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An (H)C(CO)NH-TOCSY pulse scheme for sequential assignment of protonated methyl groups in otherwise deuterated $^{15}N,^{13}C$-labeled proteins

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Summary

A biosynthetic strategy has recently been developed for the production of $^{15}N,^{13}C,^{2}H$-labeled proteins using $^{1}H_3C$-pyruvate as the sole carbon source and D_2O as the solvent. The methyl groups of Ala, Val, Leu and Ile ($\gamma 2$ only) remain highly protonated, while the remaining positions in the molecule are largely deuterated. An (H)C(CO)NH-TOCSY experiment is presented for the sequential assignment of the protonated methyl groups. A high-sensitivity spectrum is recorded on a $^{15}N,^{13}C,^{2}H,^{1}H_3C$-labeled SH2 domain at 3 °C (correlation time 18.8 ns), demonstrating the utility of the method for proteins in the 30–40 kDa molecular weight range.

Recently developed $^{15}N,^{13}C,^{2}H$ triple-resonance spectroscopy has emerged as a powerful technique for the study of proteins and protein–ligand complexes (Grzesiek et al., 1993a; Yamazaki et al., 1994a,b; Farmer and Venters, 1995,1996; Shirakawa et al., 1995; Nietlispach et al., 1996; Shan et al., 1996). The significantly smaller gyromagnetic ratio (γ) of the deuteron relative to the proton ($\gamma_H/\gamma_D \sim 6.5$) results in a substantial lengthening of spin–spin relaxation times of ^{13}C and ^{1}H nuclei in proximity to the substituted deuterons (Grzesiek et al., 1993a; Markus et al., 1994; Yamazaki et al., 1994b). It is possible, therefore, to record spectra with significantly improved resolution and/or sensitivity using either partially or fully deuterated $^{15}N,^{13}C$-labeled samples.

The optimal level of sample deuteration depends to a certain extent on the size of the molecule under study and the type of experiment that will be performed. For example, Nietlispach et al. (1996) have suggested that a compromise level of approximately 50% fractional deuteration should be employed. In this case, the level of deuterium incorporation is sufficient to allow improved resolution and sensitivity for chemical shift assignment, while significant numbers of protons are retained to provide crucial distance constraints required for a structure determination. Fesik and co-workers have employed a strategy where a number of samples are utilized in structural studies, including fully protonated $^{15}N,^{13}C$- and $^{15}N,^{13}C$,75% ^{2}H-labeled molecules (Zhou et al., 1995; Muchmore et al., 1996). Recently, four-dimensional NMR experiments have been described for separating NOEs between NH protons on the basis of ^{15}N chemical shifts; these pulse schemes were applied to structural studies of perdeuterated HIV-1 Nef and carbonic anhydrase, respectively (Grzesiek et al., 1995; Venters et al., 1995). Our laboratory has developed a suite of experiments for the assignment of backbone ^{15}N, $^{13}C^{\alpha}$ and NH resonances as well as $^{13}C^{\beta}$ chemical shifts and applied the methodology to study a $^{15}N,^{13}C$, ~70% ^{2}H-labeled 37 kDa complex of trp repressor (trpR) and a 20-base-pair consensus sequence DNA (Yamazaki et al., 1994a,b). However, further studies on a 64 kDa tandem trpR complex necessitated the use of a >90% deuterated $^{15}N,^{13}C$ trpR sample for assignment of ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and NH chemical shifts (Shan et al., 1996).

The requirement of high levels of deuteration for the study of large-molecular-weight proteins and protein–ligand complexes has forced us to reconsider an initial strategy based on obtaining structures from a single 50–70% ^{2}H-labeled sample (Yamazaki et al., 1994b). While

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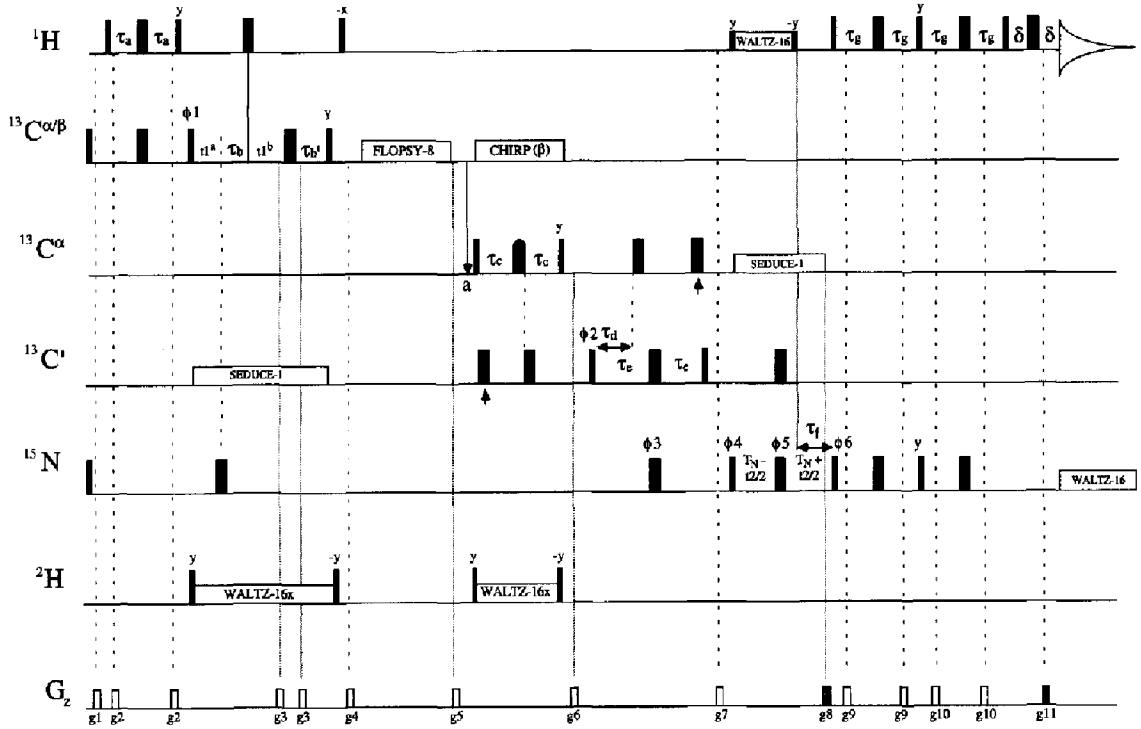

Fig. 1. Pulse sequence for the (H)C(CO)NH-TOCSY experiment, providing sequential assignments of Leu, Ile (γ2), Val and Ala methyl groups in ^{15}N,^{13}C,^{2}H,^{1}H$_3$C-labeled proteins. All narrow (wide) pulses are applied with a flip angle of 90° (180°) and are along the x-axis, unless indicated otherwise. The ^{1}H, ^{15}N, ^{13}C$^{\alpha/\beta}$, ^{13}C$^{\alpha}$, ^{13}C' (carbonyl) and ^{2}H carriers are centered at 4.98, 119, 43, 58, 177 and 3 ppm, respectively. Decoupling is interrupted prior to the application of the gradient pulses (Kay, 1993). All gradient pulses are applied in the z-direction. Proton pulses use a 26.9 kHz field, with the exception of the WALTZ-16 (Shaka et al., 1983) decoupling interval (pulses applied along the ±x-axes) and the two 90°$_y$ pulses flanking the decoupling, which employ a 5.9 kHz field. All carbon pulses are applied using a single frequency source. Prior to point a in the sequence, the carbon carrier is positioned at 43 ppm and all ^{13}C$^{\alpha/\beta}$ pulses are applied using field strengths of 19.8 kHz. Eight cycles of a FLOPSY-8 mixing sequence (Mohebbi and Shaka, 1991) are employed, using an 8 kHz field. At point a the carbon carrier is jumped to 58 ppm and all ^{13}C$^{\alpha}$ 90° (180°) rectangular pulses are applied with a field of $\Delta/\sqrt{15}$ ($\Delta/\sqrt{3}$), where Δ is the difference (Hz) between the centers of the ^{13}C$^{\alpha}$ and ^{13}C' chemical shift regions, so that application of the pulses minimizes excitation of carbonyl spins (Kay et al., 1990). The shaped 180° ^{13}C$^{\alpha}$ pulse in the center of the $2\tau_c$ period is a 370 μs REBURP pulse (Geen and Freeman, 1991) with an excitation maximum centered at 35 ppm (i.e., 23 ppm phase-modulated pulse). The phase of the REBURP pulse is adjusted to compensate for the slight phase change associated with the different power levels used for this pulse and the two rectangular pulses at the start and end of the $2\tau_c$ period. All ^{13}C' pulses are applied as 119 ppm phase-modulated pulses (i.e., the carrier is positioned at 58 ppm) (Boyd and Soffe, 1989; Patt, 1992) using a field strength of $\Delta/\sqrt{15}$. ^{13}C$^{\alpha}$ and ^{13}C' decoupling are achieved using WALTZ-16 fields with 300 μs 90° pulses having the SEDUCE-1 (McCoy and Mueller, 1992) profile. In the case of ^{13}C' decoupling, a 134 ppm cosine-modulated field is employed. Note that the principal use of this pulse scheme is in the sequential assignment of methyl groups. However, the β and γ positions of Asx and Glx, respectively, are protonated to some extent (see text) and the ^{13}C' decoupling employed during t_1 improves the quality of data from these residues. On-resonance ^{13}C$^{\alpha}$ decoupling is used during the ^{15}N t_2 evolution period. The positions of the Bloch–Siegert compensation pulses (Vuister and Bax, 1992) are indicated by vertical arrows underneath the pulses. ^{2}H 90° pulses are applied using a 2.2 kHz field, while decoupling is performed with a 0.8 kHz field. ^{13}C$^{\beta}$ decoupling is achieved using CHIRP inversion pulses (Fu and Bodenhausen, 1995), indicated by CHIRP (β) in the figure. Because the ^{13}C$^{\beta}$ chemical shifts of Ala are centered at approximately 20 ppm, while the β-carbon shifts of Ile, Val and Leu are in the range ~30–45 ppm, ^{13}C$^{\beta}$ decoupling is conveniently achieved by employing two fields. The first field is swept from 14 to 26 ppm during each τ_c period and serves to decouple the β-carbons of Ala. The second field is swept from 23 to 47 ppm and decouples ^{13}C$^{\beta}$ spins of Ile, Val and Leu. Note that the sweep time for this field is $\tau_c/2$ and that in a single sweep twice the spectral range is covered relative to the Ala decoupling field. Thus, the Ala field applied during τ_c consists of a single CHIRP inversion pulse of phase x, while the Ile, Val and Leu decoupling field consists of two successive CHIRP inversion pulses of phases x and –x. The inversion profile of each CHIRP pulse is improved by ramping the B_1 field from 0 to $B_{1,max}$ during the first 30% of the pulse and ramping the field down to 0 during the final 30%, using a ramping function with a sine profile. Finally, during the second half of the ^{13}C$^{\alpha}$, C' transfer (i.e., the second τ_c period) the phases of each of the CHIRP pulses are reversed. Since the B_1 field strength necessary for inversion is proportional to the square root of the rate of the frequency sweep (Kupce and Freeman, 1995), the maximum amplitude of the Ala decoupling field is set to 50% of that of the decoupling fields for Ile, Val and Leu. The performance of the decoupling is relatively insensitive to the B_1 field strength over a range of several dB. Field strengths of 0.47 and 0.95 kHz are employed for Ala and Ile,Val,Leu decoupling, respectively. The delays employed are the following: $\tau_a = 1.8$ ms, $\tau_b = 0.90$ ms, $\tau_c = 4.4$ ms, $\tau_d = 4.4$ ms, $\tau_e = 12.4$ ms, $\tau_f = 5.5$ ms, $\tau_g = 2.2$ ms, $\delta = 0.5$ ms, $T_N = 12.4$ ms. Additional delays are: $t1^a = t_1/2$, $t1^b = t_1/2 - n\zeta$ and $\tau_b' = \tau_b - n\zeta$, where $\zeta = [\tau_b - gt3]/(N-1)$, with N the number of complex points in the carbon dimension, n = 0,1,2,...(N–1) (Grzesiek and Bax, 1993b; Logan et al., 1993) and gt3 is the duration of gradient g3. The phase cycling is: ϕ1 = (x,–x), ϕ2 = (x,x,–x,–x), ϕ3 = 8(x),8(–x), ϕ4 = x, ϕ5 = 4(x),4(–x), ϕ6 = x, Rec = (x,–x,–x,x). Quadrature detection in F1 is achieved via States–TPPI of ϕ1 (Marion et al., 1989). Quadrature in F2 is achieved using the enhanced sensitivity gradient approach, where for each value of t_2 N- and P-type coherences are obtained by recording data sets with the sign of g8 inverted and 180° added to the phase of ϕ6 (Kay et al., 1992). Data sets, obtained in an interleaved manner for positive and negative g8 values, are recombined as described previously (Kay et al., 1992) to generate pure absorption lineshapes in F2. The durations and strengths of the gradients are: g1 = 1.0 ms, 4 G/cm, g2 = 0.1 ms, 8 G/cm, g3 = 0.2 ms, 15 G/cm, g4 = 0.3 ms, 8 G/cm, g5 = 0.5 ms, 8 G/cm, g6 = 0.5 ms, 4 G/cm, g7 = 0.3 ms, 2.5 G/cm, g8 = 1.25 ms, 30 G/cm, g9 = 0.2 ms, 5 G/cm, g10 = 0.2 ms, 5 G/cm, g11 = 0.125 ms, 29 G/cm.

perdeuteration is beneficial for assigning backbone NH, ^{15}N and ^{13}C chemical shifts, the depletion of protons results in molecules with significant levels of protonation only at exchangeable NH sites. Simulations have demonstrated that distance constraints between backbone NH protons are likely to be insufficient to produce global folds of many proteins (Gardner et al., 1996). With these problems in mind, we have recently developed a biosynthetic approach in which overexpression of proteins in >99% deuterated D_2O with protonated ^{13}C-pyruvate as the sole carbon source results in molecules that are highly deuterated at the majority of the positions, with the exception of the methyl groups of Ala, Val, Leu and Ile (γ2 only). In addition, H^{β} positions of Ser and Asx as well as H^{γ} sites on Arg, Pro and Glx retain significant levels of protonation. The labeling pattern observed and details of this method have been described previously (Rosen et al., 1996). Experimental results on the $^{15}N,^{13}C,^{2}H,^{1}H_3C$-labeled C-terminal SH2 domain from phospholipase $C_{\gamma 1}$ (PLCC SH2) and calculations performed on a number of proteins with molecular weights in the range 15–40 kDa indicate that significantly improved structures can be obtained by including methyl–methyl and methyl–NH NOEs compared to the use of NOEs connecting NH protons exclusively (Gardner et al., 1996).

A prerequisite to structural studies by NMR is that the assignment of chemical shifts is complete. While backbone ^{15}N, $^{13}C^{\alpha}$, NH and $^{13}C^{\beta}$ chemical shift assignment is straightforward using recently published $^{15}N,^{13}C,^{2}H$ methods, the success of our strategy for the determination of protein global folds from $^{15}N,^{13}C,^{2}H,^{1}H_3C$-labeled molecules is predicated on the site-specific assignment of methyl groups in the protein. In principle, (H)C(CO)NH- and H(CCO)NH-TOCSY-based pulse sequences (Logan et al., 1992,1993; Montelione et al., 1992; Grzesiek et al., 1993b) are well suited for this task. In practice, however, the sensitivity of such experiments decreases significantly with molecular weight and spectra recorded on protonated proteins in the 30 kDa molecular-weight regime are likely to be incomplete. In this communication we describe a number of improvements to the original pulse schemes and demonstrate that high-sensitivity spectra can be obtained on $^{15}N,^{13}C,^{2}H,^{1}H_3C$-labeled proteins, allowing complete or very near complete assignments of Ala, Val, Leu and Ile (γ2 only) methyl carbons. Using this approach, high-quality spectra can be generated for molecules with correlation times on the order of 20 ns. In addition, methyl ^{1}H chemical shifts can also be obtained by substituting a proton chemical shift evolution period for the carbon evolution (Grzesiek et al., 1993b) or by recording a 4D experiment (Logan et al., 1992).

Figure 1 illustrates the pulse scheme that we have employed for obtaining methyl carbon assignments on $^{15}N,^{13}C,^{2}H,^{1}H_3C$-labeled proteins. The experiment closely resembles previous sequences that were designed for application to fully protonated $^{15}N,^{13}C$-labeled samples and therefore only the differences are discussed. The magnetization transfer pathway can be described as:

$$^{1}H \xrightarrow{J_{HC}} {}^{13}C(t_1) \xrightarrow{\text{TOCSY}} {}^{13}C^{\alpha} \xrightarrow{J_{C^{\alpha}C'}}$$

$$^{13}C' \xrightarrow{J_{NC'}} {}^{15}N(t_2) \xrightarrow{J_{NH}} NH(t_3)$$

with the active couplings indicated above each arrow and t_i denoting an acquisition time. In an effort to optimize the sensitivity, a number of strategies were employed. (i) Saturation and dephasing of the water signal were minimized, as described previously (Grzesiek and Bax, 1993a; Kay et al., 1994; Stonehouse et al., 1994). Note that magnetization originates from methyl protons (not NH spins) in the present experiment and perturbation of the water magnetization from its equilibrium state is likely to be less important for the sensitivity in comparison with sequences where the transfer pathway originates from NH magnetization. However, depending on NH exchange rates, water saturation can lead to a decrease in the overall proton spectral envelope via saturation transfer (Li and Montelione, 1993) and precautions have been taken to minimize this effect. In this regard, it should be noted that immediately prior to the ^{13}C-FLOPSY (Mohebbi and Shaka, 1991) period a proton 90° pulse of phase −x is applied to ensure that the water magnetization is placed along the +z-axis. In the absence of this pulse the water signal would be in the transverse plane at the start of FLOPSY mixing and some fraction of the signal, although likely not all, would be restored to the +z-axis during the ~ 20 ms mixing period. Thus, at the conclusion of the mixing time the position of the water magnetization would be a function of the duration of mixing and the extent of radiation damping, making it difficult to restore the full complement of the water signal to the +z-axis prior to acquisition. (ii) An 'enhanced sensitivity' pulsed-field gradient strategy has been implemented (Kay et al., 1992; Schleucher et al., 1993). In a previous publication a comparison of the sensitivities of triple-resonance experiments recorded on perdeuterated proteins as a function of tumbling time in solution either with or without 'enhanced sensitivity' has been presented (Shan et al., 1996). The 'enhanced sensitivity' method shows gains in sensitivity for proteins with correlation times at least as large as 20 ns.

A further improvement in the sensitivity of the experiment can be made by employing $^{13}C^{\beta}$ decoupling during the transfer between the $^{13}C^{\alpha}$ and carbonyl (C') spins. In the absence of decoupling, the passive $^{13}C^{\alpha}$-$^{13}C^{\beta}$ scalar coupling results in a significant attenuation of the signal. In the case of protonated samples, a compromise transfer delay is chosen so that the deleterious effect of the carbon α-β coupling is minimized. For example, neglecting the effects of transverse relaxation, an optimal delay time for magnetization transfer from $^{13}C^{\alpha}$ to C' is 6.4 ms, during

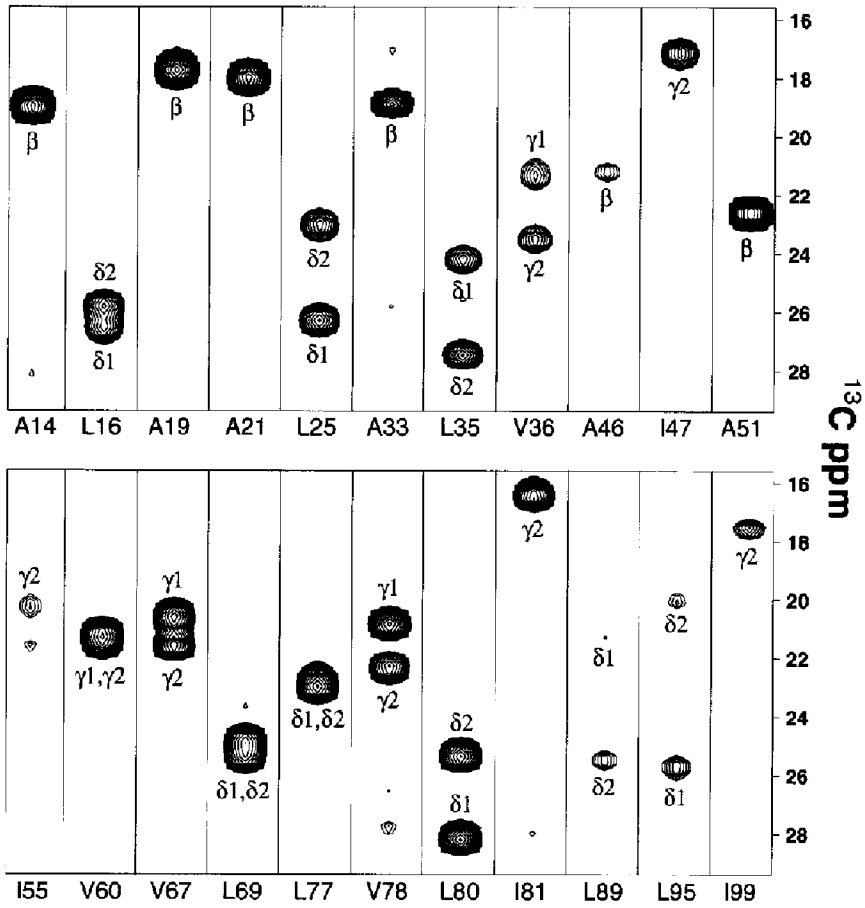

Fig. 2. Strip plots from the (H)C(CO)NH-TOCSY spectrum of a sample of the $^{15}N,^{13}C,^{2}H,^{1}H_3C$-labeled PLCC SH2 domain showing methyl correlations (residue i) with ^{15}N,NH spin pairs (residue i + 1). The spectrum was recorded on a Varian UNITY+ 600 MHz spectrometer using a 2.0 mM sample of protein with equimolar unlabeled peptide, comprising the high-affinity binding site surrounding phosphotyrosine 1021 of the platelet-derived growth factor receptor, 0.1 M phosphate, pH 6.0, at 3 °C. A 3D data set consisting of 64 × 32 × 576 complex points was recorded with acquisition times of 7.0, 24.0 and 64 ms in t_1, t_2 and t_3, respectively. A recycle delay of 0.8 s was employed, resulting in a total data acquisition time of approximately 60 h. As described briefly in the text and in detail by Rosen et al. (1996), the labeling scheme employed results in a distribution of methyl isotopomers, although for Leu, Ile(γ2) and Val the CH_3 isotopomer is dominant. The resolution in the carbon dimension is largely limited by the short t_1 acquisition time and not by the deuterium isotope shifts associated with the distribution of isotopomers.

which time ~65% of the signal is transferred. In practice, however, $^{13}C^{\alpha}$ transverse relaxation in protonated samples can be very efficient; in the case of a 37 kDa trpR–DNA complex, an average $^{13}C^{\alpha}$ T_2 value of 16.5 ms was measured (Yamazaki et al., 1994b) and the transfer efficiency is therefore reduced further.

In a previous publication, we showed that for the pyruvate-derived amino acids (Ala, Val, Leu and Ile) the use of $^{1}H_3C$-pyruvate/D_2O growth conditions results in a high degree of deuteration at all carbon positions between the methyl groups (γ2 only for Ile) and the backbone amide nitrogen (Rosen et al., 1996). The probabilities of individual residues containing only deuterium at non-methyl sites are approximately 100, 98, 90 and 99% for Ala, Val, Leu and Ile, respectively and the $^{13}C^{\alpha}$ positions of all residues are completely deuterated. In the present application, where methyl groups of Ala, Val, Leu and Ile (γ2 only) are to be assigned, it is therefore possible to extend the $^{13}C^{\alpha}$, C' transfer time to $1/(2J_{C^{\alpha}C'})$ with very limited relaxation losses (see below) and employ $^{13}C^{\beta}$ decoupling to ensure that the passive $^{13}C^{\alpha}$-$^{13}C^{\beta}$ scalar couplings do not interfere with the sensitivity of the experiment. Recently, Kupce and co-workers have developed a number of elegant decoupling schemes (Kupce and Freeman, 1995; Kupce and Wagner, 1995) which are based on the use of adiabatic CHIRP pulses (Fu and Bodenhausen, 1995). The approach employed in the present experiment is similar to WURST-1 decoupling as described by Kupce and Freeman (1995) and is discussed in the legend to Fig. 1. In principle, the use of $^{13}C^{\beta}$ decoupling during the $^{13}C^{\alpha}$, C' transfer period allows complete or near-complete transfer of magnetization during this step for the residues considered in this application (Ala, Val, Leu and Ile).

The utility of the pulse scheme for obtaining correlations linking $\{^{13}C_{methyl}(i),^{15}N(i+1),NH(i+1)\}$ chemical shifts is illustrated in Fig. 2 using a 2.0 mM sample of a ^{15}N, ^{13}C,^{2}H,$^{1}H_3C$-labeled PLCC SH2 domain. To evaluate the sensitivity of the experiment for application to proteins in the 30–40 kDa molecular-weight range, the spectrum was recorded at 3 °C. A correlation time of 18.8 ± 0.6 ns was obtained for PLCC SH2 at this temperature on the basis of ^{15}N T_1, T_2 and steady-state ^{1}H-^{15}N NOE values (Farrow et al., 1994). Note that correlation times of 14.5 and 20.5 ns were obtained for a 37 kDa trpR–DNA complex at 37 °C (Yamazaki et al., 1994b) and a 64 kDa tandem trpR–DNA complex at 45 °C (Shan et al., 1996), respectively. In Fig. 2, strip plots are illustrated for each pyruvate-derived methyl-containing residue in the molecule, with the exception of Val^{28}, which precedes a proline, and Ile^{5}. The absence of a cross peak for Ile^{5} may be due to the very weak correlation observed for His^{6} in ^{1}H-^{15}N HSQC spectra at 3 °C.

An alternative strategy for minimizing the effect of the passive $^{13}C^{\alpha}$-$^{13}C^{\beta}$ coupling on the sensitivity of the (H)C(CO)NH-TOCSY experiment is to allow the α-β coupling to evolve for a period of $1/J_{CC} \sim 28$ ms during the $^{13}C^{\alpha}$, C' transfer, while ensuring that evolution due to $J_{C^{\alpha}C'}$ proceeds for $1/(2J_{C^{\alpha}C'})$. In this way evolution due to $J_{C^{\alpha}C^{\beta}}$ is refocussed and there is no need for $^{13}C^{\beta}$ decoupling. However, despite the fact that the $^{13}C^{\alpha}$ position is highly deuterated and therefore the carbon transverse relaxation rate is greatly reduced at this position, care must still be taken to ensure that relaxation losses are minimized during the course of the sequence. We have measured $^{13}C^{\alpha}$ $T_{1\rho}$ values for a sample of ^{15}N,^{13}C,^{2}H,$^{1}H_3C$-labeled PLCC SH2 at 3 °C using an experiment proposed by Yamazaki et al. (1994b), from which an average T_2 value of 95 ± 20 ms is calculated for 48 residues in the core of the protein. It is clear, therefore, that the experimental setup proposed in Fig. 1 is preferred for obtaining correlations linking Ala, Val, Leu and Ile (γ2) methyl groups with backbone (^{15}N,NH) pairs. Indeed, a comparison of spectra recorded where either the α-β coupling is allowed to evolve for $1/J_{CC}$ or the scheme of Fig. 1 is used shows significant sensitivity gains for the latter approach.

Recently, Farmer and Venters (1995) have described a C(CO)NH-TOCSY pulse scheme for application to perdeuterated molecules in which polarization originates on ^{13}C spins. Essentially complete ^{13}C assignments have been obtained for human carbonic anhydrase, a 29 kDa protein, using this approach. In the case of sequential assignment of protonated methyl groups in otherwise highly deuterated proteins, it is advantageous to begin with polarization on the proton spins for a number of reasons. The larger gyromagnetic ratio of protons relative to carbon spins ensures that the sensitivity of the experiment is higher when magnetization originates on protons. NMR and mass spectrometric data of ^{15}N,^{13}C,^{2}H,$^{1}H_3C$-labeled proteins produced using the $^{1}H_3C$-pyruvate-D_2O strategy indicate that the methyl groups of Leu, Ile (γ2 only), Val and Ala are protonated to average levels of approximately 80, 60, 60 and 40%, respectively (Rosen et al., 1996). Neglecting relaxation, we estimate that methyl–NH correlations will be ~ two- to fourfold more sensitive, depending on the protonation level, in experiments where signal originates on protons. Moreover, the presence of multiple protons per methyl group ensures that longitudinal ^{1}H relaxation is efficient, so that reasonable recycle times can be employed. In the case of the ^{15}N,^{13}C,^{2}H,$^{1}H_3C$-labeled PLCC SH2 domain, single exponential fits of ^{1}H T_1 recovery curves gave T_1 values of 0.34 ± 0.11 and 0.58 ± 0.16 s for CH_3 and CHD_2 groups, respectively. Of course, these results are approximate, as the relaxation of methyl protons is nonexponential (Werbelow and Grant, 1977).

In summary, in this communication an (H)C(CO)NH-TOCSY sequence is presented for sequential assignment of methyl groups of Ala, Val, Leu and Ile (γ2 only) residues in ^{15}N,^{13}C,^{2}H,$^{1}H_3C$-labeled proteins. The experiment has a high sensitivity, even for proteins with correlation times on the order of 20 ns. It is straightforward to incorporate a fourth dimension so as to allow recording of methyl ^{1}H chemical shifts as well (Logan et al., 1992). The combination of this (H)C(CO)NH-TOCSY experiment and the schemes for backbone assignment presented previously will allow rapid assignment of ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, NH and side-chain methyl chemical shifts. This will pave the way for the determination of protein global folds on the basis of NH–NH, methyl–NH and methyl–methyl distance restraints obtained from 4D NOE-based experiments.

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