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Efficient assignment of methyl resonances: enhanced sensitivity by gradient selection in a DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment

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Abstract

We present a gradient selected and doubly sensitivity-enhanced DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment for the sequence-specific assignment of methyl resonances in ^{13}C,^{15}N labeled proteins. The proposed experiment provides improved sensitivity and artifact suppression relative to the phase-cycled experiments. One part of the ^{13}C chemical shift evolution takes place under heteronuclear multiple quantum coherence, whereas the other part occurs under ^{13}C single quantum coherence in a semi-constant time fashion. The feasibility of the experiment was assessed using ^{15}N,^{13}C labeled *Mus musculus* coactosin (16 kDa), having a rotational correlation time of 14.5 ns at 15 °C in D_2O. A 16-h experiment on 600 MHz ^{1}H yielded good quality data and enabled the assignment of 70 out of 72 methyl groups in coactosin. As well as being an improved approach for methyl resonance assignment, this experiment can also be highly valuable for the rapid assignment of methyl resonances in SAR by NMR studies.

Introduction

Hydrophobic amino acids are often buried within a folded protein, where they interact with similar neighbors forming a core of the protein. Thereby, they serve as a source for valuable NOE restraints for protein structure determination. Cavities in hydrophobic regions offer a non-polar environment, where many enzymatic reactions occur and where ligand-binding sites can often be localized. Assignment of the methyl groups is then of key importance for both structural and functional studies of proteins, as a prerequisite step for the characterization of long-range intra- and intermolecular NOEs. Residues comprising methyl groups are typically assigned using either ^{13}C–^{1}H detected HCCH–TOCSY/COSY (Bax et al., 1990a, b; Fesik et al., 1990; Kay et al., 1990a, b) or ^{15}N–^{1}H^N detected (H)CCNH–TOCSY/(H)CC(CO)NH–TOCSY and H(CC)NH–TOCSY/H(CC)(CO)NH–TOCSY experiments (Montelione et al., 1992; Grzesiek et al., 1993; Logan et al., 1993; Lyons and Montelione, 1993; Gardner et al., 1996; Liu and Wagner, 1999). The ^{15}N–^{1}H^N detected TOCSY experiments are widely used owing to their excellent resolution in ^{15}N and ^{1}H^N dimensions. In addition, these experiments are often more attractive than their ^{13}C–^{1}H detected counterparts since efficient water suppression can be obtained by utilizing sensitivity-enhanced gradient selection, which is optimal for ^{15}N–^{1}H^N moieties (Kay et al., 1992). Furthermore, ^{1}H^N spins also resonate far from the water signal, which facilitates the data interpretation. For larger ^{15}N,^{13}C labeled proteins, the coherence transfer efficiency

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drops dramatically in HCC(CO)NH–TOCSY type of experiments due to the inefficient ^{13}C–^{13}C TOCSY transfer followed by relatively long transfer steps from $^{13}C^{\alpha}$ (or via $^{13}C'$) to the ^{15}N,^{1}H spin pair. The transfer throughput can be significantly enhanced utilizing fractional deuteration or selective methyl protonation for branched chain amino acids on otherwise deuterated aliphatic carbon background. A procedure for selective methyl-protonation of Val, Ile and Leu residues on ^{13}C,^{15}N,^{2}H labeled proteins has been recently introduced by Kay and co-workers (Gardner et al., 1997a, b; Goto et al., 1999). By employing the TROSY approach on these (H)CCNH–TOCSY/(H)CC(CO)NH–TOCSY experiments their performance can be further improved (Hilty et al., 2002), although the gain in sensitivity is negatively counterbalanced by the rapid transverse relaxation of $^{13}C^{\alpha}$ spin or $^{13}C'$ spin at the field strength optimal for the TROSY effect (Permi and Annila, 2004). Thus, for larger proteins the use of the significantly shorter ^{13}C–^{1}H detected experiments offers a far more sensitive approach. Tugarinov and Kay proposed a ^{13}C–^{13}C COSY transfer for assigning selectively protonated methyl resonances with the aid of the (H)C(CA)–COSY experiment (Tugarinov and Kay, 2003a). They have also proposed new labeling methods for linearizing the spin systems of Leu and Val residues by replacing one of the methyl groups ($^{13}CH_3$) with $^{12}CD_3$ isotopes. Thereupon, they presented a set of ^{13}C–^{13}C COSY based experiments for assigning these specifically labeled samples (Tugarinov and Kay, 2003b).

A somewhat different approach was suggested earlier by Uhrín and co-workers (2000) and more recently by Yang et al., (2004). In their approach, methyl resonances are assigned in a sequence-specific manner by linking the ^{13}C and ^{1}H chemical shifts of methyl groups with the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts. To briefly outline the course of the $HCCH_3$–TOCSY or MQ–(H)CC_mH_m–TOCSY experiments, the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts are first labeled in F_1 dimension and afterwards the desired magnetization is relayed to the methyl carbons using the ^{13}C–^{13}C TOCSY transfer. The subsequent frequency labeling of ^{13}C methyl resonances during the relatively long (~28 ms) constant-time period, followed by proton detection, provides excellent resolution and establishes the assignment of methyl-containing residues with high sensitivity; thanks to the slow transverse relaxation of methyl resonances. Remarkably, this $HCCH_3$–TOCSY (Uhrín et al., 2000) or MQ–(H)CC_mH_m–TOCSY (Yang et al., 2004) based approach is very efficient also for large ^{15}N,^{13}C labeled proteins, as demonstrated for the 42 kDa ^{15}N,^{13}C labeled acyl carrier protein synthase (AcpS) homotrimer (Yang et al., 2004), hence providing a cost-effective alternative to the relatively expensive production of selectively methyl-protonated samples. We have further elaborated this approach by improving the sensitivity of these methyl-detected experiments. In this paper, we show that a significant improvement in sensitivity can be obtained for a protein with a rotational correlation time of 14.5 ns by using the doubly sensitivity-enhanced DE-MQ–(H)CC_mH_m–TOCSY experiment with gradient selection in both indirectly detected dimensions.

Materials and methods

The proposed DE-MQ–(H)CC_mH_m–TOCSY pulse scheme was compared experimentally with the MQ–(H)CC_mH_m–TOCSY experiment (Yang et al., 2004) on 1 mM uniformly ^{15}N,^{13}C labeled coactosin (Hellman et al.,) having a molecular mass of 16 kDa (142 amino acid residues), dissolved in D_2O, 10 mM Bis–Tris buffer (pH 6.0), 50 mM NaCl, 1 mM DTT in a 300 µl Shigemi microcell at 15 °C. Both experiments were carried out on a Varian Unity INOVA 600 NMR spectrometer, equipped with a $^{15}N/^{13}C/^{1}H$ triple-resonance probehead and an actively shielded z-axis gradient system. Spectra were acquired using four transients per FID with 60, 64, and 2048 complex points, corresponding to acquisition times of 5, 27, and 128 ms in t_1, t_2, and t_3, respectively. Delays used were $\tau_a = 1.7$ ms, $\tau_b = 1.1$ ms, $\tau_c = 1.53$ ms, $\tau_d = 0.6$ ms, $\tau_e = 2$ ms, and $T_C = 14$ ms. Isotropic DIPSI-3 (Shaka et al., 1988) TOCSY sequence with 12.2 ms mixing time was utilized for ^{13}C–^{13}C transfer (z-filtration was utilized in the MQ–(H)CC_mH_m–TOCSY experiment). The data were zero-filled to $256 \times 512 \times 2048$ points before Fourier transform and phase-shifted squared sine-bell window functions were applied in all three dimensions.

Results and discussion

Description of the pulse sequence

The pulse sequence of the doubly sensitivity enhanced (Sattler et al., 1995a), gradient selected $C^{\alpha}C^{\beta}$–TOCSY-C_mH_m(DE-MQ–(H)CC_mH_m–TOCSY) experiment is depicted in Figure 1. The general course of the DE-MQ–(H)CC_mH_m–TOCSY is identical to the original experiments (Uhrín et al., 2000; Yang et al., 2004) and consequently we will mainly emphasize the differing parts, i.e., on the key elements that enable sensitivity-enhancement in the proposed experiment.

Magnetization flows through the experiment as follows

$$^{1}H_{(i)} \xrightarrow{^{1}J_{CH}} {}^{13}C_{(i)}(t_1) \xrightarrow{^{1}J_{CC}(\mathrm{TOCSY})}$$

$$^{13}C^{m}_{(i)}(\mathrm{CT}-t_2) \xrightarrow{^{1}J_{CH}(yxz-\mathrm{ICOS}-\mathrm{CT})} {}^{1}H^{m}_{(i)}(t_3),$$

where the couplings which are facilitating the transfer are shown above the arrows, and t_i ($i = 1$–3) refer to the acquisition time for the corresponding spin.

Initially, the magnetization is transferred from the aliphatic protons ($^{1}H^{\alpha}$ and $^{1}H^{\beta}$ in particular) to the adjacent aliphatic carbon spins ($^{13}C^{\alpha}$ and $^{13}C^{\beta}$) by utilizing HMQC type transfer. The succeeding 90° pulse on ^{13}C (with phase ϕ_1) converts the antiphase ^{1}H magnetization into ^{1}H–^{13}C multiple-quantum coherence, described by the density operator $2H_xC_y$. During the subsequent t_1 period, the chemical shift of the ^{13}C spin is recorded. Yang and co-workers observed that the use of multiple-quantum coherence for the ^{13}C chemical shift labeling improves the sensitivity as compared to the ^{13}C single quantum coherence (Yang et al., 2004). In our scheme, one part of the frequency labeling occurs under the heteronuclear multiple-quantum coherence, whereas the other part of the chemical shift evolution takes place under the heteronuclear single quantum coherence (Aitio and Permi, 2000). This implementation enables us to use the delay $2\tau_b$ also for the ^{13}C chemical shift labeling, which improves the overall sensitivity to some extent (see caption of Figure 1 for details). It is worth pointing out that although this implementation resembles the familiar shared (or semi-constant) time chemical shift evolution (Grzesiek and Bax 1993; Logan et al., 1993), additional RF pulses are necessary in order to decouple the ^{1}H–^{13}C coupling during the t_1 period.

The second element, which improves the sensitivity and provides more efficient artifact

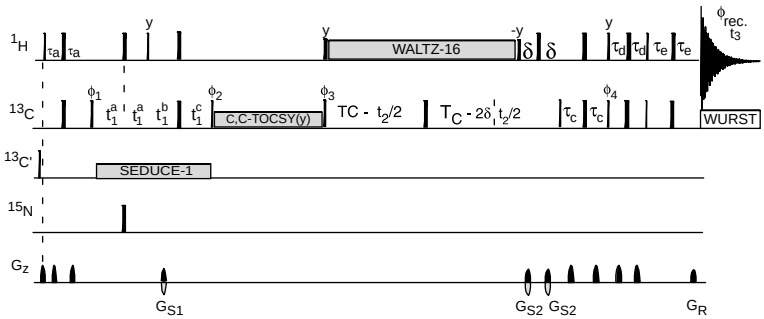

Figure 1. The doubly sensitivity-enhanced, gradient selected DE-MQ–(H)CC_mH_m–TOCSY experiment for the sequence-specific assignment of methyl resonances in ^{13}C,^{15}N labeled proteins. Narrow and wide bars correspond to 90° and 180° flip angles, respectively, applied with phase x unless otherwise stated. All 90° (180°) pulses for aliphatic carbons were applied with a strength of 20.8 kHz. The ^{1}H, ^{15}N, and $^{13}C'$ carrier positions are 4.7 (water), 120 (center of ^{15}N spectral region), and 175 ppm (center of $^{13}C'$ spectral region). The ^{13}C carrier is set initially to the middle of aliphatic ^{13}C region (39 ppm) and shifted to 20 ppm just after the 90° ϕ_3 pulse. Frequency discrimination in F_1 and F_2 is obtained using the sensitivity-enhanced gradient selection (Kay et al., 1992; Schleucher et al., 1993). The echo and antiecho signals in F_1 and F_2 dimensions are collected separately by inverting the sign of the G_{S1} and G_{S2} gradient pulses together with the inversion of ϕ_2, and ϕ_2 and ϕ_4, respectively. The nominal delay durations are: $\tau_a = 1/(4J_{HC}) \sim 1.7$ ms; $t_1^a = (1-\kappa)^* t_1/2$, $t_1^b = \tau_b + \kappa^* t_1/2$, $t_1^c = \tau_b - \kappa^* t_1/2$, $\tau_b = 1/(4J_{CH}) \sim 1.1$ ms; $\tau_c = 1/(4J_{CH}) \sim 0.76$ ms; $\tau_d = 1/(4J_{HmCm}) \sim 0.6$ ms; $\tau_e = 1/(4J_{HmCm}) \sim 2.0$ ms; $T_C = 1/(2J_{C^{\alpha}C^{\beta}}) \sim 14$ ms; δ = gradient + field recovery delay; $0 \le \kappa \le 2\tau_b/t_{1,\max}$. Gradient strengths (durations): $G_{S1} = 3.75$ G^{-1}cm (0.3 ms), $G_{S2} = 18$ G^{-1}cm (0.3 ms), $G_R = 10$ G/cm (0.3 ms). Phase cycling: $\phi_1 = y, -y$; $\phi_2 = x$; $\phi_3 = x$; $\phi_4 = y$; $\phi_{rec} = x, -x$. The DIPSI-3 spin-lock (9 kHz) was employed for ^{13}C–^{13}C transfer. The WALTZ-16 sequence (Shaka et al., 1983) was used to decouple ^{1}H spins during $2T_C$, whereas SEDUCE-1 shape (McCoy and Mueller, 1992) was applied for $^{13}C'$ decoupling during the t_1 period. The adiabatic WURST field (Kupce et al., 1995) was used to decouple ^{13}C during acquisition.

suppression, is the gradient selection (G_{S1}) during the t_1 period. As the following isotropic mixing element can successfully be used for transferring both the orthogonal magnetization components without prolonging the pulse scheme, a theoretical $\sqrt{2}$ gain in sensitivity should occur in comparison to the conventional hypercomplex frequency discrimination in which two amplitude modulated signals per t_1 increment are collected (Cavanagh and Rance, 1990; Sattler et al., 1995b; Kövér et al., 1998). The desired magnetization is then phase modulated by $\exp(i\omega_C t_1)$ after the t_1 period. The following 90° (^{13}C) pulses (with phases ϕ_2 and ϕ_3), flanking the isotropic TOCSY mixing sequence, transfer the orthogonal magnetization components along the amino acid sidechain to the methyl carbons. The chemical shift evolution of methyl carbons takes place during the t_2 period, implemented into the constant-time period ($2T_C$). The transverse relaxation time of methyl carbon is significantly longer than that of $^{13}C^{\alpha}/^{13}C^{\beta}$ spins, which enables the use of a long constant-time period (~28 ms) for the chemical shift labeling. This provides excellent resolution in the methyl carbon dimension (F_2), which is of utmost importance for the lucrative assignment strategy for methyl containing amino acids. Proton spin flips during the long $2T_C$ period will seriously deteriorate the sensitivity of the experiment in protonated samples. For this reason, proton decoupling is applied during most of the $2T_C$ period in order to attain higher sensitivity (Uhrín et al., 2000).

The third proposed element is the gradient selection (G_{S2}) in the t_2 period. An additional 180° (^1H) pulse is applied between the two gradient pulses to prevent J coupling evolution during the gradient echo. This implementation also removes the artifacts arising from the imperfect 180° (^1H) pulse between the gradients. Again, both the orthogonal magnetization components can be transferred from the methyl carbons to the methyl protons using the in-phase coherence-order selective coherence transfer (Sattler et al., 1995b). It is worth pointing out that the use of coherence-order selective transfer for the conversion of antiphase $2H_zC_m^-$ magnetization into H_m^- coherence, would actually result in sensitivity loss in comparison to the conventional INEPT transfer. In this case, however, we are aiming for the in-phase to in-phase ($C_m^- \rightarrow H_m^-$) coherence transfer using the gradient selection together with the *yxz*–ICOS–CT element (Sattler et al., 1995b). Although this transfer is less than optimal for the I_3S moieties as determined by the unitary bound on spin dynamics for Hermitian matrices (Sorensen, 1989; Sattler et al., 1995b; Untidt et al., 1998), it provides improved sensitivity with respect to refocused INEPT. Calculations are suggesting a sensitivity improvement by factor 1.22 (neglecting effects of relaxation and B_1 field inhomogeneity). The gradient selection in t_2 period is also essential for obtaining the heteronuclear gradient-echo, which in turn, offers superior suppression of non-carbon bound proton magnetization, e.g., water signal. Optimal sensitivity for the methyl moieties during the final *yxz*–ICOS–CT element can be obtained by setting the delay $2\tau_c$ to $0.196/J_{CmHm}$ (1.53 ms), whereas the delay $2\tau_d$ is set to $0.153/J_{CmHm}$ (1.2 ms, assuming $^1J_{CmHm} = 128$ Hz).

Thus, prior to acquisition, the signal of interest is phase modulated by $\exp(i\omega_C t_1)\exp(i\omega_{Cm} t_2)$. In this way, sensitivity-enhanced gradient selection can be applied to both indirectly detected ^{13}C dimensions, which in principle enables recording of three-dimensional spectrum with a single transient. In summary, for recording the doubly sensitivity-enhanced three-dimensional spectrum, four separate experiments are required with echo (E)–antiecho (A) selection in both indirectly detected dimensions;

$$\mathrm{A/A}: \quad H_m^- \exp(i\omega_C t_1)\exp(i\omega_{C_m} t_2)\exp(i\omega_{Hm} t_3), \tag{1}$$

$$\mathrm{E/E}: \quad H_m^- \exp(i\omega_C t_1)\exp(-i\omega_{C_m} t_2)\exp(i\omega_{Hm} t_3), \tag{2}$$

$$\mathrm{E/A}: \quad H_m^- \exp(-i\omega_C t_1)\exp(i\omega_{C_m} t_2)\exp(i\omega_{Hm} t_3), \tag{3}$$

$$\mathrm{A/E}: \quad H_m^- \exp(-i\omega_C t_1)\exp(-i\omega_{C_m} t_2)\exp(i\omega_{Hm} t_3). \tag{4}$$

After appropriate Fourier transform, i.e., by manipulating the data sets according to the sensitivity-enhanced echo–antiecho procedure (Kay et al., 1992) in both F_1 and F_2 dimensions, a three-dimensional spectrum results in which signal-to-noise ratio is increased by a factor of

1.73 for the desired correlations emerging at $\omega_C(i)$, $\omega_{Cm}(i)$, and $\omega_{Hm}(i)$. Typically, however, sensitivity gain is rendered somewhat smaller due to the greater number of RF pulses and additional delays employed during which the transverse relaxation will somewhat counterbalance the attainable sensitivity. The proposed DE-MQ–(H)CC$_m$H$_m$–TOCSY pulse sequence is 1.2 ms longer than the conventional, phase-cycled version of the experiment. On the contrary, the initial part of the DE-MQ–(H)CC$_m$H$_m$–TOCSY scheme is $(t_{1,\max} - 2\tau_b)$ shorter than the original experiment owing to the semi-constant time chemical shift evolution. Furthermore, homonuclear ^{13}C–^{13}C couplings and the apparent transverse relaxation rate are scaled down by the factor of $\kappa = (t_{1,\max} - 2\tau_b)/t_{1,\max}$. Relaxation during the final in-phase COS–CT element will not pose a serious problem in methyl groups since the transverse relaxation rates for $^{13}C_m$ and $^{1}H_m$ spins are relatively low even in larger proteins.

In order to assess the overall performance of the sensitivity-enhanced experiment, we applied the DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment for a 142-residue, uniformly ^{15}N,^{13}C-labeled coactosin, a cytoskeletal regulatory protein from *Mus musculus* (Hellman et al.,). The overall rotational correlation time τ_c of this protein in H_2O at 25 °C was found to be 10.1 ns, based on heteronuclear ^{15}N T_1 and T_2 relaxation measurements at 800 MHz ^{1}H. In order to mimic the assignment of methyl-containing amino acid residues on larger proteins, we dissolved coactosin in D_2O solution, and carried out the measurements at 15 °C. Under these conditions, the rotational correlation time for coactosin is approximately 14.5 ns, corresponding to a globular protein with a molecular mass of 30 kDa in H_2O at room temperature.

Figure 2 shows representative ω_1–ω_3 strip plots from the DE-MQ–(H)CC$_m$H$_m$–TOCSY spectrum recorded from ^{15}N,^{13}C enriched coactosin. It is easy to realize from Figure 2 that the assignment of methyl resonances can be established with the aid of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts. Owing to the inherently good dispersion of the methyl resonances together with high digital resolution attainable in the orthogonal ω_2 dimension, a minimal resonance overlap in the three-dimensional spectrum is guaranteed. Out of 142 residues in coactosin a total of 72 methyl resonances, belonging to 46 methyl-containing residues (10 Ala, 7 Leu, 8 Ile, 11 Val, and 10 Thr), are visible in the ^{13}C–CT–HSQC spectrum. We were able to assign 70 out of these 72 methyl groups, based on the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts and former sequential assignment of coactosin (Hellman et al.), which were established from HNCA/HN(CO)CA/iHNCA and HNCACB/HN(CO)CACB experiments (Grzesiek and Bax, 1992a, b; Wittekind and Mueller, 1992; Yamazaki et al., 1994; Permi, 2002). As already pointed out by Uhrín and coworkers, the (H)CC$_m$H$_m$–TOCSY type experiments are superior to the (H)CC(CO)NH–TOCSY type experiments, in the case of overlapping ^{15}N,^{1}H^{N} cross peaks, for the assignment of residues with two methyl groups since in the proposed approach the same $^{13}C^{\alpha}/^{13}C^{\beta}$ chemical shifts are observable in two different strips unless both $^{13}C_m$ and $^{1}H_m$ shifts are degenerate in the two methyl groups. This is exemplified for a pair of methyl groups found in V27, L58, and I82 residues in coactosin (Figure 2.).

In order to assess the sensitivity of the proposed DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment with respect to the conventional phase cycled pulse scheme, we compared the intensities of $^{13}C^{\alpha/\beta}$, $^{13}C_m$, $^{1}H_m$ cross peaks between these two experiments. For both experiments, DIPSI-3 mixing sequence was used as a ^{13}C-spin lock. In general, the sensitivity of the proposed scheme is excellent, enabling collecting of good quality data in 16 h on a 1 mM protein sample at 600 MHz ^{1}H without cryoprobe. The representative one-dimensional cross-sections from the MQ–(H)CC$_m$H$_m$–TOCSY (dashed line) and the proposed DE-MQ–(H)CC$_m$H$_m$–TOCSY (solid line) spectra are shown overlaid in Figure 3. As can be appreciated from the figure, DE-MQ–(H)CC$_m$H$_m$–TOCSY exhibits superior sensitivity with respect to the MQ–(H)CC$_m$H$_m$–TOCSY experiment. The sensitivity is improved with a factor of 1.4–2.0, with an average at 1.6, suggesting that the proposed implementation can afford a twofold reduction in the experimental time. It is plausible when moving towards higher magnetic fields (800 and 900 MHz) that the attainable gain in sensitivity decreases as DIPSI-3 becomes more inefficient on larger offsets than for instance FLOPSY-8 (Mohebbi and Shaka, 1991).

Side chain methyl groups have been found to exist more frequently in the walls of the

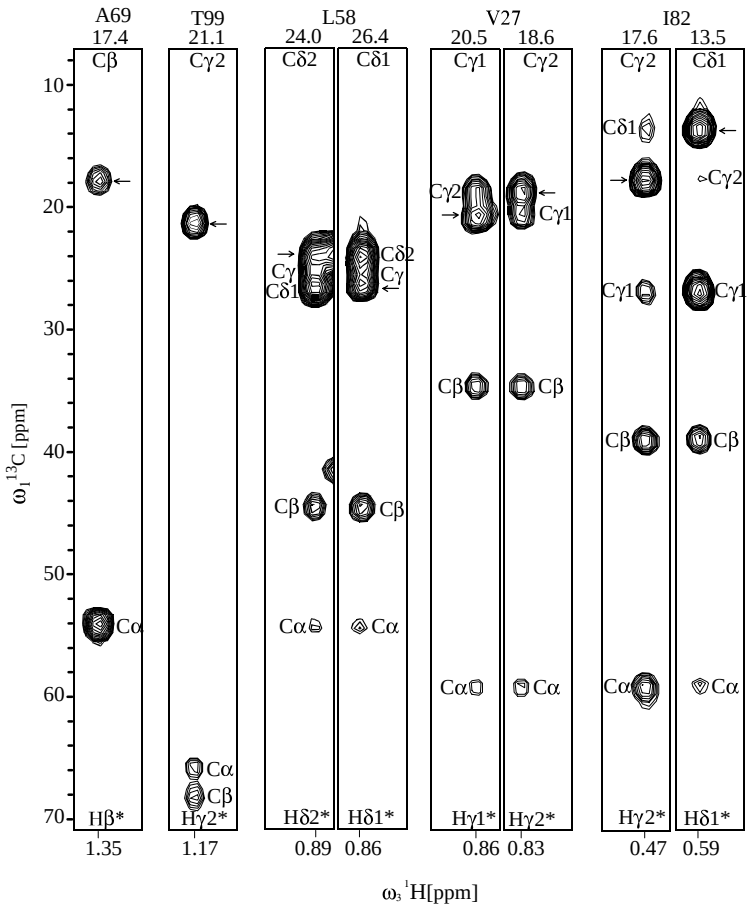

Figure 2. Representative strip plots from the ^{13}C–$^{1}H_m$ region of the DE–MQ–(H)CC$_m$H$_m$–TOCSY spectrum acquired on a 1 mM, ^{15}N,^{13}C-labeled coactosin on 600 MHz ^{1}H at 15 °C, using the pulse sequence in Figure 1. The acquisition time of the spectrum was 16 h. For each residue, cross peak identity is labeled. The ω_2 chemical shift is shown above the corresponding strip. The 'diagonal' peaks are marked with vertical arrows, and their corresponding ω_2 and ω_3 chemical shifts for $^{13}C_m$ and $^{1}H_m$ are shown above and below the corresponding strip, respectively.

hydrophobic cavities than backbone amide protons. Based on this observation, Hajduk et al. (2000) extended the application of chemical-shift-based method from commonly utilized perturbation in amide proton chemical shift (Hajduk et al. 1997) to detection of differences in side chain methyl group shifts. Chemical-shift based screening method for ligand binding reported by Hajduk et al. (2000) as well as protein structure determination based on distance restraints derived from methyl groups (Gardner et al., 1997a, b), can be applied to high molecular weight proteins when uniformly ^{15}N,^{13}C,^{2}H labeled, but selectively methyl protonated samples are available. Unfortunately, this is not economically viable particularly when protein expression levels are low or even moderately low. The proposed DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment is a generally applicable method for acquiring NMR data from hydrophobic methyl groups, e.g. chemical shift changes and intermolecular NOEs, for aiding not only structural but also interaction studies of protein–ligand complexes.

Conclusions

We have introduced a gradient selected, doubly sensitivity-enhanced DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment for the assignment of structurally and biologically important methyl resonances in ^{13}C labeled proteins. The proposed experiment

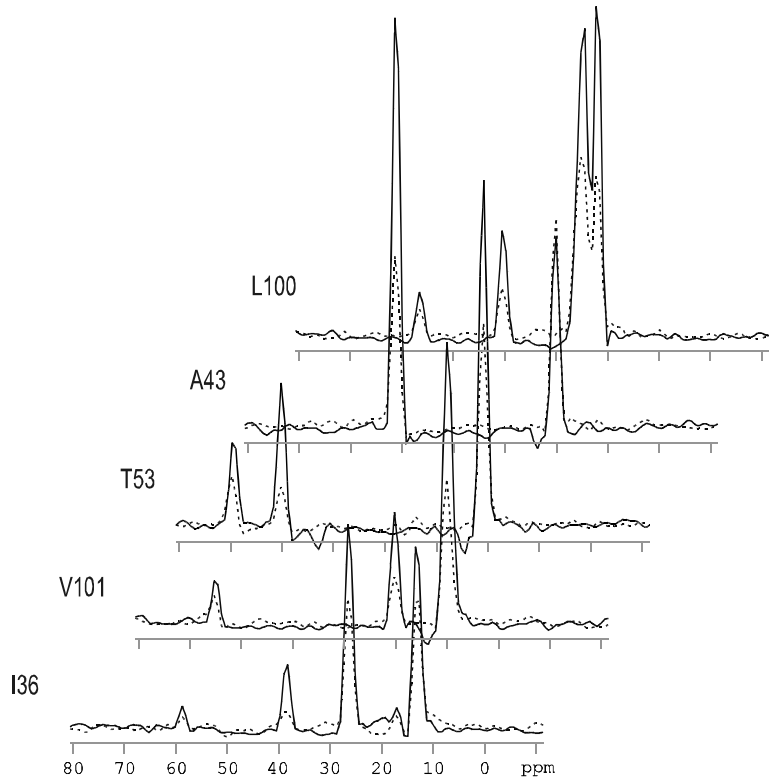

Figure 3. Five illustrating F_1 cross sections from the three dimensional MQ–(H)CC$_m$H$_m$–TOCSY and DE-MQ–(H)CC$_m$H$_m$–TOCSY spectra measured from coactosin. Data from the MQ–(H)CC$_m$H$_m$–TOCSY (Yang et al., 2004) is represented with dashed lines, and data from the DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment with solid lines. The spectra are normalized to have an identical noise level in each spectrum.

provides improved sensitivity and artifact suppression with respect to the previously described MQ–(H)CC$_m$H$_m$–TOCSY scheme. Basically, the new experiment is able to provide sequence-specific assignment of methyl resonances whenever HNCACB/HN(CO)CACB correlations can be established on ^{15}N,^{13}C enriched proteins. The gradient selection enables recording of three-dimensional spectrum with minimal phase cycling and offers superior elimination of unwanted coherences, e.g., water signal. The DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment is also ideal for rapidly re-establishing the sequence-specific assignment of methyl resonances if the assignment is lost owing to complex formation in SAR by NMR studies (Hajduk et al., 2000). This might occur when high-affinity ligands are in a slow exchange with the protein. We believe that the gradient selected DE-MQ–(H)CC$_m$H$_m$–TOCSY experiment offers an advantageous, general tool for ligand binding site mapping, protein and protein–ligand structure determination of even high molecular weight systems by using only ^{15}N,^{13}C labeled protein samples.

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