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Side chain and backbone assignments in isotopically labeled proteins from two heteronuclear triple resonance experiments

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Two multi-dimensional heteronuclear NMR experiments are described for assigning the resonances in uniformly ^{15}N- and ^{13}C-labeled proteins. In one experiment (HCNH-TOCSY), the amide nitrogen and proton are correlated to the side-chain protons and carbons of the same and preceding residue. In a second triple resonance experiment (HC(CO)NH-TOCSY), the amide nitrogen and proton of one residue is correlated exclusively with the side-chain proton and carbon resonances of the preceding residue by transferring magnetization through the intervening carbonyl. The utility of these two experiments for making sequential resonance assignments in proteins is illustrated for [U-^{15}N,^{13}C]FKBP (107 residues) complexed to the immunosuppressant, ascomycin.

Assignment; Protein; FKBP; Heteronuclear multi-dimensional NMR

1. INTRODUCTION

Obtaining unambiguous assignments is central to the determination of high-resolution solution structures of proteins using NMR spectroscopy. The assignment strategy involves three steps [1]: the correlation of the individual resonances of the amino acid spin systems from scalar connectivities, identification of the spin systems by amino acid type, and the linking together of neighboring amino acids from either ^1H,^1H NOESY [1] or recently developed triple resonance scalar correlation experiments [2]. Although a number of NMR experiments have been developed for each of these steps in the assignment process, in many cases ambiguities still arise due to spectral overlap. For example, in 3D [3] and 4D HCCH-TOCSY experiments [4] used to correlate the ^1H and ^{13}C resonances of the amino acid side chains in large, uniformly ^{13}C-labeled proteins, complications can occur when the H^α and C^α frequencies overlap. Likewise, ambiguities arise when linking together the neighboring amino acid spin systems using the 3D HNCA [2] and HN(CO)CA experiments [5] when the C^α spins overlap. Some of these ambiguities can be resolved in 4D HNCAHA and HN(CO)CAHA experiments [6,7] or in CBCANH [8] and CBCA(CO)NH [9] experiments in which the identification of neighboring amino acids is improved by correlating more frequencies in the same experiment.

In this paper, we describe a new set of heteronuclear triple resonance experiments which extends the number of side-chain resonances that are correlated with the backbone amides, thereby minimizing ambiguities in linking together adjacent amino acid spin systems. In addition, in the same set of experiments, the individual amino acid spin systems are identified by amino acid type from the characteristic ^{13}C chemical shifts of the amino acid side chains [4,10], allowing the sequence-specific assignments to be made solely from these two NMR experiments. The utility of these experiments for assigning proteins is demonstrated on [U-^{15}N,^{13}C]FKBP (107 residues) [11,12] complexed to ascomycin [13].

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Abbreviations: FKBP, FK506 binding protein; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; INEPT, insensitive nucleus enhancement by polarization transfer.

2. MATERIALS AND METHODS

2.1. *NMR sample preparation*

[U-^{15}N,^{13}C]FKBP was isolated and prepared as previously described [14]. The final sample buffer consisted of 50 mM potassium phosphate (pH 6.5), 100 mM NaCl, and 1 mM deuterated dithiothreitol in H_2O(90%)/D_2O(10%). The FKBP/ascomycin complex was prepared by adding an excess of ascomycin to FKBP and stirring the sample at room temperature for 48 h. Excess ascomycin was removed by centrifugation prior to the NMR experiments. The final protein concentration was 3 mM.

2.2. *NMR spectroscopy*

All NMR spectra were acquired on a Bruker AMX600 NMR spectrometer at 30°C. The two 3D versions of the experiments were collected under identical conditions with 64 × 40 × 1,024 complex points using sweep widths of 6,250 Hz (^1H,t_1), 2,128 Hz (^{15}N,t_2), and 10,000 Hz (^1H,t_3). The data were processed on Silicon Graphics computers using in-house written software. In the two indirect dimensions, the data were extended using linear prediction [15]. The final processed

3D data sets consisted of 256 (^{1}H,ω_1) × 128 (^{15}N,ω_2) × 1,024 (^{1}H,ω_3) real points. The 4D HC(CO)NH-TOCSY experiment was collected with 40 × 8 × 8 × 1,024 complex points using sweep widths of 6,250 Hz (^{1}H,t_1), 3,290 Hz (^{13}C,t_2), 2,128 Hz (^{15}N,t_3), and 10,000 Hz (^{1}H,t_4). The indirect dimensions were extended by linear prediction, and the final data set size of the 4D experiment was 256 (^{1}H,ω_1) × 64 (^{13}C,ω_2) × 32 (^{15}N,ω_3) × 1,024 (^{1}H,ω_4) real points.

3. RESULTS AND DISCUSSION

Fig. 1 shows the pulse sequences used in these experiments. Based on the magnetization transfer pathway, the experiments are called HCNH-TOCSY and HC(CO)NH-TOCSY. For the HC(CO)NH-TOCSY experiment (Fig. 1A), the magnetization follows the path:

$$^1H(\omega_1) \rightarrow {}^{13}C(\omega_2) \rightarrow {}^{13}C^{\alpha} \rightarrow {}^{13}CO \rightarrow {}^{15}N(\omega_3) \rightarrow {}^1H^N(\omega_4)$$

The magnetization pathway for the HCNH-TOCSY experiment is:

$$^1H(\omega_1) \rightarrow {}^{13}C(\omega_2) \rightarrow {}^{13}C^{\alpha} \rightarrow {}^{15}N(\omega_3) \rightarrow {}^1H^N(\omega_4)$$

All magnetization transfers occur via large one-bond hetero- and homo-nuclear couplings which are, to a first approximation, independent of local geometry.

Both experiments begin with a refocused-INEPT transfer of proton magnetization to their attached carbons. The experiments have been optimized by concatenation of 180° pulses [16]. The 4D experiments are recorded by independent incrementation of both t_1 and t_2 to obtain the ^{1}H and ^{13}C chemical shifts, respectively. In the 4D experiment, 8 complex carbon evolution points were acquired in the constant time C–H scalar coupling refocusing period as shown in Fig. 1. In 3D versions of this experiment, either the ^{1}H or ^{13}C chemical shifts are obtained by incrementing t_1 or t_2, respectively. For 3D versions of the experiment with carbon evolution, the $2\tau_2$ period was modified to give:

^{1}H 90(ϕ2) 180

^{13}C 90(ϕ3)-Δ_1- -Δ_2- -Δ_3-180(ϕ4)-Δ_4-90(y)

^{13}CO 180

where $\Delta_1 = \Delta_3$ = the initial t_2 delay and $\Delta_2 = \Delta_4 = 1.1$ ms. For subsequent t_2 increments, Δ_1 is incremented by dwell/2 with Δ_3 incremented and Δ_4 decremented so that the change in $\Delta_3 + \Delta_4$ = dwell/2.

After the refocused-INEPT portion of the experiment, the ^{13}C magnetization of the amino acid side chains is transferred to C$^{\alpha}$ using a z-filtered FLOPSY-8 mixing sequence [17]. In the HC(CO)NH-TOCSY experiment, transverse C$^{\alpha}$ magnetization generated by the C$^{\alpha}$ 90° pulse is transferred to its attached CO via the large $^1J_{C^{\alpha},CO}$ coupling during the $2\tau_3$ period. Carbonyl magnetization is then refocused with respect to its attached C$^{\alpha}$ ($2\tau_4$) and simultaneously dephased with respect to its attached ^{15}N (τ_4, τ_5, and τ_6). During the constant time ^{15}N evolution period ($2\tau_7$), antiphase ^{15}N magnetization is rephased with respect to its attached carbonyl and dephased with respect to its amide proton (τ_8). Observable $^1H^N$ magnetization is generated by a reverse-INEPT sequence and detected during t_4.

The HCNH-TOCSY experiment (Fig. 1B) can be described in a similar fashion except that the C$^{\alpha}$ magnetization present following the FLOPSY-8 mixing sequence [16] is transferred directly to its attached amide nitrogen and subsequently to the amide proton. Transfer of C$^{\alpha}$ magnetization to the CO is avoided in this experiment by decoupling of the carbonyls.

Fig. 2 depicts $^1H(\omega_1)$,$^1H^N(\omega_3)$ planes from 3D H(C)NH-TOCSY (Fig. 2a,c,e) and 3D H(C)(CO)NH-TOCSY (Fig. 2b,d,f) spectra extracted at the ^{15}N amide chemical shifts (ω_2) of the FKBP residues shown to the right of the spectra. The spectra illustrate the utility of these experiments for making the assignments of three sequential residues (K17-R18-G19) of FKBP. In the first step, the amide proton and nitrogen signals are correlated to the aliphatic side-chain protons of the same residue as shown for K17 in the H(C)NH-TOCSY spectrum of Fig. 2a. Although correlations between the amide proton and nitrogen with the side-chain protons of the preceding residue are also expected in this experiment, these signals were generally not observed or very weak due to the small $^2J_{N,C^{\alpha}}$ coupling constant. In principle, a 3D ^{15}N-resolved TOCSY experiment (3) could also be used to correlate the amide and side-chain resonances of the same residue. However, for larger proteins, the 3D ^{15}N-resolved TOCSY experiment rarely provides all of the side-chain resonances, and in many cases, only correlations between the amides and H$^{\alpha}$ are observed [19].

In the next step of the assignment procedure, the amide ^{1}H and ^{15}N of the next residue (i+1) is identified by matching the proton signals (ω_1) in the 3D H(C)NH- and H(C)(CO)NH-TOCSY experiments as shown in Fig. 2b. Since several signals are used in the matching procedure, ambiguities are rarely encountered. The process is continued by repeating these two steps as demonstrated in the remaining spectra of Fig. 2.

In the above examples, 3D versions of the HCNH- and HC(CO)NH-TOCSY experiments were described in which the proton signals are indirectly detected in ω_1. The same assignment procedure could be applied using 3D versions of the experiments in which the ^{13}C chemical shifts are obtained in ω_2. Alternatively, 4D experiments could be recorded in which *both* the ^{1}H *and* ^{13}C chemical shifts are obtained. Fig. 3 depicts a series of $^1H(\omega_1)$,$^{13}C(\omega_2)$ planes from a 4D HC(CO)NH-TOCSY spectrum of [U-^{15}N,^{13}C]FKBP/ascomycin showing the side chain resonances of the indicated residues detected at the $^{15}N(\omega_3)$,$^1H^N(\omega_4)$ chemical shifts of the i+1 residues. The ^{1}H and ^{13}C chemical shifts of the side chains

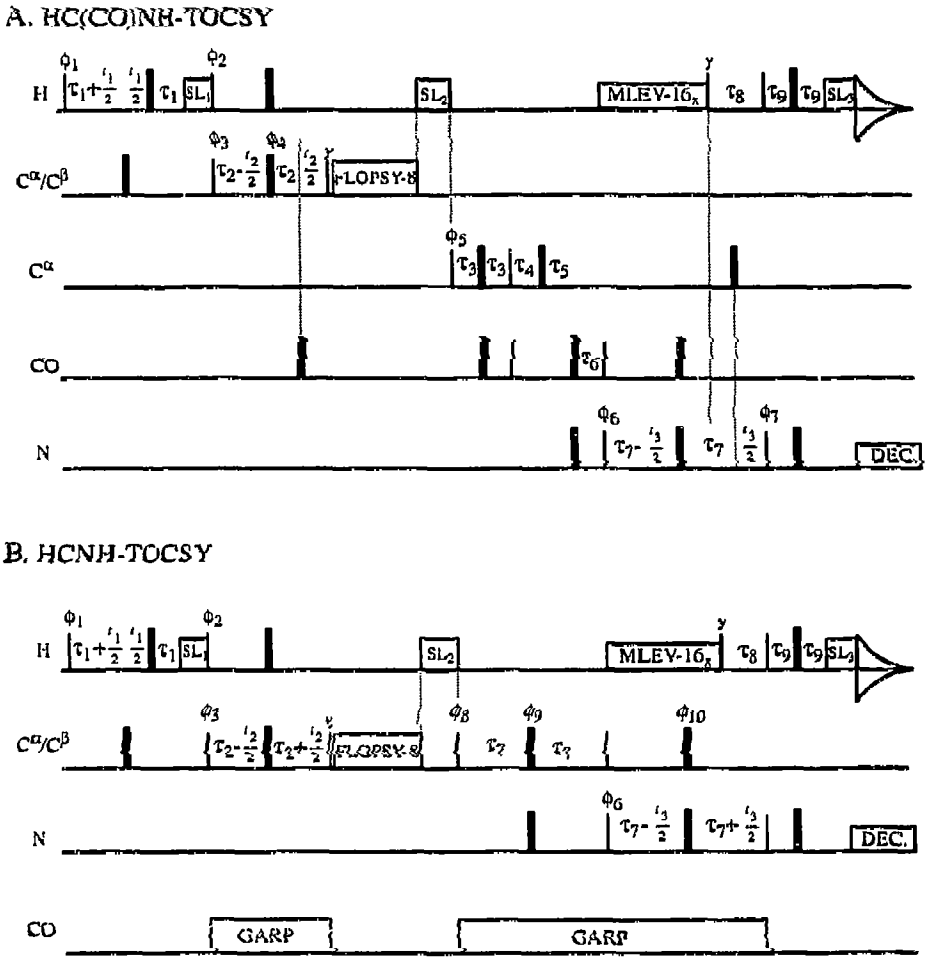

Fig. 1. The pulse sequences for (A) the 4D HC(CO)NH-TOCSY and (B) 4D HCNH-TOCSY experiments. The thin and thick solid lines represent 90° and 180° pulses, respectively. The proton carrier was set at the water frequency (4.8 ppm) and the nitrogen carrier was at 117 ppm. The carbon carrier frequency was placed at 37 ppm for the pulses applied to C^{α}/C^{β} using a 90° pulse width of 13.5 μs. The carrier was switched to 59 ppm for pulses applied to the C^{α} region using a 90° pulse width of 57 μs. During the TOCSY portion of the sequence, a 90° pulse width of 37.5 μs was employed. The C^{α} (57 μs) and CO (58 μs) 90° pulse widths in the HC(CO)NH-TOCSY experiment were adjusted to provide a null in their excitation profiles at the CO and C^{α} frequency, respectively. Spin lock purge pulses (SL_1 = 1.0, SL_2 = 3.0, SL_3 = 0.5 ms) were used for solvent suppression. Proton decoupling during the nitrogen evolution period was accomplished with a synchronous MLEV-16 decoupling scheme (3.8 kHz RF field), and ^{15}N-decoupling was employed during the acquisition period using GARP [21] with a 1.8 kHz RF field. Carbonyl decoupling was accomplished in sequence 1B by continuous low-power GARP decoupling [21] (1.04 kHz RF field). The phase cycling for both sequences was as follows: $\phi_1 = x,-x$; $\phi_2 = 8(y),8(-y)$; $\phi_3 = 2(x),2(-x)$; $\phi_4 = 8(x),8(-x)$; $\phi_5 = 8(y),8(-y)$; $\phi_6 = 4(x),4(-x)$; $\phi_7 = 8(x),8(-x)$; $\phi_8 = 16(y),16(-y)$; $\phi_9 = 16(x),16(-x)$; $\phi_{10} = 8(x),8(-x)$. In both 3D and 4D versions of experiment (A) 16 scans/increment were used with the receiver = $x,2(-x),x,-x,2(x),-x,-x,2(x),-x,x,2(-x),x$ (total acquisition times of 2.2 and 4.5 days, respectively). For sequence (B), 32 scans/increment were used (total acquisition time of 4.5 days) and the receiver phase in sequence (B) was the same as for experiment (A) except for the inversion of the receiver phase for the second 16 scans required by the phase cycling of ϕ_8. Pulses for which no phase is indicated were applied along the x axis. Quadrature detection in the indirect dimensions was obtained using the States-TPPI method [22] by phase cycling ϕ_1 for t_1, ϕ_3 for t_2, and ϕ_6 for t_3. The delays were: τ_1 = 1.5 ms, τ_2 = 1.1 ms, τ_3 = 3.5 ms, τ_4 = 4.5 ms, τ_5 = 6.5 ms, τ_6 = 11.0 ms, τ_7 = 11.2 ms, τ_8 = 5.6 ms, and τ_9 = 2.3 ms.

are readily obtained and used to assign the individual spin systems by amino acid type. In the 4D version of the experiment, overlap of the proton signals (e.g. Hγ^1 and Hγ^2 of V68, Fig. 3) can be resolved by the different chemical shifts of their attached carbons. The ^{1}H and ^{13}C frequencies are then used to link together the amino acid spin systems. A particular advantage of this experiment is that the side-chain signals are correlated to the amide ^{15}N/^{1}H^N frequencies which are more well-resolved compared to the C^{α}/H^{α} chemical shifts typically used as starting points to assign the resonances of the side chains. For example, overlap of the H^{α}/C^{α} chemical shifts occurs for L104/K34, D32/N43, and K105/Q65 which complicates the assignments of these residues. However, as shown in Fig. 3, these spin systems are cleanly resolved in the 4D HC(CO)NH-TOCSY experiment.

From the two 4D NMR experiments, the sequence-

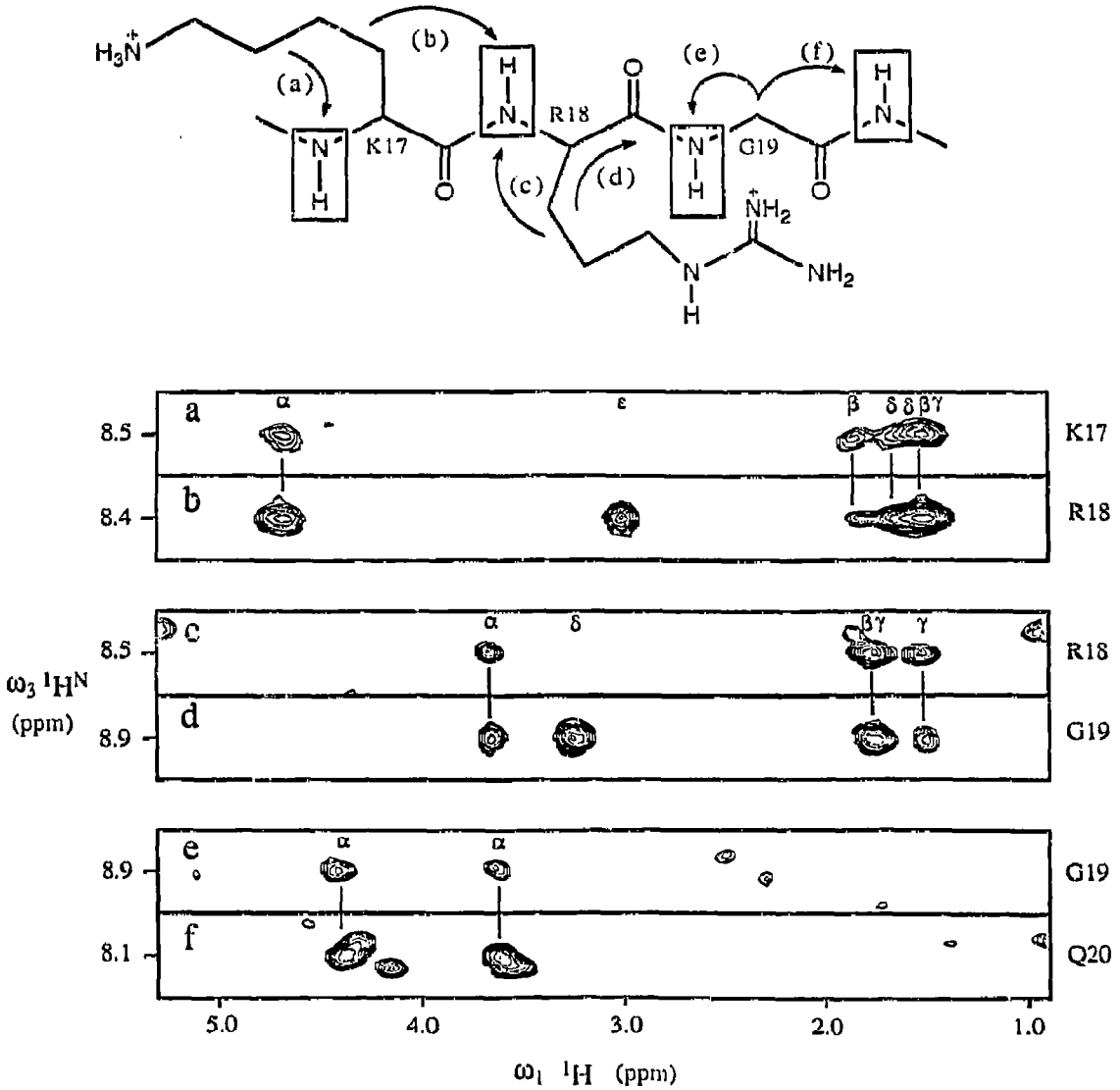

Fig. 2. (Top) Schematic illustration of the correlations observed (arrows) in the HCNH- and HC(CO)NH-TOCSY experiments for three adjacent residues (K17-R18-G19) of FKBP. (a,c,e) $^1H(\omega_1),^1H^N(\omega_3)$ planes from a 3D H(C)NH-TOCSY spectrum of [U-^{15}N,^{13}C]FKBP/ascomycin extracted at the ^{15}N chemical shifts (ω_2) of K17, R18, and G19. (b,d,f) $^1H(\omega_1),^1H^N(\omega_3)$ planes from a 3D H(C)(CO)NH-TOCSY spectrum of [U-^{15}N,^{13}C]FKBP/ascomycin extracted at the ^{15}N chemical shifts (ω_2) of R18, G19, and Q20. The εCH_2 of K17 and δCH_2 signals of R18 are missing in the 3D H(C)NH-TOCSY spectra (Fig. 2a,c) due to the shorter TOCSY mixing time (15.1 ms) used in this experiment compared to that used in the 3D H(C)(CO)NH-TOCSY experiment (18.8 ms).

specific assignments of nearly all of the ^{1}H, ^{15}N, and ^{13}C resonances for small, isotopically labeled proteins can be assigned. This approach avoids the need to compare NMR data from several 3D experiments acquired on different samples prepared in H_2O or D_2O. In addition, the HCNH- and HC(CO)NH-TOCSY experiments are particularly valuable for assigning proteins in the unfolded or partially folded state. For denatured proteins many of the C^α/H^α signals overlap; whereas, the amide $^{15}N/^1H^N$ signals typically provide the best spectral dispersion. Thus, the side-chain signals for unfolded proteins can be assigned using these experiments by correlating them to the well-resolved amide signals (manuscript in preparation).

The HCNH- and HC(CO)NH-TOCSY experiments couple two commonly used pulse sequences: the HCCH-TOCSY and the HNCA or HN(CO)CA experiments. The signal detected is thus dependent on the transfer efficiency of these two steps. The $C^\alpha \rightarrow CO \rightarrow N$ transfer is very efficient, making the HC(CO)NH-TOCSY experiment more sensitive than the HCNH-TOCSY experiment. This is especially true for larger proteins where short C^α T_2 values strongly attenuate the $C^\alpha \rightarrow N$ transfer step [20].

For larger proteins, resonance overlap and missing data present serious problems in making the sequential assignments. Missing data allows only relatively short stretches of sequentially assigned resonances to be ob-

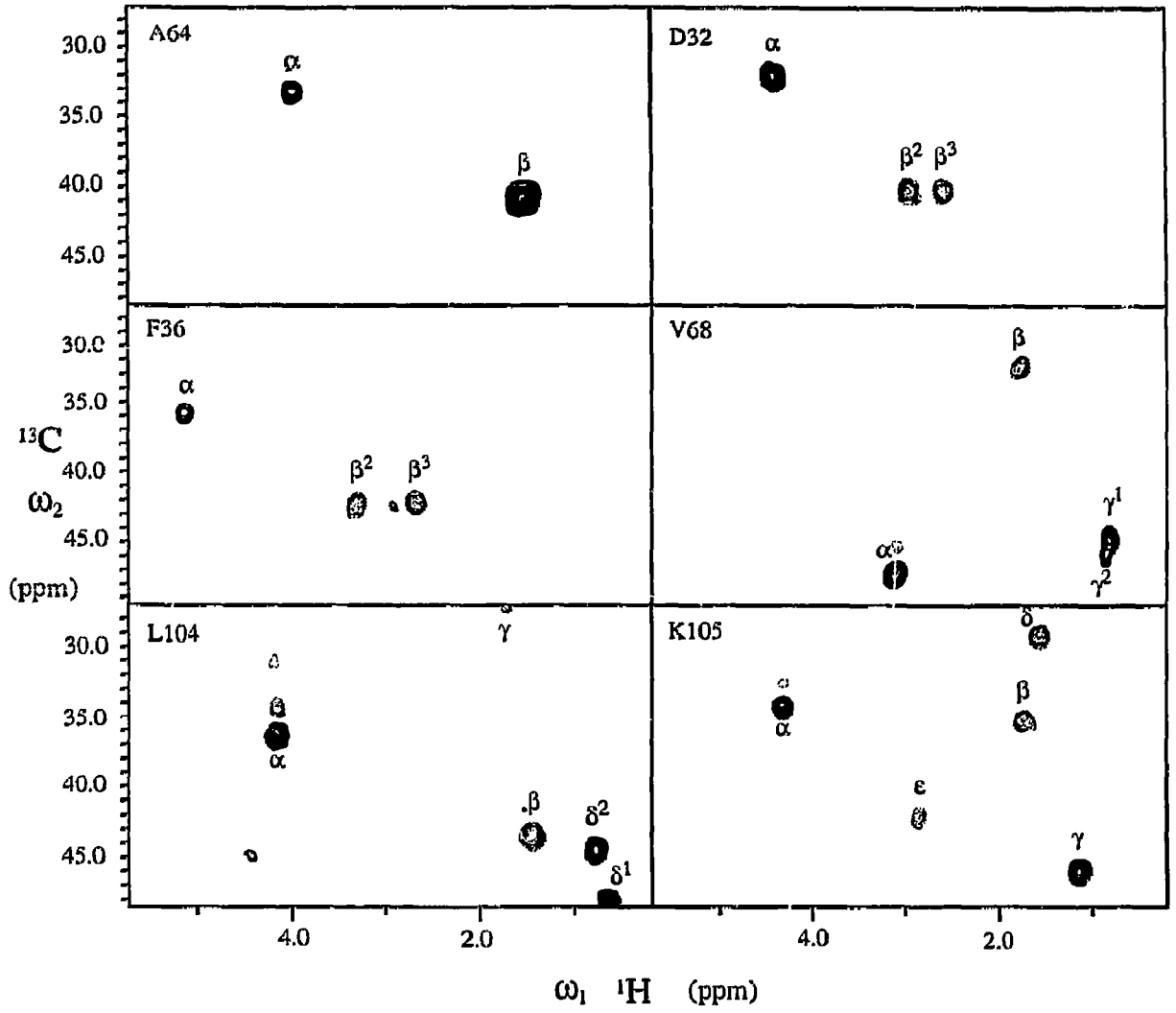

Fig. 3. $^1H(\omega_1)$,$^{13}C(\omega_2)$ planes from a 4D HC(CO)NH-TOCSY spectrum of [U-^{15}N,^{13}C]FKBP/ascomycin showing the indicated side chain resonances detected at the $^{15}N(\omega_3)$,$^1H^N(\omega_4)$ chemical shifts of the i+1 residues. The TOCSY mixing time was 19.2 ms. The phase ramp in ω_2 (180°) was chosen so that the folded (black) and unfolded (gray) resonances have opposite signs. Folded resonances with proton chemical shifts of less than 3.0 ppm can generally be unfolded by subtracting the carbon sweep width (21.8 ppm) from the peak position while all other folded resonances are unfolded by adding the carbon sweep width to their peak position.

tained which cannot be placed in the protein sequence due to ambiguities in assigning the spin systems by amino acid type. The 4D HC(CO)NH-TOCSY is ideally suited for overcoming these problems, since residue types are assigned by inspection due to the characteristic ^{13}C chemical shifts. The data is also much less ambiguous to interpret than data obtained in HCCH-TOCSY experiments, since the side chain ^1H and ^{13}C chemical shifts are already correlated to the amide nitrogen. For larger proteins, we have found the data obtained in the HC(CO)NH-TOCSY experiment, combined with the 4D HNCAHA and HN(CO)CAHA experiments, are generally sufficient for complete sequential assignments.

4. CONCLUSIONS

The two-pulse sequences presented in this paper greatly facilitate the identification of adjacent amino acid spin systems by correlating the side-chain resonances with the backbone proton and nitrogen. In addition, from the side-chain ^1H and ^{13}C resonances, which are unambiguously obtained using the well-resolved amide $^{15}N/^1H^N$ frequencies, the spin systems can be assigned by amino acid type. Taken together, this information rapidly leads to the sequence-specific assignments in small proteins. These experiments are also particularly valuable in NMR studies of unfolded proteins in which only the amide $^{15}N/^1H^N$ can be resolved and C^α T_2 values are long. For larger proteins, the utility of the

HCNH-TOCSY experiment is more limited due to the short C^{α} relaxation times. However, the HC(CO)NH-TOCSY experiment is a valuable tool for resolving ambiguities in assigning the resonances of larger proteins.

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