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Classification of amino acid spin systems using PFG HCC(CO)NH-TOCSY with constant-time aliphatic ^{13}C frequency labeling

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Summary

We have developed a useful strategy for identifying amino acid spin systems and side-chain carbon resonance assignments in small ^{15}N-,^{13}C-enriched proteins. Multidimensional constant-time pulsed field gradient (PFG) HCC(CO)NH-TOCSY experiments provide side-chain resonance frequency information and establish connectivities between sequential amino acid spin systems. In PFG HCC(CO)NH-TOCSY experiments recorded with a properly tuned constant-time period for frequency labeling of aliphatic ^{13}C resonances, phases of cross peaks provide information that is useful for identifying spin system types. When combined with ^{13}C chemical shift information, these patterns allow identification of the following spin system types: Gly, Ala, Thr, Val, Leu, Ile, Lys, Arg, Pro, long-type (i.e., Gln, Glu and Met), Ser, and AMX-type (i.e., Asp, Asn, Cys, His, Phe, Trp and Tyr).

Sequence-specific resonance assignments provide the basis for interpretation of multidimensional NMR spectra and for determination of 3D structures of proteins. A key step in this assignment procedure is the identification of amino acid spin systems. Recently, ^{13}C-^{13}C COSY (Grzesiek and Bax, 1992a,b,1993; Wittekind and Mueller, 1993; Muhandiram and Kay, 1994; Wang et al., 1994) and TOCSY (Logan et al., 1992; Montelione et al., 1992; Clowes et al., 1993; Grzesiek et al., 1993; Lyons et al., 1993; Lyons and Montelione, 1993; Richardson et al., 1993; Farmer and Venters, 1995) experiments have been described to correlate the aliphatic ^{1}H or ^{13}C resonances of a given amino acid directly with the intraresidue backbone amide group, or with the backbone amide group of the following residue in the sequence. These data allow identification of some spin system types based on $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts and cross-peak phases (Grzesiek and Bax, 1993), chemical shifts of aliphatic β, γ, δ and ε resonances (Grzesiek et al., 1993; Logan et al., 1993; Lyons et al., 1993), and identification of cross peaks between aliphatic and side-chain amide resonances of asparagine and glutamine residues (Montelione et al., 1992; Lyons et al., 1993; Wittekind and Mueller, 1993). Experiments have also been developed that allow identification of glycine α resonances, based on suppression of methine groups (Wittekind et al., 1993), of aromatic (Yamazaki et al., 1993) or arginine (Yamazaki et al., 1995) spin systems using coherence transfer between side-chain resonances, and of aromatic spin systems using phase effects due to scalar coupling of $^{13}C^{\beta}$ and aromatic $^{13}C^{\gamma}$ nuclei (Grzesiek and Bax, 1993).

Spectral data characteristic of spin system types are very helpful in algorithms for automated analysis of resonance assignments (Zimmerman and Montelione, 1995). In order to obtain additional information that is useful for classifying amino acid spin systems in small proteins, we have developed an improved version of the 3D HCC-(CO)NH-TOCSY experiment (Logan et al., 1992; Montelione et al., 1992; Clowes et al., 1993; Grzesiek et al., 1993) which utilizes pulsed-field gradients (PFGs) and constant-time ^{13}C frequency labeling. In this PFG HCC-(CO)NH-TOCSY experiment, the constant-time ^{13}C frequency labeling period is tuned to generate cross-peak phases that depend on the number of directly coupled aliphatic carbons, providing information that is useful for the identification of many spin system types.

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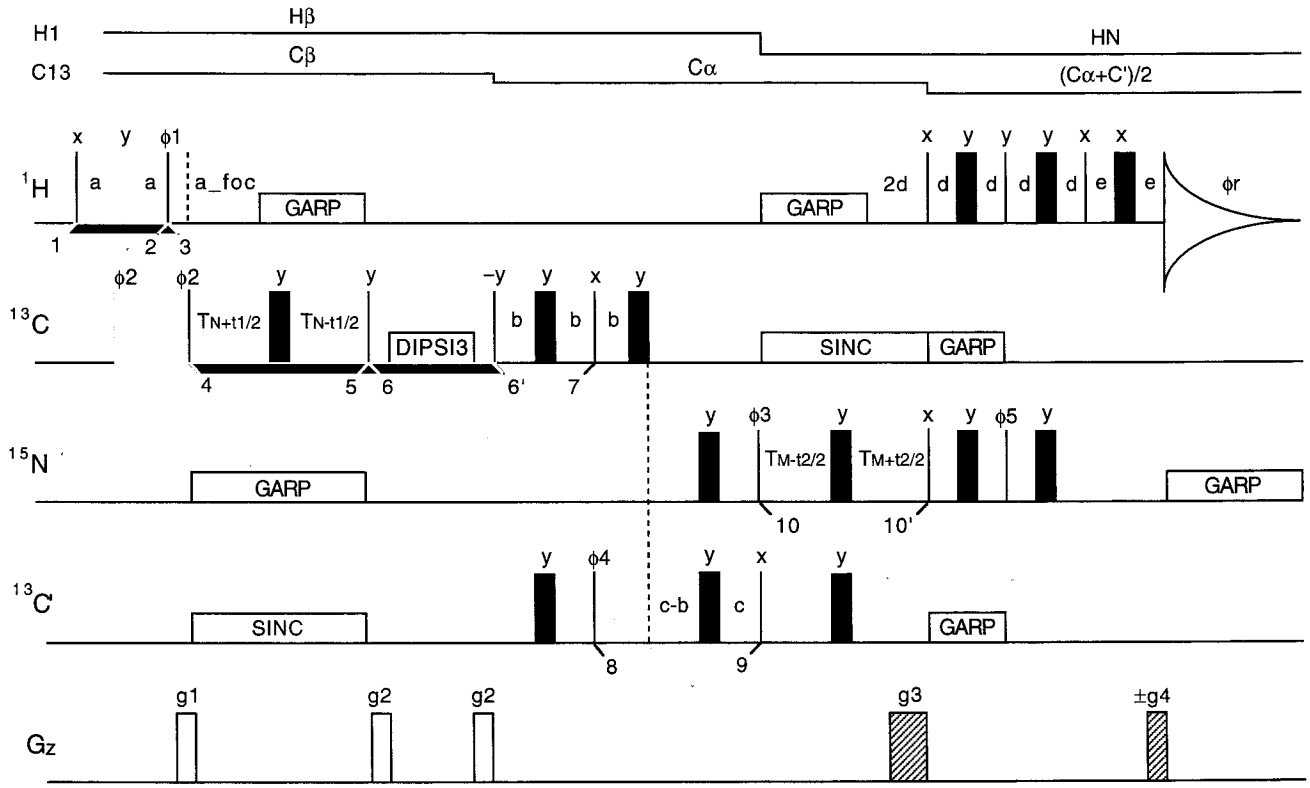

Fig. 1. Pulse sequence of the 3D PFG HCC(CO)NH-TOCSY experiment with constant-time aliphatic ^{13}C frequency labeling. For an 8 kDa protein, coherence transfer delays are tuned to a = 1.5 ms, a_foc = 2.5 ms, b = 3.2 ms, c = 13.5 ms, d = 2.7 ms, and e = 650 μs, respectively; the constant-time evolution periods are T_N = 14 ms and T_M = 14 ms. ^{13}C isotropic mixing is achieved using either DIPSI-3 (Shaka et al., 1988) or FLOPSY-8 (Mohebbi and Shaka, 1991), with variable mixing times. During aliphatic ^{13}C frequency labeling, ^{1}H and ^{15}N spins are decoupled from aliphatic ^{13}C using GARP (Shaka et al., 1985) and the carbonyl $^{13}C'$ spins are decoupled using band-selective SINC (S.D. Emerson and G.T. Montelione, unpublished results) waveforms. During t_2, the $^{1}H^N$ and $^{13}C^\alpha$ spins are decoupled from ^{15}N using GARP and SINC, respectively, and during t_3, ^{15}N spins are decoupled from $^{1}H^N$ using GARP. GARP was also used to simultaneously decouple $^{13}C^\alpha$ and carbonyl $^{13}C'$ spins from ^{15}N during the reverse-refocused INEPT transfer step from ^{15}N to ^{1}H following the t_2 evolution time. These multiple spin-lock and decoupling schemes were executed using waveform generators. The GARP ^{1}H decoupling during T_N and T_M requires lower decoupler powers and results in less sample heating than other decoupling sequences like WALTZ-16. The SINC narrow-band decoupling sequence exhibits smaller off-resonance effects than several other tested schemes (S.D. Emerson and G.T. Montelione, unpublished results). Gradients represented by open vertical bars are optional z and zz filters, while those shown as hatched vertical bars are used for ^{15}N coherence selection. Quadrature detection in the t_1 domain was obtained in the States-TPPI manner (Marion et al., 1989). The pulse phases were cycled as follows: φ1 = 4(+y),4(−y); φ2 = +x,−x; φ3 = +x,+x,−x,−x; φ4 = 8(+x),8(−x); φ5 = +y,−y; and the receiver phase φr = +x,−x,−x,+x,−x,+x,+x,−x,−x,+x,+x,−x,+x,−x,+x,−x,−x,+x. All 180° pulses were applied with phase y. For each t_2 value, two FIDs were recorded with φ5 = +y, g4 = +z and φ5 = −y, g4 = −z, and stored separately. Postacquisition processing involved adding and subtracting these two time domain data sets, storing the results in separate memory locations, Fourier transforming the two data sets, and then applying 90° zero-order phase corrections in both t_2 and t_3 to one data set before adding them together to yield pure phase in the ^{15}N dimension with sensitivity enhancement (Palmer et al., 1991; Kay et al., 1992; Muhandiram and Kay, 1994; Schleucher et al., 1994). The values of coherence transfer delays b and c were determined empirically and depend on the relaxation properties of the protein system. Their optimum values depend on the time required for development (or refocusing) of antiphase magnetization and the attenuation of these coherences due to passive couplings and relaxation. Accordingly, it can be advantageous to tune the b delays before and after the $C^\alpha \rightarrow C'$ coherence transfer pulses separately. The pulse sequence source code for this PFG HCC(CO)NH-TOCSY experiment is available by anonymous ftp at nmrlab.cabm.rutgers.edu.

The pulse scheme for a 3D PFG HCC(CO)NH-TOCSY experiment is shown in Fig. 1. Conceptually, this experiment is similar to non-phase versions of these experiments that have already been published (Logan et al., 1992; Montelione et al., 1992; Clowes et al., 1993; Grzesiek et al., 1993; Farmer and Venters, 1995). The key differences are the use of PFGs for ^{15}N coherence selection and solvent suppression, the addition of an optional sensitivity enhancement scheme (Palmer et al., 1991; Kay et al., 1992), optimized concatenation of coherence defocusing and refocusing periods with constant-time frequency labeling of ^{13}C and ^{15}N, and the use of SINC sinc-type band-selective decoupling of $^{13}C'$ carbonyl and $^{13}C^\alpha$ resonances during constant-time periods $2T_N$ and $2T_M$, respectively. The SINC decoupling scheme, which will be described in detail elsewhere, provides uniform and highly selective decoupling of the $^{13}C'$ (or $^{13}C^\alpha$) resonance, with minimal perturbation of $^{13}C^\alpha$ (or $^{13}C'$) spin states. Furthermore, in the following discussion we describe how the constant-time ^{13}C frequency labeling period $2T_N$ can be tuned to develop phase and frequency information that is characteristic of different spin system types.

In the following description of the HCC(CO)NH-TOCSY experiment, product operators (Sørensen et al., 1983) for the spins of the aliphatic backbone and side-chain proton, the amide proton, the ^{13}C aliphatic side-chain carbon, the $^{13}C^{\alpha}$ carbon, the $^{13}C'$ carbonyl carbon, and the ^{15}N backbone amide are identified with the notation **H**, $\mathbf{H^N}$, **C**, $\mathbf{C^{\alpha}}$, **C'**, and **N**, respectively. For simplicity, the effects of relaxation are not included here; they will be discussed elsewhere. The first step of the pulse scheme involves an INEPT transfer to enhance the nuclear spin polarization of aliphatic carbons. The resulting spin operators at times 1 through 4 are $\mathbf{H}_z$, $2\mathbf{H}_x\mathbf{C}_z$, $-2\mathbf{H}_z\mathbf{C}_z$, and $2\mathbf{H}_z\mathbf{C}_y$, respectively. An optional short (< 500 μs) zz filter (Bruhwiler and Wagner, 1986; Montelione and Wagner, 1989; John et al., 1992) can be applied between time points 3 and 4 by using a z gradient pulse. During the delay a_foc, the $2\mathbf{H}_z\mathbf{C}_y$ term rephases with respect to its attached proton(s), and can be tuned to distinguish methines, methylenes, and methyls (Ernst et al., 1987; Wittekind et al., 1993). In this work, the delay a_foc was set to 2.5 ms to simultaneously optimize positive coherence for these three kinds of aliphatic carbons. With this value of a_foc, the phase of the ^{13}C magnetization at the end of the constant-time evolution period $2T_N$ (point 5 in Fig. 1) depends only on the number of direct ^{13}C-^{13}C couplings (Vuister and Bax, 1992; Grzesiek and Bax, 1993). Accordingly (ignoring relaxation), the magnetization develops differently during t_1 for different ^{13}C coupling topologies:

$$
\begin{aligned}
-\mathbf{C}_x &\rightarrow -\mathbf{C}_x \cos(\omega_c t_1) && 0\ ^{13}C\text{-}^{13}C \text{ couplings}\\
&\rightarrow -\mathbf{C}_x \cos(\omega_c t_1)\cos(2\pi J_{CQ} T_N) &&\\
&\quad + \ldots && 1\ ^{13}C\text{-}^{13}C \text{ coupling}\\
&\rightarrow -\mathbf{C}_x \cos(\omega_c t_1)\cos(2\pi J_{CQ} T_N) && \qquad (1)\\
&\quad \times\cos(2\pi J_{CR} T_N) + \ldots && 2\ ^{13}C\text{-}^{13}C \text{ couplings}\\
&\rightarrow -\mathbf{C}_x \cos(\omega_c t_1)\cos(2\pi J_{CQ} T_N) &&\\
&\quad \times\cos(2\pi J_{CR} T_N)\cos(2\pi J_{CS} T_N) &&\\
&\quad + \ldots && 3\ ^{13}C\text{-}^{13}C \text{ couplings}
\end{aligned}
$$

where . . . denotes terms that do not result in observable magnetization, ω_c is the ^{13}C Larmor frequency, and the aliphatic carbons interacting with carbon spin C by one-bond ^{13}C-^{13}C scalar coupling are denoted Q, R and S. Assuming $J = J_{CQ} = J_{CR} = J_{CS}$, the terms of Eq. 1 can be rewritten generally as:

$$-\mathbf{C}_x \cos(\omega_c t_1)\cos^m(2\pi J T_N) \qquad (2)$$

where the exponent m corresponds to the number of aliphatic carbons with active one-bond coupling to carbon spin C. The resulting modulation of coherences during the constant-time period $2T_N$ is shown in Fig. 2A. For short T_N values, all topologies result in cross peaks with identical phases, while for values $1/2J < 2T_N < 3/2J$, topologies with odd and even m values result in cross peaks with opposite phases. This modulation provides a general approach for distinguishing different spin system types.

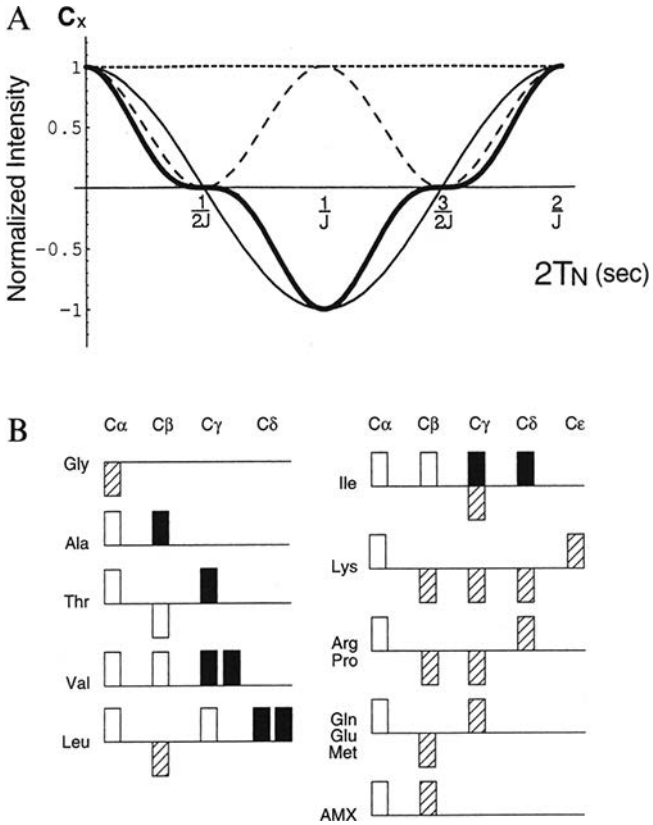

Fig. 2. (A) Coherence transfer function for different values of constant-time ($2T_N$) evolution. The modulation of coherence transfer depends on the number m of directly coupled aliphatic carbons (Ernst et al., 1987) as follows: m = 0 (e.g. glycine C^{α}), dotted line; m = 1 (e.g. all other C^{α}, and AMX-type C^{β}), thin line; m = 2 (e.g. long-type C^{β}), dashed line; m = 3 (e.g. valine C^{β}, leucine C^{γ}, and isoleucine C^{β}), thick line. (B) Schematic diagram of phase patterns of the aliphatic carbon cross peaks for 10 amino acid spin system classes identified in the PFG HCC(CO)NH-TOCSY experiment. Open, hatched and solid bars denote methine, methylene and methyl carbons, which can be distinguished by their characteristic ^{13}C chemical shift ranges. The peak phases are designated by the direction (positive or negative) of the bars; by convention, the non-glycine C^{α} cross peaks are plotted with positive contours.

Considering the structures of the amino acids, the phase patterns of side-chain cross peaks can provide valuable information for identification of spin system types in HCC(CO)NH-TOCSY spectra. As shown in Fig. 2B, seven amino acid types (Gly, Ala, Thr, Val, Leu, Ile and Lys) exhibit unique phase patterns. This information is further complemented by the characteristic ^{13}C chemical shift ranges of aliphatic methine, methylene and methyl resonances. Although both proline and arginine spin systems exhibit identical phase patterns, these can be distinguished from statistical differences in their $^{13}C^{\delta}$ chemical shift values, and from the fact that in other intraresidue triple resonance experiments, proline spin systems have no backbone amide connections, while arginine aliphatic resonances can be correlated with both backbone and side-chain ^{15}N resonances. Spin systems of glutamine and asparagine can also be identified in HCC-

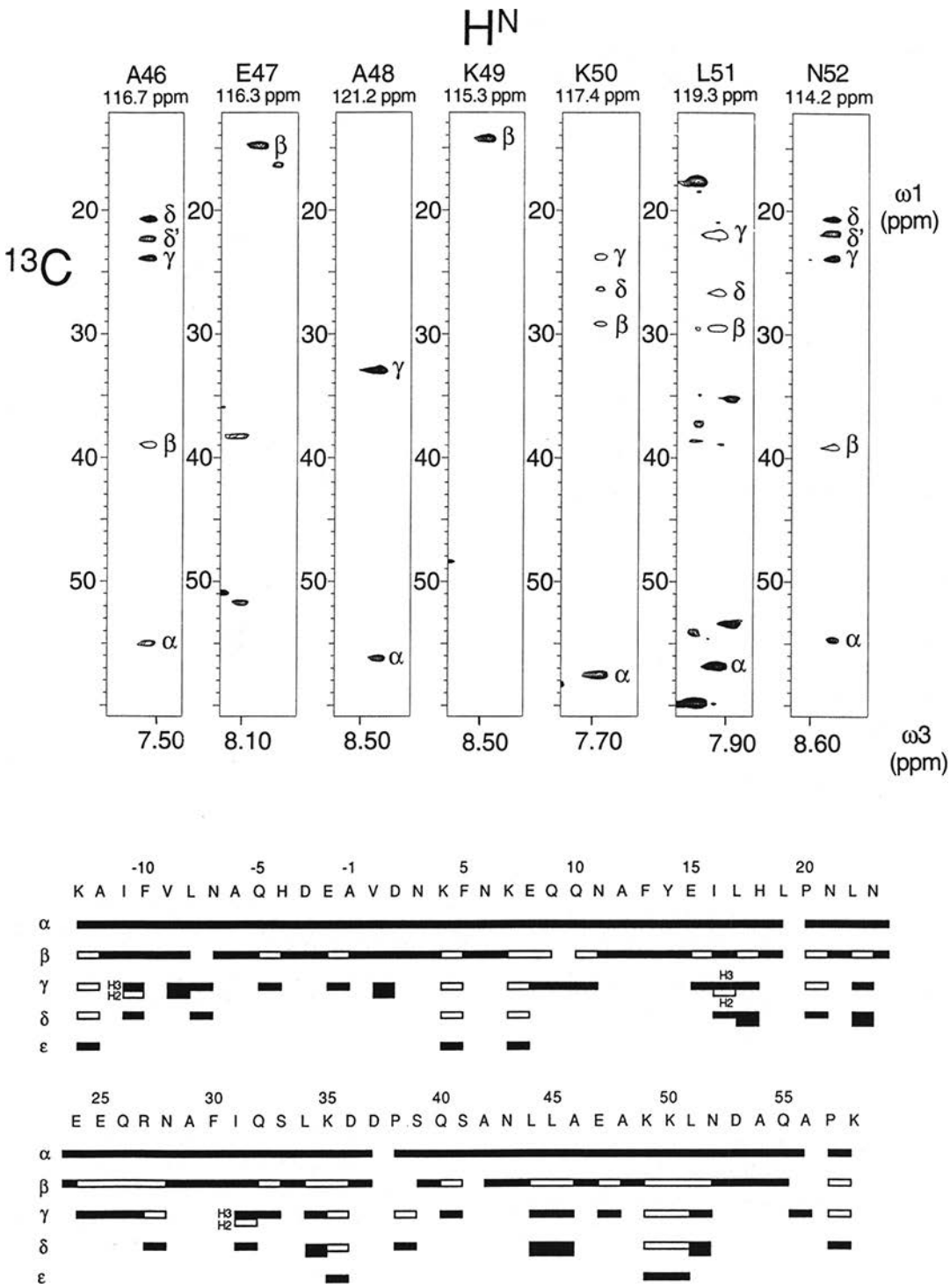

Fig. 3. (A) Strip plots of ^{13}C-H^N planes from the 3D PFG HCC(CO)NH-TOCSY spectrum (TOCSY mixing time = 24 ms) for the α-helical polypeptide segment Leu^{45}-Asn^{52} of Z-Domain. Each amide shows correlations to carbon resonances of side chains in the preceding residue. Negative peaks are displayed with a single contour, and positive peaks with several contours. Data collection included 120 complex points in t_1, 44 complex points in t_2, and 512 complex points in t_3. The total collection time was approximately 36 h. The data were extended in the ^{15}N dimension using linear prediction, and were zero-filled prior to Fourier transformation, resulting in final digital resolutions of 31 Hz/point in ω_1, 13 Hz/point in ω_2 and 5 Hz/point in ω_3. (B) Survey of sequential connectivities identified by analysis of 2D and 3D PFG HCC(CO)NH-TOCSY data for Z-Domain, recorded with TOCSY mixing times of 14 and 24 ms. Sequential cross peaks from the aliphatic carbon C^α, C^β, C^γ, C^δ, and C^ε resonances of residue i to the ^{15}N and H^N resonances of residue i+1 are indicated by horizontal bars labeled α, β, γ, δ, and ε, respectively. Cross-peak phases are distinguished by solid (positive) or open (negative) bars. The 300 µl sample of uniformly ^{13}C-,^{15}N-enriched Z-Domain was prepared in a Shigemi NMR tube at a protein concentration of 1.2 mM. The solvent was 95% H_2O/5% D_2O containing 10 mM K_2HPO_4 and 0.2 mM NaN_3, pH 6.5, at a temperature of 30 ± 0.1 °C. NMR spectra were obtained on a Varian Unity 500 spectrometer, modified to provide a second ^{13}C synthesizer frequency for pulsing and decoupling of carbonyl resonances. Data processing was carried out using VNMR (Varian Associates), Triad (Tripos, Inc.), and NMRCompass (Molecular Simulations, Inc.) software.

(CO)NH-TOCSY spectra using characteristic cross peaks to side-chain amide protons (Montelione et al., 1992; Lyons et al., 1993), while serine residues are distinguished from other AMX spin systems with the same side-chain phase patterns using their characteristic C^{β} resonance frequencies.

Following the constant-time ^{13}C frequency labeling period in Fig. 1, the transverse C_x magnetization is flipped onto the z-axis for ^{13}C-^{13}C isotropic mixing, which is typically 10–50 ms. In our experience, when short (< 50 ms) mixing times are used, similar results are obtained employing either DIPSI-3 (Shaka et al., 1985) or FLOPSY-8 (Mohebbi and Shaka, 1991) for this TOCSY transfer. Optional z gradients (< 500 μs) before and after the isotropic mixing step can also be used for improved H_2O solvent suppression. Following the TOCSY transfer step, the ^{13}C carrier frequency is reset to the center of the C^{α} region, and the resulting C_z^{α} magnetization is flipped into the transverse plane at point 6'. Next, magnetization is transferred from C^{α} nuclei (point 7) to C' nuclei (point 8) and then to the backbone amide nitrogen of the following residue N_{i+1} (point 9), using two concatenated INEPT-type sequences. During the subsequent constant-time ^{15}N evolution period $2T_M$ (beginning at point 10), nitrogen magnetization antiphase to C' ($2\mathbf{N}_y\mathbf{C}'_z$) is refocused while nitrogen magnetization antiphase to H^N is developed ($-2\mathbf{N}_y\mathbf{H}^N_z$). ^{15}N chemical shift evolution during the constant-time period $2T_M$ gives rise to:

$$-2\mathbf{N}_y\mathbf{H}^N_z \cos(\omega_N t_2) + 2\mathbf{N}_x\mathbf{H}^N_z \sin(\omega_N t_2) \qquad (3)$$

at point 10'. These two terms are directed into the receiver with 90° phase shifts in both the t_2 and t_3 dimensions using reverse-INEPT with sensitivity enhancement, as described elsewhere (Palmer et al., 1991; Kay et al., 1992; Muhandiram and Kay, 1994; Schleucher et al., 1994).

The pulse sequence was optimized and tested on Z-Domain, a predominantly α-helical, 8.0 kDa protein domain derived from the immunoglobulin-binding Protein A of *Staphylococcus aureus* (Nilsson et al., 1987; Lyons et al., 1993). Figure 3A shows ^{13}C-H^N strips from a typical 3D PFG HCC(CO)NH-TOCSY spectrum recorded on a 1.2 mM sample of ^{13}C-,^{15}N-enriched Z-Domain. The sequential connectivities identified by analysis of spectra recorded with different DIPSI-3 mixing times are summarized in Fig. 3B. In most cases, multiple sequential connectivities with phase information were established between each amino acid spin system in the sequence, including many C_i^{δ}-H^N_{i+1} and C_i^{ε}-H^N_{i+1} connectivities (Fig. 3B). Similar results were obtained using a 3.0 mM ^{13}C-, ^{15}N-enriched sample of the 8.0 kDa major cold shock protein from *Escherichia coli* (data not shown).

The HCC(CO)NH-TOCSY experiment can be recorded either without (using $2T_N < 1/2J$) or with (using $2T_N \sim 1/J$) side-chain topology phase information. Of course, the phase version of the experiment exhibits lower sensitivity due to nuclear relaxation during the lengthened $2T_N$ period. This difference in sensitivity depends on the relevant coherence relaxation rates, which for larger protein systems may preclude useful signal-to-noise ratios. For this reason, it is generally best to record both the non-phase (higher sensitivity) and phase (lower sensitivity) versions of the experiment. In addition, the efficiency of ^{13}C-^{13}C TOCSY transfer depends on several factors, and is optimal for different side-chain carbons using different isotropic mixing times. It is our experience that the most complete data sets are obtained by recording several spectra with different isotropic mixing times. In very poorly resolved regions of HCC(CO)NH-TOCSY spectra, some confusion in classifying spin system types can also arise due to partial cancellation of overlapping positive and negative peaks.

In summary, these results demonstrate that the phase version of the PFG HCC(CO)NH-TOCSY experiment is quite efficient for small proteins. The resulting phase patterns can be combined with ^{13}C chemical shift information to uniquely characterize many spin system types, and are highly amenable to automated analysis. Similar results have been obtained on small (< 10 kDa) proteins using related PFG HCCNH-TOCSY, PFG HCCNH-COSY and PFG HCC(CO)NH-COSY pulse sequences (C.B. Rios, M. Tashiro and G.T. Montelione, unpublished results), with either carbon or proton frequency evolution in the t_1 period. Modified versions of these constant-time triple resonance experiments that are useful for unique identification of aromatic or Glx/Asx spin systems, exploiting ideas inherent in the 2D-edited constant-time HSQC experiment (Grzesiek and Bax, 1993) are also being developed in our laboratory. For larger proteins with short transverse ^{13}C relaxation times, the PFG HCC(CO)NH-TOCSY experiment described here does not work very well. However, it may be possible to obtain side-chain ^{13}C phase information for larger proteins using randomly or uniformly deuterated samples and beginning with aliphatic carbon polarization, as has recently been demonstrated for the non-phase CC(CO)NH-TOCSY experiment (Farmer and Venters, 1995).

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References

Bruhwiler, D. and Wagner, G. (1986) *J. Magn. Reson.*, **69**, 546–551.

Clowes, R.T., Boucher, W., Hardman, C.H., Domaille, P.J. and Laue, E.D. (1993) *J. Biomol. NMR*, **3**, 349–354.

Ernst, R.R., Bodenhausen, G. and Wokaun, A. (1987) *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, Clarendon Press, Oxford.

Farmer II, B.T. and Venters, R.A. (1995) *J. Am. Chem. Soc.*, **117**, 4187–4188.

Grzesiek, S. and Bax, A. (1992a) *J. Magn. Reson.*, **99**, 201–207.

Grzesiek, S. and Bax, A. (1992b) *J. Am. Chem. Soc.*, **114**, 6291–6293.

Grzesiek, S., Anglister, J. and Bax, A. (1993) *J. Magn. Reson. Ser. B*, **101**, 114–119.

Grzesiek, S. and Bax, A. (1993) *J. Biomol. NMR*, **3**, 185–204.

John, B.K., Plant, D. and Hurd, R. (1992) *J. Magn. Reson. Ser. A*, **101**, 113–117.

Kay, L.E., Keifer, P. and Saarinen, T. (1992) *J. Am. Chem. Soc.*, **114**, 10663–10665.

Logan, T.M., Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1992) *FEBS Lett.*, **314**, 413–418.

Logan, T.M., Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1993) *J. Biomol. NMR*, **3**, 225–231.

Lyons, B.A. and Montelione, G.T. (1993) *J. Magn. Reson. Ser. B*, **101**, 206–209.

Lyons, B.A., Tashiro, M., Cedergren, L., Nilsson, B. and Montelione, G.T. (1993) *Biochemistry*, **32**, 7839–7845.

Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **84**, 393–399.

Mohebbi, A. and Shaka, A.J. (1991) *Chem. Phys. Lett.*, **178**, 374–378.

Montelione, G.T. and Wagner, G. (1989) *J. Am. Chem. Soc.*, **111**, 3096–3098.

Montelione, G.T., Lyons, B.A., Emerson, S.D. and Tashiro, M. (1992) *J. Am. Chem. Soc.*, **114**, 10974–10975.

Muhandiram, D.R. and Kay, L.E. (1994) *J. Magn. Reson. Ser. B*, **103**, 203–216.

Nilsson, B., Moks, T., Jansson, B., Abrahmsen, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T.A. and Uhlen, M. (1987) *Protein Eng.*, **1**, 107–113.

Palmer, A.G., Cavanagh, J., Wright, P.E. and Rance, M. (1991) *J. Magn. Reson.*, **93**, 151–170.

Richardson, J.M., Clowes, R.T., Boucher, W., Domaille, P.J., Hardman, C.H., Keeler, J. and Laue, E.D. (1993) *J. Magn. Reson. Ser. B*, **101**, 223–227.

Schleucher, J., Schwendinger, M., Sattler, M., Schmidt, P., Schedletzky, O., Glaser, S.J., Sørensen, O.W. and Griesinger, C. (1994) *J. Biomol. NMR*, **4**, 301–306.

Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.

Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.

Sørensen, O.W., Eich, G.W., Levitt, M.H., Bodenhausen, G. and Ernst, R.R. (1983) *Prog. NMR Spectrosc.*, **16**, 163–192.

Vuister, G.W. and Bax, A. (1992) *J. Magn. Reson.*, **98**, 428–435.

Wang, A.C., Lodi, P.J., Qin, J., Vuister, G.W., Gronenborn, A.M. and Clore, G.M. (1994) *J. Magn. Reson. Ser. B*, **105**, 196–198.

Wittekind, M. and Mueller, L. (1993) *J. Magn. Reson. Ser. B*, **101**, 201–205.

Wittekind, M., Metzler, W.J. and Mueller, L. (1993) *J. Magn. Reson. Ser. B*, **101**, 214–217.

Yamazaki, T., Forman-Kay, J.D. and Kay, L.E. (1993) *J. Am. Chem. Soc.*, **115**, 11054–11055.

Yamazaki, T., Pascal, S.M., Singer, A.U., Forman-Kay, J.D. and Kay, L.E. (1995) *J. Am. Chem. Soc.*, **117**, 3556–3564.

Zimmerman, D. and Montelione, G.T. (1995) *Curr. Opin. Struct. Biol.*, in press.