Journal of Biomolecular NMR, **11:** 191–198, 1998.
KLUWER/ESCOM

A spin system labeled and highly resolved ed-H(CCO)NH-TOCSY experiment for the facilitated assignment of proton side chains in partially deuterated samples

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Received 29 August 1997; Accepted 23 October 1997

Key words: multi-quantum, partially deuterated proteins, proton side-chain assignment, spin system editing

Abstract

The introduction of deuterated and partially deuterated protein samples has greatly facilitated the ^{13}C assignment of larger proteins. Here we present a new version of the HC(CO)NH-TOCSY experiment, the ed-H(CCO)NH-TOCSY experiment for partially deuterated samples, introducing a multi-quantum proton evolution period. This approach removes the main relaxation source (the dipolar coupling to the directly bound ^{13}C spin) and leads to a significant reduction of the proton and carbon relaxation rates. Thus, the indirect proton dimension can be acquired with high resolution, combined with a phase labeling of the proton resonances according to the C-C spin system topology. This editing scheme, independent of the CH_n multiplicity, allows to distinguish between proton side-chain positions occurring within a narrow chemical shift range. Therefore this new experiment facilitates the assignment of the proton chemical shifts of partially deuterated samples even of high molecular weights, as demonstrated on a 31 kDa protein.

Introduction

Recently developed NMR spectroscopy of fractionally deuterated [U-^{15}N, ^{13}C]-labeled samples 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; Nietlispach et al., 1996; Shan et al., 1996). The spin-spin relaxation times of ^{13}C and ^{1}H nuclei in proximity to hydrogen positions substituted by deuterium increase significantly due to the substantially smaller gyromagnetic ratio γ of the deuteron relative to the proton ($\gamma_H/\gamma_D \sim 6.5$; Grzesiek et al., 1993a; Markus et al., 1994; Yamazaki et al., 1994b). This results in spectra with improved resolution and/or sensitivity for either partially or fully deuterated $^{15}N^{13}C$-labeled samples. Previously developed triple-resonance experiments for highly or completely deuterated samples have focused on the peptide backbone (Yamazaki et al., 1994a, b; Shirakawa et al., 1995; Shan et al., 1996; Ikegami et al., 1997) and on the ^{13}C-assignment of the side chains (Farmer and Venters, 1995). Several other experiments rely only on the exchangeable amide protons, taking advantage of the reduction of their linewidth without loss of intensity (Torchia et al., 1988; Grzesiek et al., 1995; Venters et al., 1995; Farmer and Venters, 1996).

Various approaches have been proposed to deal with the lack of side-chain distance constraints from highly deuterated protein samples, which could even prevent the determination of the global fold. At least additional constraints from selectively protonated residue types (Gardner et al., 1996; Metzler et al., 1996; Smith et al., 1996) or from selective protonation of methyl groups (Rosen et al., 1996) should be incorporated. A more refined structure determination can be achieved at lower deuteration levels, therefore a level of ca. 50% fractional deuteration has been suggested as a compromise. Thus, the advantages of deuteration for resolution and sensitivity and, on the other hand, the need for a sufficient number of non-exchangeable

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protons for the generation of crucial distance constraints can be combined (Nietlispach et al., 1996). Fesik and co-workers have employed a strategy utilizing a set of differently labeled samples, including both fully protonated [U-^{15}N, ^{13}C]- and [U-^{15}N, ^{13}C, 75% ^{2}H]-labeled samples (Zhou et al., 1995; Huang et al., 1996; Muchmore et al., 1996).

In these approaches with a deuteration level of 50–75%, the chemical shift assignment of the side-chain protons is mainly obtained from HC(CO)NH-TOCSY experiments (Logan et al., 1992; Montelione et al., 1992; Grzesiek et al., 1993b). Here we present a new ed-H(CCO)NH-TOCSY experiment for the facilitated assignment of proton side-chain resonances in partially deuterated proteins. The proposed pulse sequence combines a spin system labeling scheme based on C-C multiplicity with a multi-quantum proton evolution period providing high resolution *and* good sensitivity.

Even with partially deuterated protein samples, the incorporation of an evolution period for carbon-bound protons always leads to a significant reduction of sensitivity due to relaxation processes, regardless of the specific experiment. The reason is that, although the relaxation rate of the evolving proton spin is reduced due to removal of most of the ^{1}H-^{1}H interactions, the dipolar couplings to the directly bound ^{13}C spins remain as a major relaxation source. Especially in combination with the required high resolution in the indirect proton dimension, this leads to a dramatic loss of signal intensity for larger proteins.

However, in the slow tumbling limit, the decay of ^{1}H-^{13}C or ^{1}H-^{15}N two-spin coherence is, in a first approximation, not affected by this dipolar coupling (Griffey and Redfield, 1987). Although this feature has found useful application in ^{1}H-^{15}N NMR (e.g., Bax et al., 1989) and in some triple-resonance experiments (e.g., Seip et al., 1992), ^{13}C linewidths obtained from heteronuclear multiple quantum correlation (HMQC) experiments of proteins did not show any improvement over the corresponding heteronuclear single-quantum correlation (HSQC) spectra (Bax et al., 1990). As has been pointed out in that study, the presence of homonuclear ^{1}H-^{1}H J-modulations in the HMQC experiment leads to unresolved multiplet patterns in the ^{13}C dimension, cancelling any gain in resolution from the reduced relaxation rate. So far, two approaches have been suggested to overcome this problem. Grzesiek and Bax (1995) used a spin-locked heteronuclear multi-quantum constant time experiment to achieve a line narrowing of the ^{13}Cα spins, while Swapna et al. (1997) introduced a constant time evolution period. Lately, multi-quantum techniques have been applied successfully to molecules with less complex ^{1}H spin systems than normal proteins, i.e., a reduced number of ^{1}H–^{1}H scalar and dipolar couplings. Multi-quantum techniques were shown to be superior for ^{1}H-^{13}C correlations in RNA (Marino et al., 1997), as well as for triple-resonance experiments in perdeuterated proteins selectively protonated at the α position (Yamazaki et al., 1997).

In this paper we will demonstrate that an intensity gain of ca. 18% can be achieved in the HMQC over the HSQC experiment for 75% fractionally deuterated protein samples. This effect is due to the dilution of the protons in the fractionally deuterated samples, which leads to a reduction of the disadvantageous homonuclear scalar and dipolar couplings. In the following we will discuss the advantages of multi-quantum evolution periods for partially deuterated proteins in the context of a specific pulse sequence, the new ed-H(CCO)NH-TOCSY experiment.

The unambiguous assignment of proton side chains solely based on typical HC(CO)NH-TOCSY experiments can be a difficult task, especially in the case of amino acid residues with small chemical shift differences within the spin system. A classification of amino acid spin systems by phase labeling of the ^{13}C resonances according to the C-C and/or C-H spin system topologies was recently proposed by Montelione and co-workers (Tashiro et al., 1995; Rios et al., 1996; Feng et a., 1996). However, these approaches are limited to small proteins between 7 and 14 kDa due to relaxation problems. Here we present a novel modification of the HC(CO)NH-TOCSY experiment, the *edited* ed-H(CCO)NH-TOCSY experiment, for partially deuterated samples. The editing of the ^{1}H resonances according to the ^{13}C-^{13}C multiplicity of the directly bound carbon facilitates the assignment of the side-chain proton chemical shifts. The proposed pulse sequence contains a multi-quantum proton evolution period, leading to a significant reduction of the proton relaxation rate. Therefore this new technique allows the acquisition of spectra with a highly resolved indirect proton dimension even for partially deuterated proteins of high molecular weights.

The pulse sequence for the ed-H(CCO)NH-TOCSY experiment is shown in Figure 1. Conceptually, this experiment is similar to previous sequences for ^{1}H or ^{13}C side-chain assignment that were designed for [U-^{15}N, ^{13}C]-labeled samples ranging from natural isotopic hydrogen composition to full deutera-

tion (e.g., Logan et al., 1992; Montelione et al., 1992; Farmer and Venters, 1995). Therefore we will only discuss the relevant differences.

The magnetization transfer pathway can be described as follows:

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

$$^{13}C' \xrightarrow{^1J_{C'N}} {}^{15}N(t_2) \xrightarrow{^1J_{NH}} {}^1N_H(t_3)$$

The active couplings are indicated above each arrow; t_i denotes an acquisition time. The essential part of this new pulse sequence for partially deuterated samples is the multi-quantum ^{1}H evolution period combined with a ^{13}C editing delay (marked by the box in Figure 1). For a two-spin system in the slow tumbling limit, the mutual relaxation is slowest when the two nuclei are active in multi-quantum rather than single-quantum coherences (Norwood, 1992). However, in fully protonated protein samples this two-spin approximation breaks down due to strong ^{1}H-^{1}H dipolar interactions. But in the case of partially deuterated samples, the two-spin model is again a valid approximation. Due to the dilution of the protons, we have observed enhancement factors of 1.0–1.4 (on average 1.18) in the HMQC compared to the HSQC spectrum of a 0.9 mM 75% deuterated [U-^{15}N, ^{13}C]-labeled sample of the 31 kDa IIA^{Man} domain homodimer of *E. coli* (Seip et al., 1994). These enhancements are independent of the proton multiplicity and the location in the spin systems. Representative examples are shown in Figure 2.

These results demonstrate that the so-called *fixed part* (i.e., the part not affected by deuteration) of the single-quantum relaxation of a ^{13}C-^{1}H spin pair (ca. 70% of the total relaxation for $^1H^\alpha$, ca. 50% for a ^{13}CHD carbon; Nietlispach et al., 1996) can be further reduced by the generation of ^{1}H-^{13}C multi-quantum coherence, leading to a further increase in signal intensity. Furthermore, the number of passive ^{1}H-^{1}H scalar couplings is greatly reduced by the partial deuteration, as simulated in Figure 3. Therefore the multi-quantum proton evolution period (cf. Figure 1a) leads to an intensity gain of 15% compared to the single-quantum version. Due to the length of the editing delay Δ_{ED} (28 ms), an unusually high resolution can be achieved in the indirect proton dimension.

The unresolved line splittings due to the remaining homonuclear scalar couplings can be easily removed by the use of a constant time evolution period, leading in principle to a higher signal intensity (pulse sequence 1b). While this is true for short constant time multi-quantum evolution periods up to ca. 12 ms (yielding 112% intensity compared to a non-constant time experiment according to Figure 1a with $t_{tmax} = 12$ ms), for longer constant time periods evolution of the remaining homonuclear scalar and dipolar couplings leads to a decrease in the signal intensity down to 75% for 28 ms. Therefore, in the constant time version a gain in intensity can only be achieved in combination with a significant loss of resolution caused by the short 12 ms constant time delay. Considering the small chemical shift differences within the proton spin systems and the possible mutual extinction of neighbouring signals with opposite sign (see below), we recommend the non-constant time version as the standard experiment. For the 75% deuterated sample of the 31 kDa IIA^{Man} homodimer the ed-H(CCO)NH-TOCSY experiment (sequence 1a) reaches 80% intensity, and with 12 ms constant time proton evolution (sequence 1b) even 90% intensity compared to a non-edited standard H(CCO)NH-TOCSY with 'shared time' evolution (Logan et al., 1993). Therefore the gain in information from the editing procedure can be obtained with an almost neglectable trade-off in signal intensity.

In addition, for higher deuteration levels the refocusing delay Δ_1 can be optimized for the dominant isotopomer CHD_n (e.g., for 75% deuterated methylene groups: CHD 38%, CH_2 6%; methyl groups: CHD_2 42%, CH_2D 14%, CH_3 2%). Since the ed-H(CCO)NH-TOCSY experiment starts with magnetization on non-exchanging protons, saturation of the water resonance is only of minor importance. However, a water flip-back version (Grzesiek and Bax, 1993a; Jahnke and Kessler, 1994; Kay et al., 1994) can be easily implemented by placing the ^{1}H carrier on the water frequency and adding an additional selective pulse (cf. Figure 1).

A second essential aspect of our experiment is the facilitated assignment of the proton chemical shifts due to phase labeling according to the C-C spin system topology. For ^{13}C spin systems this kind of phase labeling – based on the number of directly bound carbon atoms – was first demonstrated by Tashiro et al. (1995). During the delay Δ_{ED} the ^{13}C couplings between directly bound ^{13}C nuclei evolve; the ^{13}C magnetization at the end of Δ_{ED} (marked with *a* in Figure 1) is labeled with the proton chemical shift and its phase depends on the number of direct ^{13}C-^{13}C-couplings (Vuister and Bax, 1992; Grzesiek and

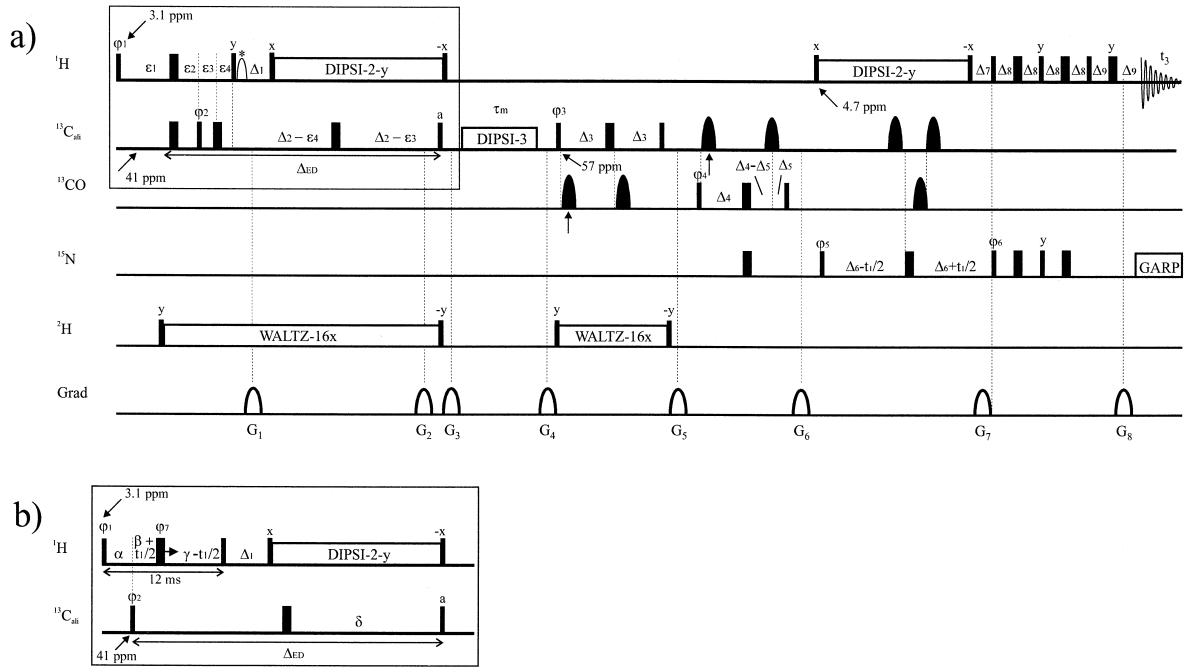

Figure 1. (a) Pulse sequence for the multi-quantum phase ed-H(CCO)NH-TOCSY experiment, providing spin system edited sequential assignments with high resolution of partially deuterated and ^{15}N,^{13}C-labeled proteins. All narrow (wide) pulses correspond to flip angles of 90 ° (180 °) and are applied along the x-axis, unless indicated otherwise. All gradient pulses are applied in the z-direction. Proton pulses use a 28.2 kHz field, with the exception of the DIPSI-2 (Shaka et al., 1988) decoupling interval and the two 90 ° y-pulses flanking the decoupling, which employ a 8.3 kHz field. Since the ed-H(CCO)NH-TOCSY experiment starts from non-exchanging protons, saturation of the water resonance is only of minor importance. However, a water flip-back version (Grzesiek and Bax, 1993a; Jahnke and Kessler, 1994; Kay et al., 1994) can be easily implemented by placing the ^{1}H carrier on the water frequency and adding an additional selective pulse (marked by an asterisk). All carbon pulses are applied using a single frequency source. The rectangular pulses on $^{13}C_{ali}$/$^{13}C\alpha$ and ^{13}CO were calibrated to provide a null in their excitation profiles at the ^{13}CO and $^{13}C\alpha$ frequency, respectively. Alternatively, for the $^{13}C_{ali}$ resonances hard pulses combined with a selective ^{13}CO decoupling (e.g. with SEDUCE-1 profiles) can be used. HERMITE-shaped (Warren, 1984) selective inversion pulses (220 μs) were chosen because of their low power integral (shown with sine-bell shape). The positions of the Bloch–Siegert compensation pulses are indicated by vertical arrows below the pulses. ^{13}C isotropic mixing was achieved using either DIPSI-3 (Shaka et al., 1988) or FLOPSY-8 sequences (Mohebbi and Shaka, 1991), with variable mixing times (here: 16.3 ms) and a 10 kHz field. Selected acquisition parameters were as follows: $\epsilon_1 = 2.0$ ms, $\epsilon_2 = 1.5$ ms, $\epsilon_3 =$ μs, $\epsilon_4 = 3$ μs, $\Delta_1 = 3.5$ ms, $\Delta_2 = 14$ ms, $\Delta_3 = 3.0$ ms, $\Delta_4 = 13.5$ ms, $\Delta_5 = 4.55$ ms, $\Delta_6 = 13.5$ ms, $\Delta_7 = 5.6$ ms, $\Delta_8 = 2.65$ ms, $\Delta_9 = 1.4$ ms. All delays were corrected with regard to pulse lengths and switching delays to avoid the need for phase corrections in the indirect dimensions. The phase cycle was $\varphi_2 = 4$(y), 4(−y); $\varphi_3 = 8$(x), 8(−x); $\varphi_4 = 2$(x), 2(−x); $\varphi_5 =$x, −x; and $\varphi_r =$x, 2(−x), x, −x, 2(x), 2(−x), 2(x), −x, x, 2(−x), x. Quadrature detection in F1 was achieved via States-TPPI of φ_1 (Marion and Bax, 1989) and shared time evolution (Logan et al., 1993) with ϵ_1 decremented by 20 μs; ϵ_2, ϵ_3 and ϵ_4 were incremented by 20 μs, 118 μs and 118 μs, respectively. The duration of the total ^{13}C editing delay Δ_{ED} was fixed at 28 ms by simultaneously decrementing Δ_2. Quadrature detection in F2 was achieved using the enhanced sensitivity gradient approach: for each value of t_2, N- and P-type coherences are obtained separately by alternately recording data sets with inversion of the sign of G_8 and phase φ7 (Kay et al., 1992). The strength of the gradients is given in percent of full strength (70 G/cm): $G_1 = 80\%$, $G_2 = 80\%$, $G_3 = 40\%$, $G_4 = 40\%$, $G_5 = 30\%$, $G_6 = 70\%$, $G_7 = 98.7\%$, $G_8 = 10/-10\%$. (b) Alternative version with a constant time multi-quantum delay, trading ^{1}H resolution for increased sensitivity. A 10% intensity gain can be achieved over version a with the constant time delay limited to 12 ms. This version differs in the following acquisition parameters from version a: $\alpha = 3.5$ ms, $\beta = 2.5$ ms, $\gamma = 6.0$ ms, $\delta = 14.0$ ms; $\varphi_7 = 4$(y), 4(−y).

Bax, 1993b). The relevant magnetization at this point is given by

$$C_x \cos(\omega_H t_1) \cos^m(2\pi J \Delta_{ED})$$

where m equals the number of $^{1}J_{CC}$ couplings of the carbon spin C to other aliphatic carbons. For Δ_{ED} values of $1/(2J) < \Delta_{ED} < 3/(2J)$, topologies with odd and even values of m result in cross peaks with opposite sign. Detecting C-C phase information on the resonances of the directly bound H atoms has already been described for a CT-HSQC version (Vuister and Bax, 1992) and for H^{α} resonances of small proteins for HACANH or HACACONH versions (Feng et al., 1996). However, our new approach can be used even with large proteins and much less sensitive experiments, since the slow relaxation rates of the

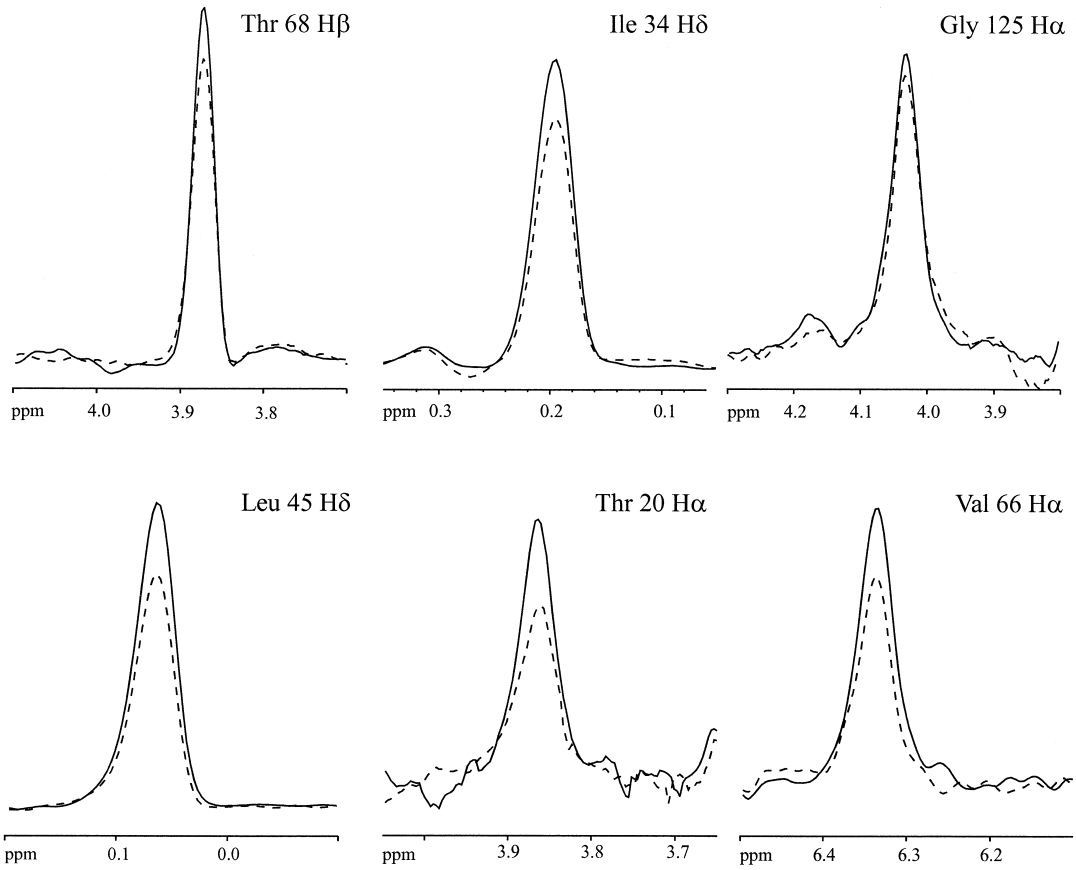

Figure 2. Intensity enhancement of the HMQC over the HSQC spectrum using 75% fractionally deuterated samples. Shown are selected cross sections of the IIA^{Man} domain of *E. coli* (31 kDa, 0.9 mM). The HMQC spectrum is represented by a solid line, the HSQC spectrum by a dashed line. Averaged over all signals, an 18% intensity gain was observed for the HMQC spectrum. Selected acquisition parameters for both spectra are: NS = 48; TD(F1) = 270; TD(F2) = 2048; quadrature detection in F1 was achieved via States-TPPI of φ1 (Marion and Bax, 1989). The final acquisition time in F1 was 16.5 ms, the acquisition time in F2 was 123 ms. ^{2}H decoupling during transverse magnetisation on ^{13}C was achieved using WALTZ-16 (Shaka et al., 1983) with a 0.8 kHz field, ^{13}C decoupling during the acquisition time (F2) using GARP (Shaka et al., 1985) with a 3.1 kHz field.

multi-quantum states due to partial deuteration allow the use of the necessary long delay Δ_{ED} required for the C-C topology labeling. Whereas the CT-HSQC and HACANH versions only allow editing of single proton signals, the ed-H(CCCO)NH-TOCSY generates an editing pattern for the whole proton side-chain spin system. Figure 4 shows a schematic representation of the resulting pattern in the indirect proton dimension, combining the proton random coil shifts for the different side chains with the sign pattern from the respective carbon spin system topologies. This editing pattern allows a facilitated amino acid recognition and can be easily implemented into automated assignment strategies. In addition, in spin systems with overlapping chemical shift regions, this phase labeling can be a very useful tool for an unambiguous assignment of the proton side-chain signals. For example, the H^{α} chemical shifts of Gly, the methylene group of Ile and the H^{β} chemical shifts of Leu and Thr are easily identified by their characteristic phase. Likewise, in the spin system topology of Glu, Gln, and Met, the H^{γ} chemical shifts can be unambiguously distinguished from the H^{β} chemical shifts, regardless of their similar chemical shift range. While the possibility of mutual extinction between overlapping peaks with different sign exists, it is reduced by the high resolution and the narrower linewidth of the signals in this experiment.

The pulse sequence presented here has been optimized and tested on a 0.9 mM [U-^{13}C, ^{15}N, 75% ^{2}H]-labeled sample of the 31 kDa IIA^{Man} domain homodimer of *E. coli* (Seip et al., 1994). Representative

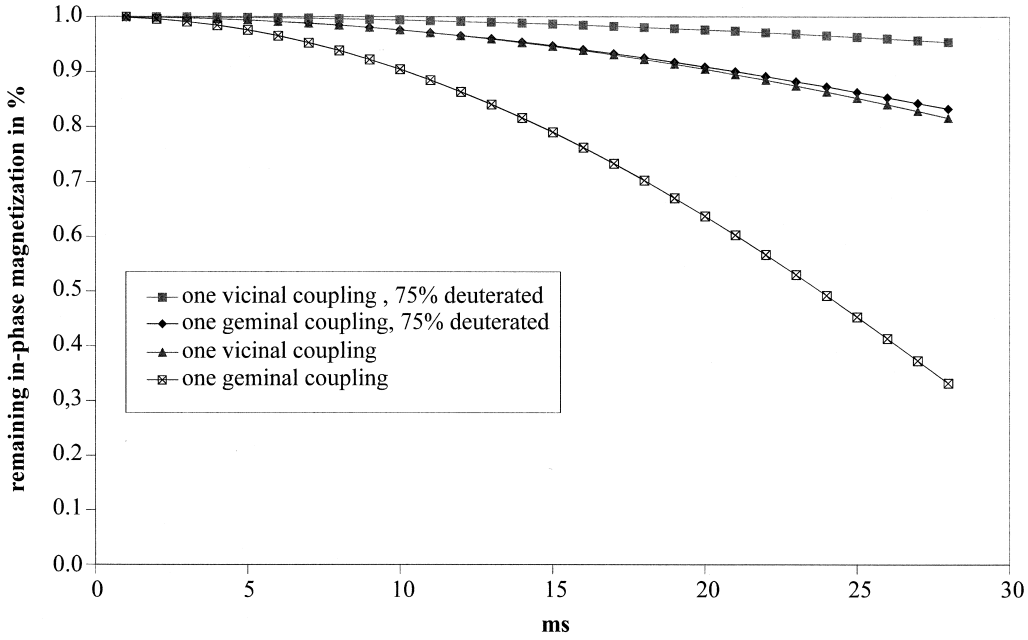

Figure 3. Simulated loss of in-phase magnetization due to proton homonuclear scalar couplings for different constant time delays, shown for the case of one vicinal and one geminal coupling for fully protonated and 75% fractionally deuterated samples. The reduced decrease of magnetization in the deuterated sample due to the replacement of protons by deuterons is especially pronounced for geminal couplings.

strip plots from the 28 ms constant time version for the different amino acid spin system classes are shown in Figure 5. These examples demonstrate that it is possible to obtain high resolution and spin system labeling in the indirect proton dimension, even for proteins with a high molecular weight. The efficiency of the ^{13}C-^{13}C TOCSY transfer depends on several factors and reaches an optimum at different isotropic mixing times for different side-chain carbons (Eaton et al., 1990). Therefore, for a complete proton assignment a combination of different mixing times should be used.

Obviously, this approach of transferring information about the carbon spin system topology to the proton resonances can also be applied to non-deuterated samples. However, for such samples a single-quantum approach combined with a 'shared time' evolution (Logan et al., 1993) would be more suitable. Because of the short relaxation times such an application is restricted to proteins with molecular weights below 8–10 kDa (Tashiro et al., 1995).

In summary, the ed-H(CCO)NH-TOCSY experiment for partially deuterated samples combines high resolution in the indirect proton dimension with phase labeling according to the carbon spin system topology. This results in a proton editing pattern independent of the CH_n multiplicity, a valuable tool for the unambiguous assignment of the proton side-chain signals. The multi-quantum proton evolution period makes it applicable to partially deuterated proteins even of high molecular weight. Therefore this experiment should be a useful addition to the common assignment strategy for partially deuterated samples based on CC(CO)NH-TOCSY spectra (Farmer and Venters, 1995) and conventional H(CCO)NH-TOCSY experiments.

Acknowledgements

We wish to thank Prof. B. Erni and Regula Gutknecht (both of the University of Bern) for providing the partially deuterated sample of IIA^{Man} and Dr. Ralph Peteranderl for carefully reading the manuscript. Financial support from the Deutsche Forschungsgemeinschaft, the Sonderforschungsbereich 369, the Fonds der Chemischen Industrie and the Dr.-Ing. Leonhard-Lorenz-Stiftung is gratefully acknowledged.

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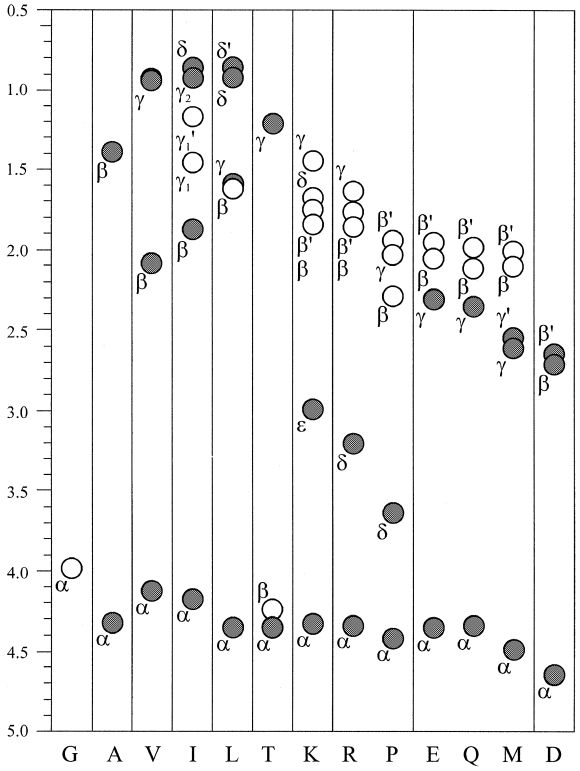

Figure 4. Schematic phase pattern for the 10 amino acid spin systems identified in the ed-H(CCO)NH-TOCSY experiment, using ^{1}H random coil chemical shift values. Black and white circles indicate positive and negative peaks, respectively. For consistency the random coil chemical shifts of the amino acid residues when followed by alanine (Wishart et al., 1995) were used. All AMXY-type amino acid spin systems (e.g. Phe, Asn, Asp, Tyr) show the same phase labeling and are therefore represented by Asp.

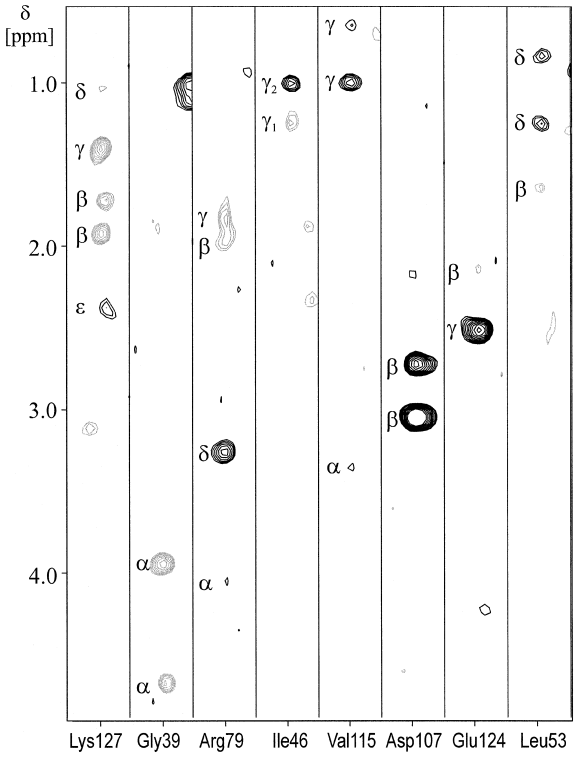

Figure 5. Representative strip plots for the different amino acid spin system classes of the ^{1}H-$^{1}H_{N}$ planes from the 3D ed-H(CCO)NH-TOCSY spectrum (TOCSY mixing time = 16.3 ms). The spectrum was obtained on a uniformly $^{13}C/^{15}N$ and 75% ^{2}H-labeled sample of the IIA^{Man} domain of *E. coli* (31 kDa, 0.9 mM), dissolved in 90% H_2O/10% D_2O with 50 mM potassium phosphate buffer, pH 7.0, at a temperature of 310 K on a Bruker DMX750 spectrometer. Each amide shows correlation peaks to proton resonances of the side chains in the preceding residue. Negative peaks are displayed with gray and positive peaks with black contour lines. The TOCSY mixing time used here is not suited for alanine or threonine spin systems, which are therefore omitted in the strip plots. Data collection included 64 complex points in the remote proton dimension, 32 complex points in the nitrogen dimension and 512 complex points in the direct proton dimension. The total experimental time was approximately 130 h. The data were extended in the ^{15}N dimension using linear prediction and then zero-filled prior to Fourier transformation, resulting in final digital resolutions of ca. 20 Hz/point in the indirect dimensions and ca. 10 Hz/point in the direct dimension.

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