH_3 groups in fractionally ^{13}C-labelled proteins using GFT (3, 2)D CT-HCCH-COSY: stereospecific assignments of CH_3 groups of Val and Leu residues

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Abstract We propose a (3, 2)D CT-HCCH-COSY experiment to rapidly collect the data and provide significant dispersion in the spectral region containing ^{13}C–^{1}H cross peaks of CH_3 groups belonging to Ala, Ile, Leu, Met, Thr and Val residues. This enables one to carry out chemical shift based editing and grouping of all the ^{13}C–^{1}H cross peaks of CH_3 groups belonging to Ala, Ile, Leu, Met, Thr and Val residues in fractionally (10%) ^{13}C-labelled proteins, which in turn aids in the sequence-specific resonance assignments in general and side-chain resonance assignments in particular, in any given protein. Further, we demonstrate the utility of this experiment for stereospecific assignments of the *pro*-R and *pro*-S methyl groups belonging to the Leu and Val residues in fractionally (10%) ^{13}C-labelled proteins. The proposed experiment opens up a wide range of applications in resonance assignment strategies and structure determination of proteins.

Keywords Isotope labeling · NMR · Automated assignments · Sequence specific resonance assignments · Stereo-specific resonance assignments

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Introduction

In protein NMR, there have been several attempts to derive different types of structural restraints for elucidating the protein structures. The constraints thus derived for structural studies include distance and torsion angle constraints, chemical shifts, dipolar couplings etcetera (Wüthrich 1986; Barnwal et al. 2007, 2008a, b; Chary and Govil 2008). One another class of restraints is derived from the stereospecific assignment of diastereotopic methyl (CH_3) groups of Val and Leu residues in proteins. These assignments provide the most important information concerning the orientation of isopropyl groups in Val and Leu residues about the C^{α}–C^{β} and C^{β}–C^{γ} bonds, respectively, and as a result have significant influence on the precision of derived 3D structures. To date, the most widely used approach for stereospecific assignment of CH_3 groups in Val and Leu residues relies on fractional ^{13}C labeling (Neri et al. 1989). However, in the case of large sized proteins (Mr > 20 kDa), severe spectral overlap poses a formidable problem. This is evident from Fig. 1A, which indicates the extent of spectral overlap in regions containing ^{13}C–^{1}H cross peaks of CH_3 groups in Val and Leu residues with those of other amino acid residues that have cross peaks in their vicinity. This characterization is based on a statistical analysis of chemical shift data of 235 proteins taken from the BioMagResBank (BMRB; http://www.bmrb.wisc.edu). As evident in this Fig. 1A, the cross peaks arising from Ala(C^{β}–H^{β}), Lys(C^{γ}–H^{γ}), Ile($C^{\delta 1}$–$H^{\delta 1}$ and $C^{\gamma 2}$–$H^{\gamma 2}$), and Thr(C^{γ}–H^{γ}) correlations have significant spectral overlap with those of those of Val($C^{\gamma 1/\gamma 2}$–$H^{\gamma 1/\gamma 2}$) or/and Leu($C^{\delta 1/\delta 2}$–$H^{\delta 1/\delta 2}$) correlations, hampering the stereospecific assignments of the latter. Further, about 20% of the Val(C^{γ}–H^{γ}) cross peaks are seen to overlap with Leu(C^{δ}–H^{δ}) correlations. These overlap problems have been previously overcome (Atreya and Chary 2001) by using a methodology

in which specific amino-acid residues in a given protein are selectively 'unlabeled' while simultaneously allowing the fractional ^{13}C labeling of other residues. Thus, the absence of cross peaks belonging to the selectively unlabeled amino acids in a 2D [^{13}C–^{1}H]-HSQC (Kay et al. 1992) results in an enhanced spectral simplification, leading to unambiguous stereospecific assignments of diastereotopic methyl groups of Val and Leu residues in a straightforward manner.

This methodology was first demonstrated on a 15 kDa (134 residues) calcium binding protein from the protozoan *Entamoeba histolytica* (*Eh*-CaBP) (Atreya et al. 2001; Atreya and Chary 2001). Based on Fig. 1A, residues whose ^{13}C–^{1}H cross peaks overlap extensively with those of Val($C^{\gamma 1/\gamma 2}$–$H^{\gamma 1/\gamma 2}$) or/and Leu($C^{\delta 1/\delta 2}$–$H^{\delta 1/\delta 2}$) were chosen for selective unlabeling. This needed preparation of two different protein samples, one in which Ile, Leu, Lys and Thr were selectively unlabeled and the remaining amino acids were fractionally (10%) ^{13}C-labeled. This resulted in the suppression of all cross peaks arising from unlabeled residues, and observation of only Ala(C^{β}–H^{β}) and Val($C^{\gamma 1/\gamma 2}$–$H^{\gamma 1/\gamma 2}$) correlations. Though Ala(C^{β}–H^{β}) and Val($C^{\gamma 1/\gamma 2}$–$H^{\gamma 1/\gamma 2}$) cross peaks partially overlap with one another, cross metabolism of Ala to Val prevented us to unlabel Ala residues. In the second sample, only Ile, Lys and Thr residues were selectively unlabeled, resulting in the observation of only Ala(C^{β}–H^{β}), Val($C^{\gamma 1/\gamma 2}$–$H^{\gamma 1/\gamma 2}$) and Leu($C^{\delta 1/\delta 2}$–$H^{\delta 1/\delta 2}$) correlations. Owing to the fact that Val($C^{\gamma 1/\gamma 2}$–$H^{\gamma 1/\gamma 2}$) and Leu($C^{\delta 1/\delta 2}$–$H^{\delta 1/\delta 2}$) cross peaks partially overlap with one another, cross metabolism of Val to Leu prevented us to unlabel Val residues. However, with the aid of above mentioned two protein preparations, stereospecific assignments for all the CH_3 groups of Val and Leu residues were obtained unambiguously. This methodology was successfully utilized even in the case of a malate synthase G (723 residues enzyme) for the stereospecific assignments of all the CH_3 groups of 223 Val and Leu residues present in it (Tugarinov and Kay 2003).

Other strategies proposed to achieve such a stereospecific distinction between the CH_3 groups in proteins include both chemical as well as biochemical methods (Ostler et al. 1993; Tate et al. 1995). Alternatively, new pulse sequences have been proposed. In one method, magnitude of the $^{3}J(H^{\gamma}$–$C^{\delta})$ for Leu residues were measured and utilized to distinguish its *pro-R* CH_3 group from that of *pro-S* (Sattler et al. 1992). In this method, stereospecific assignments of β-methylene groups are needed as a prerequisite along with the values of $^{3}J(H^{\beta}$–$C^{\delta})$ and $^{3}J(H^{\beta}$–$H^{\gamma})$ in Leu residues, which cannot be obtained easily for large sized proteins. In another attempt, a 2D spin-echo difference experiment has been proposed for stereospecific assignments of Val CH_3 groups (Vuister et al. 1993). This method is based on the measurement of $^{3}J(^{13}C^{\gamma}$–$^{15}N)$ or $^{3}J(^{13}C^{\gamma}$–$^{13}C')$ coupling constants. However, this experiment does not throw light on the stereospecific assignment of Leu CH_3 groups. In yet

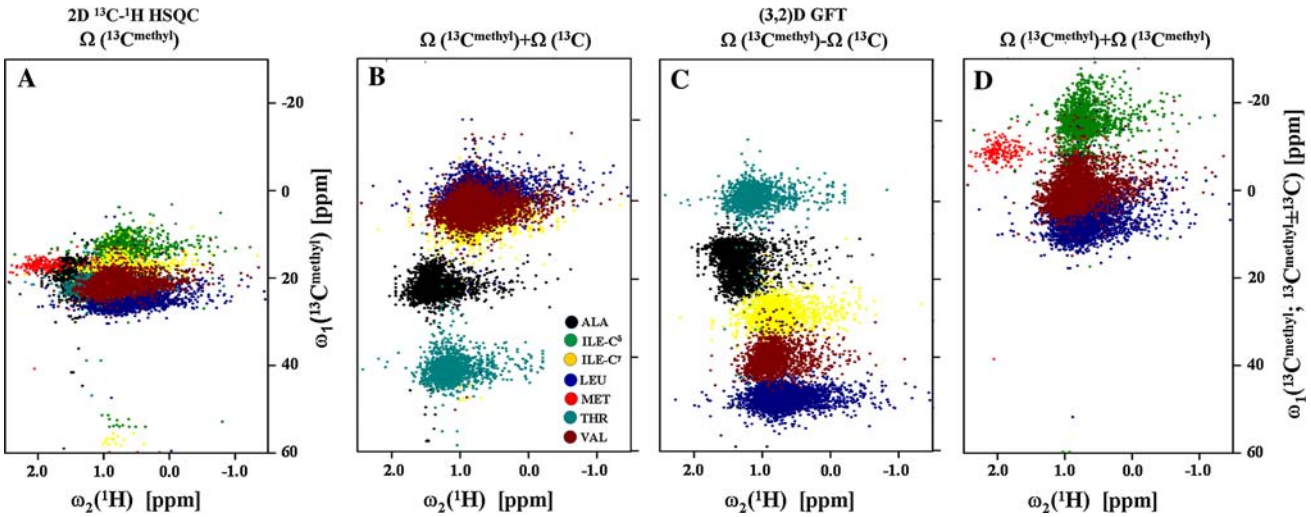

Fig. 1 Simulation and comparison of two sub-spectra of (3, 2)D CT-HCCH-COSY (**B**, **C**, **D**) with a regular 2D [^{13}C–^{1}H] HSQC spectrum. (**A**) containing ^{13}C–^{1}H cross peaks of CH_3 groups in Ala($^{13}C^{\beta}$–$^{1}H^{\beta}$), Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Ile($^{13}C^{\gamma}$–$^{1}H^{\gamma}$), Leu($^{13}C^{\delta}$–$^{1}H^{\delta}$), Met($^{13}C^{\varepsilon}$–$^{1}H^{\varepsilon}$), Thr($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) and Val($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) (**B**) the subspectra of (3, 2)D CT-HCCH-COSY each comprising of peaks at (ω_1, ω_2) [$\Omega(^{13}C^{Me}) + \kappa^{*}\Omega(^{13}C)$, $\Omega(^{1}H)$] for all the Ala($^{13}C^{\beta}$–$^{1}H^{\beta}$), Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Ile($^{13}C^{\gamma}$–$^{1}H^{\gamma}$), Leu($^{13}C^{\delta}$–$^{1}H^{\delta}$), Thr($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) and Val($^{13}C^{\gamma}$–$^{1}H^{\gamma}$). (**C**) the subspectra of (3, 2)D CT-HCCH-COSY each comprising of peaks at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) - \kappa^{*}\Omega(^{13}C)$, $\Omega(^{1}H)$] for all the Ala($^{13}C^{\beta}$–$^{1}H^{\beta}$), Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Ile($^{13}C^{\gamma}$–$^{1}H^{\gamma}$), Leu($^{13}C^{\delta}$–$^{1}H^{\delta}$), Thr($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) and Val($^{13}C^{\gamma}$–$^{1}H^{\gamma}$). (**D**) the subspectra of (3, 2)D CT-HCCH-COSY each comprising of peaks at (ω_1, ω_2) [$\Omega(^{13}C^{Me}) + \kappa^{*}\Omega(^{13}C^{Me})$, $\Omega(^{1}H)$] for all Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Leu($^{13}C^{\delta 2}$–$^{1}H^{\delta 2}$), Met($^{13}C^{\varepsilon}$–$^{1}H^{\varepsilon}$), and Val($^{13}C^{\gamma 2}$–$^{1}H^{\gamma 2}$). The scaling factor κ is set to 1.0. This characterization is based on the statistical analysis of chemical shift data of proteins available in the BioMagResBank (BMRB; http://www.bmrb.wisc.edu) which includes a database of 235 proteins

another method, a 3D CT-HCCH-COSY pulse sequence has been proposed to resolve the overlapping ^{1}H–^{13}C cross peaks arising from Val and Leu residues in a 2D-HSQC, by spreading them in the third dimension (Hu and Zuiderweg 1996), which is very time consuming.

As an alternate to these methodologies, we looked at the feasibility of modulating the chemical-shifts of the ^{13}C nucleus directly attached to various $^{13}CH_3$ groups present in a given protein, using (3,2)D CT-HCCH-COSY, an experiment based on the principle of G-matrix Fourier transform (GFT) NMR spectroscopy (Atreya and Szyperski 2004, 2005; Kim and Szyperski 2003; Szyperski and Atreya 2006). This results in significant dispersion in the spectral region containing ^{13}C–^{1}H cross peaks of CH_3 groups belonging to Ala, Ile, Leu, Met, Thr and Val residues. This lead us to carry out serendipitous identification/grouping of all the ^{13}C–^{1}H cross peaks of CH_3 groups belonging to Ala, Ile, Leu, Met, Thr and Val residues. Further, this also enabled to unambiguously carry out stereospecific resonance assignment of Leu and Val methyl groups in partially (10%) ^{13}C-labelled proteins. The experiment is basically the same as that of constant-time version of 3D HCCH-COSY experiment, which provides all the expected ^{13}C–^{1}H correlations.

The proposed new methodology is demonstrated with M-crystallin, a 85 amino-acid residue long protein (Barnwal et al. 2006). For this purpose, a fractionally (10%) ^{13}C-labeled M-crystallin sample was prepared as described by Barnwal et al. (2006) All NMR experiments with such sample were performed at 25 °C on a Bruker Avance 800 and a 700 MHz spectrometers both equipped with a cryogenic RF-probes.

The radio-frequency (r.f.) pulse scheme used for recording (3, 2)D CT-HCCH-COSY experiment is shown in Fig. 2. For the nuclei shown underlined (the two ^{13}C spins), the chemical shifts are jointly sampled, thereby providing 3D spectral information in the form of a 2D spectrum rapidly with high precision (Atreya and Szyperski 2004, 2005; Kim and Szyperski 2003; Szyperski and Atreya 2006). During the delay period τ_2 magnetization on *pro*-R $^{13}C^{Methyl}$ spin (abbreviated as $^{13}C^{Me}$) becomes anti-phase with respect to the directly attached ^{13}C spin, which is then frequency labeled during the chemical shift evolution period, t_1. During the reverse transfer period, $^{13}C^{Me}$ spins are frequency labeled in a constant-time manner. The delay, $2\tau_2$, is adjusted to $1/2J_{CC}$ ($\sim$14.0 ms). In the case of *pro*-S $^{13}C^{Me}$ groups, due to the absence of any neighbouring ^{13}C spin in fractionally ^{13}C-labeled proteins, the $^{13}C^{Me}$ magnetization remains in-phase throughout the sequence resulting in a signal opposite in sign compared to those arising from the *pro*-R methyl groups. Phase-sensitive joint-sampling of chemical shifts of $^{13}C^{Me}$ and neighbouring ^{13}C spins directly attached to $^{13}C^{Me}$ is achieved by co-incrementing their respective chemical-shift evolution periods with the ^{13}C shifts scaled by a factor 'κ' relative to $^{13}C^{Me}$ (Atreya and Szyperski 2004, 2005; Szyperski and Atreya 2006). This results, after G-matrix transformation, in two sub-spectra each comprising of peaks at a given linear combination of chemical shifts along the indirect dimension (t_1): ω_1: $\Omega(^{13}C^{Me}) \pm \kappa^*\Omega(^{13}C)$, where the two ^{13}C spins belong to a directly coupled pair.

As discussed earlier (Atreya and Szyperski 2005), the scaling factor, κ, not only allows one to increase the dispersion of peaks but also to restrict the chemical shift evolution of ^{13}C to avoid loss in sensitivity due to transverse relaxation during the t_1 (Atreya and Szyperski 2004, 2005; Szyperski and Atreya 2006). An additional 2D [^{13}C, ^{1}H] CT-HSQC recorded with the same sample provides central peak information (ω_1: $\Omega(^{13}C)$) needed to analyze the data (Atreya and Szyperski 2004, 2005; Szyperski and Atreya 2006). In (3, 2)D CT-HCCH-COSY experiment, the observed cross-peaks are stereospecifically characterized by the chemical shift information of $^{13}C^{Me}$ and its directly attached ^{13}C and their relative sign in the 2D spectrum (Atreya and Chary 2001).

To illustrate this point, we have simulated the two sub-spectra of (3, 2)D CT-HCCH-COSY spectrum (Fig. 1B, C) each comprising of peaks at $(\omega_1, \omega_2) = [\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C), \Omega(^{1}H)]$ and $[\Omega(^{13}C^{Me}) - \kappa^*\Omega(^{13}C), \Omega(^{1}H)]$ for all the methyl groups of Ala, Ile, Leu, Thr, and Val. The correlations arising from Arg(C^{γ}–H^{γ}), Ile($C^{\delta 1}$–$H^{\delta 1}$), Leu($C^{\delta 2}$–$H^{\delta 2}$), Lys(C^{δ}–H^{δ}), Met(C^{ε}–H^{ε}) and Val($C^{\gamma 2}$–$H^{\gamma 2}$) are excluded in this simulation. Peaks corresponding to Arg(C^{γ}–H^{γ}) and Lys(C^{δ}–H^{δ}) correlations are not observed in the spectrum, owing to the fact that the corresponding carbon nuclei (Arg(C^{γ}) and Lys(C^{δ})) have two neighboring ^{13}C partners and hence their transfer amplitude during the delay, $2\tau_2$, is modulated as $\cos(2\pi^{1}J_{CC}\tau_2)^*\sin(2\pi^{1}J_{CC}\tau_2)$ which is zero, when $2\tau_2$ is set to 14.0 ms (Fig. 2). On the other hand, the Ile($C^{\delta 1}$), Leu($^{13}C^{\delta 2}$), Met($^{13}C^{\varepsilon}$) and Val($^{13}C^{\gamma 2}$) have no directly attached ^{13}C in fractionally ^{13}C labeled proteins. Hence, corresponding correlations appear as diagonal peaks in the sub-spectrum comprising of peaks at $(\omega_1, \omega_2) = [\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C), \Omega(^{1}H)]$ with an opposite sign, with their individual chemical shifts at $(\omega_1, \omega_2) = [\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C^{Me}), \Omega(^{1}H)]$. This characterization is based on the statistical analysis of degenerate chemical shift data of 235 proteins taken from the BioMagResBank (BMRB; http://www.bmrb.wisc.edu). As is evident from the simulations presented here (Fig. 1), modulating the chemical shift information of the directly attached ^{13}C on to the indirectly detected $^{13}C^{Me}$ spins results in a significant resolution in the cross peaks arising from Ala($C^{\beta} + C^{\alpha}$, H^{β}), Ile($C^{\gamma} + C^{\beta}$, $H^{\gamma Me}$), Leu($C^{\delta 1} + C^{\gamma}$, $H^{\delta 1}$), Val($C^{\gamma 1}$–C^{β}, $H^{\gamma 1}$) and Thr($C^{\gamma} + C^{\beta}$, H^{γ}) correlations (Fig. 1B, C) as compared to that seen in Fig. 1A. Besides, we

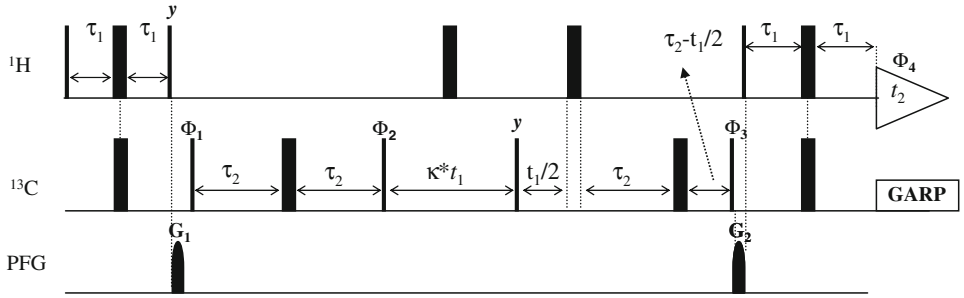

Fig. 2 R.f. pulse scheme of GFT (3, 2)D CT-HCCH-COSY for stereospecific identification of various methyl groups in fractionally ^{13}C-labeled protein. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no r.f. phase is marked, the pulse is applied along x. High-power 90° pulse lengths are: 8.7 μs for ^{1}H, 37 μs for ^{15}N and 15.8 μs for ^{13}C. $\kappa = 0.5$ (see text). The ^{1}H r.f. carrier is placed at the position of the solvent line at 4.7 ppm. The ^{15}N carrier position is set to 118.5 ppm. The ^{13}C r.f. carrier is kept at 20 ppm throughout the sequence. GARP (Shaka et al. 1985) is employed to decouple ^{13}C during acquisition. All pulsed z-field gradients (PFGs) are rectangular shaped with gradient recovery delay of 100 μs. The duration and strengths of the PFGs are: G1 (0.5 ms, 22 G/cm); and G2 (0.5 ms, 22 G/cm). The delays are: $\tau_1 = 1.7$ ms, $\tau_2 = 7.0$ ms. Phase cycling: $\phi_1 = x, -x$; $\phi_2 = y$; ϕ_4(receiver) $= x, -x$. Quadrature detection of $t_1(^{13}C^{Me})$ is carried out in States-TPPI manner using $\phi_3 = x, y$. GFT NMR phase-cycle: $\phi_1 = x, y$ and $\phi = y, -x$ in concert yields, in conjunction with quadrature detection in $t_1(^{13}C^{Me})$, 2 data sets which are linearly combined employing a G-matrix transformation with the G-matrix (Atreya and Szyperski 2005; Kim and Szyperski 2003)

could also resolve the diagonal peaks (discussed later) arising from Ile($C^{\delta 1} + C^{\delta 1}$, $H^{\delta 1}$), Leu($C^{\delta 2} + C^{\delta 2}$, $H^{\delta 2}$), Met($C^{\varepsilon} + C^{\varepsilon}$, H^{ε}) and Val($C^{\gamma 2} + C^{\gamma 2}$, $H^{\gamma 2}$) (Fig. 1D) correlations.

In the sub-spectra comprising of peaks at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C)$, $\Omega(^{1}H)$] (Fig. 1B), one can distinctly identify Ala($C^{\beta} + \kappa^*C^{\alpha}$, H^{β}) and Thr($C^{\gamma 2} + \kappa^*C^{\beta}$, $H^{\gamma 2}$) correlations, though the peaks arising from Val($C^{\gamma 1} + \kappa^*C^{\beta}$, $H^{\gamma 1}$), Ile($C^{\gamma 2} + \kappa^*C^{\beta}$, $H^{\gamma 2}$) and Leu($C^{\delta 1} + \kappa^*C^{\gamma}$, $H^{\delta 1}$) show significant spectral overlap. This overlap problem however could be resolved in the sub-spectra comprising of peaks at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) - {}^*\Omega(^{13}C)$, $\Omega(^{1}H)$] (Fig. 1C), wherein one can uniquely identify Ala($C^{\beta} - \kappa^*C^{\alpha}$, H^{β}), Ile($C^{\gamma 2} - \kappa^*C^{\beta}$, $H^{\gamma 2}$), Leu ($C^{(\delta 1} - \kappa^*C^{\beta}$, $H^{\delta 1}$), Thr($C^{\gamma 2} - \kappa^*C^{\beta}$, $H^{\gamma 2}$) and Val($C^{\gamma 1} - \kappa^*C^{\beta}$, $H^{\gamma 1}$) correlations as they all appear in non-overlapping spectral regions.

As mentioned earlier, the Met($^{13}C^{\varepsilon}$), Ile($^{13}C^{\delta}$) and *pro*-S methyl groups of Leu($^{13}C^{\delta 2}$), Val($^{13}C^{\gamma 2}$) have no directly attached ^{13}C, and hence, no coherence transfer takes place during the delay τ_2. This results in co-incrementing of the same coherence during two consecutive t_1 periods. Hence, corresponding correlations appear in the sub-spectrum comprising of peaks at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C^{Me})$, $\Omega(^{1}H)$], with an opposite sign and their individual chemical shifts are seen at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C^{Me})$, $\Omega(^{1}H)$] (Fig. 3D). As is evident from Fig. 3D, one can distinctly identify the correlations arising from Ile($C^{\delta 1} + \kappa^*C^{\delta 1}$, $H^{\delta 1}$) and Met($C^{\varepsilon} + \kappa^*C^{\varepsilon}$, H^{ε}), though the other two Leu($C^{\delta 2} + \kappa^*C^{\delta 2}$, $H^{\delta 2}$) and Val($C^{\gamma 2} + \kappa^*C^{\gamma 2}$, $H^{\gamma 2}$) correlations show partial spectral overlap.

The (3, 2)D CT-HCCH-COSY experiment described herein further provides several other advantages. (i) The spectral dispersion thus obtained enables one to identify various spin systems which in turn aids in the sequence specific resonance assignments in general and side-chain resonance assignments in particular, in any given protein. (ii) The spectral information can be obtained rapidly with a good S/N ratio. The total measurement time can be further reduced in combination with other methods used in fast data collection. (iii) Data are acquired in the form of a 2D spectrum facilitating higher spectral/digital resolution. (iv) Spectra have higher dispersion due to joint sampling of two coupled ^{13}C spin-pair. And, (v) most importantly, there is no need to for any special means of spin labeling or unlabeling of proteins to suppress unwanted peaks.

The data were pre-processed with the G-matrix using an algorithm outlined in refs. (Atreya and Szyperski 2004, 2005; Szyperski and Atreya 2006) and subsequently processed with NMRPipe (Delaglio et al. 1995) and analyzed using XEASY (Bartels et al. 1995) and CARA (Keller 2004).

Results and discussion

Figure 3A shows the selected region of 2D CT-HSQC spectrum recorded with a fractionally (10%) ^{13}C-labeled M-Crystallin protein sample. Figure 3B and C show the sub-spectra (positive contours only) of (3, 2)D CT-HCCH-COSY of the same sample each comprising of peaks at a given linear combination of chemical shifts along the indirect dimension (t_1): ω_1: $\Omega(^{13}C) \pm \kappa^*\Omega(^{13}C)$, where the chemical shifts of the two ^{13}C spins belonging to a directly coupled pair appear. Figure 3D shows the diagonal peaks seen in a selected region of the sub-spectrum (negative

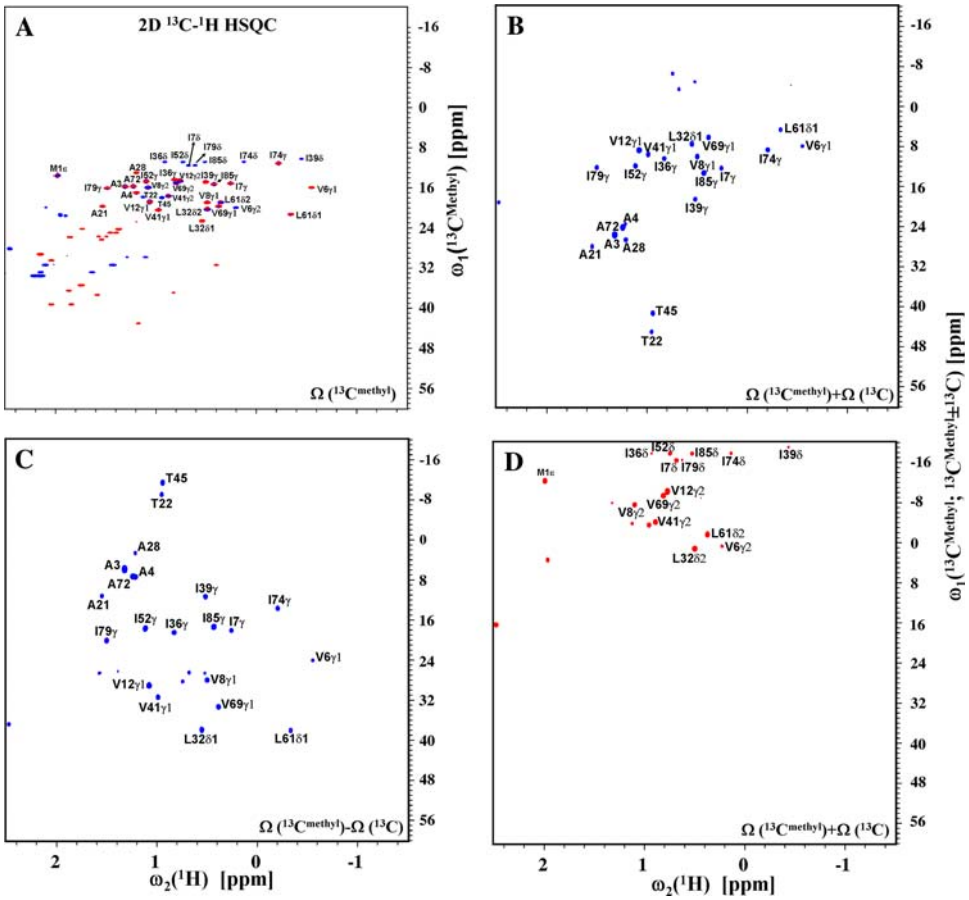

Fig. 3 Plot for M-crystallin, two sub-spectra of (3, 2)D CT-HCCH-COSY (**B**, **C**, **D**) with normal 2D [^{13}C-^{1}H] HSQC spectrum (**A**). (**A**) containing ^{13}C–^{1}H cross peaks of CH_3 groups in Ala($^{13}C^{\beta}$–$^{1}H^{\beta}$), Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Ile($^{13}C^{\gamma}$–$^{1}H^{\gamma}$), Leu($^{13}C^{\delta}$–$^{1}H^{\delta}$), Met($^{13}C^{\varepsilon}$–$^{1}H^{\varepsilon}$), Thr($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) and Val($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) (**B**) the subspectra of (3, 2)D CT-HCCH-COSY each comprising of peaks at (ω_1, ω_2) [$\Omega(^{13}C^{Me})\kappa^*\Omega(^{13}C)$, $\Omega(^{1}H)$] for all the Ala($^{13}C^{\beta}$–$^{1}H^{\beta}$), Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Ile($^{13}C^{\gamma}$–$^{1}H^{\gamma}$), Leu($^{13}C^{\delta}$–$^{1}H^{\delta}$), Thr($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) and Val($^{13}C^{\gamma}$–$^{1}H^{\gamma}$). The scaling factor κ is set to 0.5. (**C**) the subspectra of (3, 2)D CT-HCCH-COSY each comprising of peaks at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) - \kappa^*\Omega(^{13}C)$, $\Omega(^{1}H)$] for all the Ala($^{13}C^{\beta}$–$^{1}H^{\beta}$), Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Ile($^{13}C^{\gamma}$–$^{1}H^{\gamma}$), Leu($^{13}C^{\delta}$–$^{1}H^{\delta}$), Thr($^{13}C^{\gamma}$–$^{1}H^{\gamma}$) and Val($^{13}C^{\gamma}$–$^{1}H^{\gamma}$). (**D**) the subspectra of (3, 2)D CT-HCCH-COSY each comprising of peaks at (ω_1, ω_2) [$\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C^{Me})$, $\Omega(^{1}H)$] for all Ile($^{13}C^{\delta}$–$^{1}H^{\delta}$), Leu($^{13}C^{\delta 2}$–$^{1}H^{\delta 2}$), Met($^{13}C^{\varepsilon}$–$^{1}H^{\varepsilon}$), and Val($^{13}C^{\gamma 2}$–$^{1}H^{\gamma 2}$)

contours) comprising of peaks at (ω_1, ω_2) = [$\Omega(^{13}C^{Me}) + \kappa^*\Omega(^{13}C^{Me})$, $\Omega(^{1}H)$], with a sign opposite to those seen in panel 3B.

The 85 amino-acid residue long Cys-free M-crystallin protein has 5 Ala, 7 Ile, 2 leu, 1 Met, 3 Thr and 5 Val residues. As is evident from Fig. 3B, we could readily identify all the expected connectivities arising from Ala($C^{\beta} + \kappa^*C^{\alpha}$, $H^{\beta Me}$) and Thr($C^{\gamma 2} + \kappa^*C^{\beta}$, $H^{\gamma 2}$) correlations. On the other hand, with the use of Fig. 3C, we could uniquely identify all the expected connectivities arising from Ala($C^{\beta} - \kappa^*C^{\alpha}$, $H^{\beta Me}$), Ile($C^{\gamma 2} - \kappa^*C^{\beta}$, $H^{\gamma 2}$), Leu($C^{\delta 1} - \kappa^*C^{\gamma}$, $H^{\delta 1}$), Thr($C^{\gamma 2} - \kappa^*C^{\beta}$, $H^{\gamma 2}$) and Val($C^{\gamma 1} - \kappa^*C^{\beta}$, $H^{\gamma 1Me}$) correlations. Further, Fig. 3D was used to distinctly identify the correlations arising from Ile($C^{\delta 1} + \kappa^*C^{\delta}$, $H^{\delta 1}$) and Met($C^{\varepsilon} + \kappa^*C^{\varepsilon}$, H^{ε}), though the other two Leu($C^{\delta 2} + \kappa^*C^{\delta 2}$, $H^{\delta 2}$) and Val($C^{\gamma 2} + \kappa^*C^{\gamma 2}$, $H^{\gamma 2}$) correlations show partial spectral overlap. However, earlier distinct identification of all the connectivities arising from Leu($C^{\delta 1} + \kappa^*C^{\delta 1}$, $H^{\delta 1}$) and Val($C^{\gamma 1} + \kappa^*C^{\gamma 1}$, $H^{\gamma 1}$) correlations aided in an unambiguous stereospecific resonance assignment of all the Leu and Val methyl groups present in the protein. Further, one may record CCC/HCC-TOCSY-NNH experiments for the identification of the ^{15}N and $^{1}H^{N}$ resonances of the corresponding methyl containing residues distinctly.

Conclusion

In conclusion, we could successfully modulate the chemical-shifts of the ^{13}C spin that is directly attached to various $^{13}CH_3$ groups present in proteins with the proposed (3, 2)D CT-HCCH-COSY experiment. This propelled significant dispersion in the spectral region containing ^{13}C–^{1}H cross peaks of CH_3 groups belonging to Ala, Ile, Leu, Met, Thr and Val residues. This aided in rapid identification and grouping of all the ^{13}C–^{1}H cross peaks of CH_3 groups belonging to Ala, Ile, Leu, Met, Thr and Val residues, which in turn aids in the sequence-specific resonance assignments in general and side-chain resonance assignments in particular, in any given protein. Further, this experiment enabled us to distinguish between the pro-R and pro-S methyl groups belonging to the Leu and Val residues in this partially ^{13}C-labelled protein. The measurement times for these experiments can be further reduced in combination with other fast data collection methods, such as longitudinal ^{1}H relaxation optimization (Atreya and Szyperski 2004). In the case of large proteins, the experiments presented here can be combined with partial deuteration (Gardner and Kay 1998) and TROSY (Pervushin 2000) for increased resolution/sensitivity. Taken together, the proposed experiment will have wide

range of applications in sequence specific resonance assignments in general and side-chain resonance assignment strategies in particular and structure determination of proteins.

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