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A general method for assigning NMR spectra of denatured proteins using 3D HC(CO)NH-TOCSY triple resonance experiments

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SUMMARY

A general approach for assigning the resonances of uniformly ^{15}N- and ^{13}C-labeled proteins in their unfolded state is presented. The assignment approach takes advantage of the spectral dispersion of the amide nitrogen chemical shifts in denatured proteins by correlating side chain and backbone carbon and proton frequencies with the amide resonances of the same and adjacent residues. The ^{1}H resonances of the individual amino acid spin systems are correlated with their intraresidue amide in a 3D ^{15}N-edited $^{1}H,^{1}H$-TOCSY-HSQC experiment, which allows the spin systems to be assigned to amino acid type. The spin systems are then linked to the adjacent i–1 spin system using the 3D H(C)(CO)NH-TOCSY experiment. Complete ^{13}C assignments are obtained from the 3D (H)C(CO)NH-TOCSY experiment. Unlike other methods for assigning denatured proteins, this approach does not require previous knowledge of the native state assignments or specific interconversion rates between the native and denatured forms. The strategy is demonstrated by assigning the ^{1}H, ^{13}C, and ^{15}N resonances of the FK506 binding protein denatured in 6.3 M urea.

The stability and structure of native proteins reflects the difference in free energy of various interactions present in the native and denatured states (Dill and Shortle, 1991). Therefore, to understand the forces governing protein structure and folding, detailed knowledge of the interactions in both the native and denatured states is required. While the properties and structures of native proteins have been systematically studied, comparable studies on denatured proteins have been limited by the intractability of the problem to X-ray crystallographic analysis and the difficulties associated with NMR studies of denatured proteins.

A prerequisite for detailed structural characterization of denatured proteins by high-resolution NMR spectroscopy is to obtain unambiguous sequential resonance assignments. Direct assignment of the NMR spectra of denatured proteins is severely limited by extensive overlap of the ^{1}H and ^{13}C chemical shifts (Evans et al., 1991). To overcome this problem, the chemical shift disper-

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sion of the native state has been used to obtain assignments of denatured proteins via magnetization transfer experiments when the native and denatured states are in rapid equilibrium (Dobson and Evans, 1984; Dobson et al., 1984; Fox et al., 1986; Baum et al., 1989; Evans et al., 1989, 1991; Miranker et al., 1991). Recently, Wüthrich and coworkers used this method to obtain the complete backbone amide $^1H^N$ and ^{15}N assignments of the amino terminal domain of the phage 434 repressor protein in 7.0 M urea by taking advantage of the relatively high ^{15}N chemical shift dispersion in the denatured form (Wider et al., 1991). The H^α and side chain ^1H signals were then assigned by correlation with $^1H^N$ via ^{15}N-edited 2D scalar correlation experiments (Neri et al., 1992).

The magnetization transfer approach can be useful, but its dependence on native state assignments and on specific native ↔ denatured interconversion rates limits its widespread application. In this communication we demonstrate a more general method for obtaining the sequential ^1H, ^{13}C and ^{15}N assignments of denatured proteins. The assignments are obtained entirely via scalar correlations and are not dependent on previous native state assignments or favorable interconversion rates between the native and denatured forms. The key to this approach is to correlate all side chain and backbone proton and carbon resonances to the ^{15}N and amide ^1H frequencies which have the highest spectral dispersion in denatured proteins. In the first step of the assignment procedure, the ^1H resonances comprising the individual amino acid spin systems are identified in a 3D ^{15}N-edited ^1H, ^1H-TOCSY-HSQC experiment (Marion et al., 1989a; Fesik and Zuiderweg, 1990). In denatured proteins, all the non-labile protons in each spin system are correlated with the intraresidue amide proton, which facilitates assignment of the spin system by residue type. The individual spin systems are then sequentially linked, using the recently developed 3D H(C)(CO)NH-TOCSY experiment (Logan et al., 1992; Montelione et al., 1992) in which the entire spin system of the i–1 residue is correlated with the amide ^1H and ^{15}N of the i residue. Using such a large number of resonances to link the individual spin systems minimizes assignment ambiguity arising from the limited ^1H chemical shift dispersion in denatured proteins and facilitates making the sequential assignments. Finally, the ^{13}C chemical shift assignments are obtained from the 3D (H)C(CO)NH-TOCSY (Logan et al., 1992), which helps to confirm the identification of the spin systems by amino acid type made from the ^1H data.

The pulse sequences for the 3D HC(CO)NH-TOCSY experiments with ^1H or ^{13}C evolution in ω_1 are shown in Fig. 1. The H(C)(CO)NH- and (H)C(CO)NH-TOCSY sequences are nearly identical, differing only in the refocused INEPT portion of the sequence to allow for either ^1H or ^{13}C chemical shift evolution. Both experiments begin with a refocused-INEPT transfer of proton magnetization to give in-phase ^{13}C magnetization at point a, which is transferred to C^α using a z-filtered FLOPSY mixing sequence (Mohebbi and Shaka, 1991). ^{13}C trim pulses are applied before and after the z-filter to dephase unwanted ^{13}C magnetization. A proton spin-lock purge pulse is applied for water suppression, and the ^{13}C carrier frequency is changed to the C^α region before transverse C^α magnetization is regenerated by the 90° C^α pulse at point b. During the $2\tau_3$ period, C^α-CO scalar couplings evolve to give C^α magnetization that is antiphase with respect to CO prior to point c. The small bandwidth of the C^α pulses effectively decouples C^α–C^β scalar coupling for most amino acids during the $2\tau_3$ period except for threonine and serine, whose C^β chemical shifts fall within the bandwidth of the C^α pulses. The effects of these passive couplings are minimized by keeping $2\tau_3$ shorter than the optimal value of $(2J_{C^\alpha,CO})^{-1}$. The simultaneous

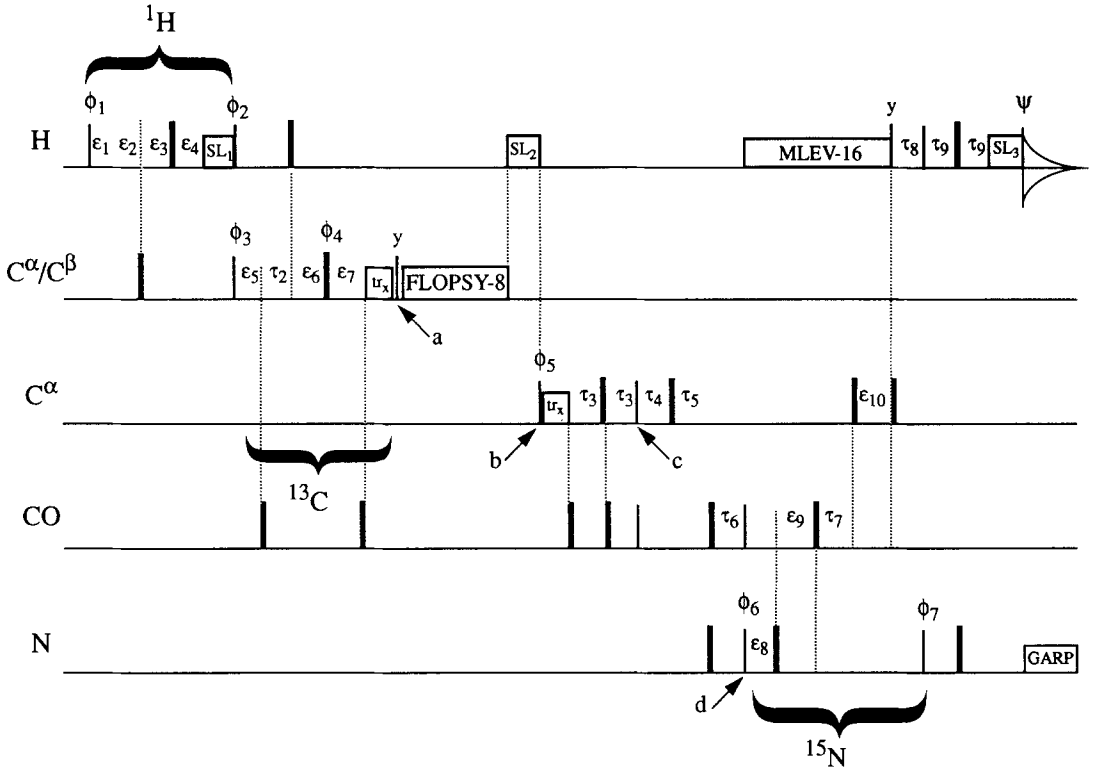

Fig. 1. The pulse sequences for the H(C)(CO)NH- and (H)C(CO)NH-TOCSY experiments. The thin and thick solid lines represent 90° and 180° pulses, respectively. All variable delays are denoted by ε and constant delays by τ. The ^{1}H carrier was set to 4.82 ppm and was on-resonance with the water frequency. The ^{15}N carrier was centered at 117 ppm and the ^{13}C carrier frequency was placed at 32.0 ppm for the pulses applied to C^{α}/C^{β} and then switched to the C^{α} region (55 ppm) for the remainder of the sequence. The ^{13}C 90° pulse widths were 12.5 and 32 μs for the refocused-INEPT and TOCSY portions of the sequence, respectively. The C^{α} (64 μs) and CO (66 μs) 90° pulse widths were calibrated to provide a null in their excitation profiles at the CO and C^{α} frequency, respectively. The first and second ^{13}C spin-lock trim pulses were applied along the x-axis (tr_x) for 2 ms and 1 ms, respectively. ^{1}H spin-lock purge pulses of 2 ms (SL_1), 4 ms (SL_2) and 0.75 ms (SL_3) were applied along the x-axis. Proton decoupling during the ^{15}N evolution period was accomplished with a synchronous MLEV scheme (4.5-kHz RF field), and nitrogen was decoupled during the acquisition period using GARP modulation with a 1.6-kHz RF field. The phase cycling for both sequences was as follows: ϕ_1 = x,−x; ϕ_2 = 4(y),4(−y); ϕ_3 = x,−x; ϕ_4 = 4(x),4(−x); ϕ_5 = 4(y),4(−y); ϕ_6 = 2(x),2(−x); ϕ_7 = 4(x),4(−x); ψ = x,2(−x),x,−x,2(x),−x. In the H(C)(CO)NH-TOCSY experiment ϕ_3 = x, and in the (H)C(CO)NH-TOCSY experiment ϕ_1 = x. Pulses for which no phase is indicated were applied along the x axis. Quadrature detection in the two indirect dimensions was obtained by incrementing ϕ_1 for ^{1}H or ϕ_3 for ^{13}C in t_1 and ϕ_6 in t_2, using the States-TPPI method (Marion et al., 1989b). Constant delay durations were τ_2 = 1.1 ms, τ_3 = 2.5 ms − 180° CO pulse, τ_4 = 4.5 ms, τ_5 = 7.0 ms − 180° C^{α} pulse, τ_6 = 11.5 ms − 180° C^{α} pulse, τ_7 = 5.7 ms − 180° CO pulse, τ_8 = 5.6 ms − 180° C^{α} pulse − 90° H pulse, and τ_9 = 2.5 ms. In the H(C)(CO)NH-TOCSY experiment, $\varepsilon_1 = \varepsilon_4$ = 1.5 ms, ε_2 (initial) = ε_3 (initial) = 31.5 μs, ε_7 = 1.1 ms and $\varepsilon_5 = \varepsilon_6$ = 0. ε_2 and ε_3 were incremented by 72.4 μs, ε_1 was incremented by 15.6 μs, and ε_4 decremented by 15.6 μs for each t_1 value. In the (H)C(CO)NH-TOCSY experiment, $\varepsilon_1 = \varepsilon_4$ = 1.5 ms, $\varepsilon_2 = \varepsilon_3$ = 0, ε_7 (initial) = τ_2 = 968 μs, ε_5 (initial) = 66.3 μs, and ε_6 (initial) = 21.2 μs. ε_5 and ε_6 were incremented by 132.5 μs and 117.6 μs, respectively, and ε_7 was decremented by 14.9 μs in subsequent t_1 times. In both experiments, ε_8 (initial) = 11.3 ms, ε_9 (initial) = ε_{10} (initial) = 163.2 μs with ε_8 decremented by 176.0 μs and ε_9 and ε_{10} incremented by 272.4 and 448.4 μs, respectively, for each t_2 increment.

C^{α}/CO 90° pulses at point c effect magnetization transfer from C^{α} to CO, which is subsequently refocused while CO–^{15}N couplings are evolved.

At point d, CO and ^{15}N 90° pulses generate transverse ^{15}N magnetization antiphase with respect to CO, which evolves simultaneously with refocusing of the attached carbonyl magnetization and dephasing with respect to its attached $^{1}H^{N}$ during τ_8. C^{α} decoupling during the ^{15}N evolution period is achieved by two 180° C^{α} pulses to avoid timing problems with our Bruker pulse programmer. $^{1}H^{N}$-^{15}N coupling is removed using a synchronous MLEV scheme to minimize scalar relaxation of the second kind (Bax et al., 1990). The sequence ends with a reverse-INEPT transfer of ^{15}N magnetization into observable amide proton magnetization.

The pulse sequences in Fig. 1 are improved over our previously published versions (Logan et al., 1992) as a result of utilizing the heteronuclear scalar transfer delays for part of the ^{1}H, ^{13}C, and ^{15}N chemical shift evolution periods. In order to understand the optimization of these time periods, the net durations of the scalar and chemical shift evolution periods must be considered. In the H(C)(CO)NH-TOCSY experiment (Fig. 1), the ^{1}H chemical shifts evolve during $(\varepsilon_1 + \varepsilon_2 + \varepsilon_3 - \varepsilon_4)$ while ^{1}H–^{13}C scalar couplings evolve during $(\varepsilon_1 + \varepsilon_2 - \varepsilon_3 + \varepsilon_4)$. For the initial t_1 increment, ε_1 and ε_4 = 1.5 ms, and the initial t_1 delay ($\varepsilon_2 + \varepsilon_3$ + 180° C^{α}/C^{β} pulse) is calculated to give a −180° linear phase correction (Bax et al., 1990). In subsequent t_1 increments, ε_2 and ε_3 are incremented by (dwell/2-ε_4 (initial)/k_H)), while ε_1 is incremented and ε_4 is decremented by (ε_4 (initial)/k_H) where k_H is one more than the number of points collected in the ^{1}H dimension. Using the ^{1}H/^{13}C scalar transfer time as part of the ^{1}H evolution time shortens this period by 3 ms. Because the chemical shift and scalar coupling evolution times are shared, we refer to this method as 'shared time' evolution. The shared time evolution approach is a general one that can be implemented in other multidimensional NMR experiments, and is especially useful in NMR experiments on large proteins with short relaxation times.

This approach is readily adapted to the (H)C(CO)NH-TOCSY experiment (Logan et al., 1992), saving 2.2 ms of scalar transfer evolution time. ^{13}C chemical shift evolves during $(\varepsilon_5 + \tau_2 + \varepsilon_6 - \varepsilon_7)$, and refocusing of ^{1}H–^{13}C scalar evolution occurs during $(\varepsilon_5 + \tau_2 - \varepsilon_6 + \varepsilon_7)$. ^{13}C chemical shift evolution is obtained by incrementing ε_5 by dwell/2 and ε_6 by (dwell/2 − ε_7 (initial)/k_C), while decrementing ε_7 by (ε_7 (initial)/k_C). In the initial ^{13}C increment, τ_2 and ε_7 = (1.1 ms − 180° CO pulse), and the initial delay ($\varepsilon_5 + \varepsilon_6$ + ^{1}H 180° pulse) = dwell/2 to provide a −180° linear phase correction. During the ^{13}C chemical shift evolution, CO is decoupled from C^{α} by the application of a CO 180° refocusing pulse. This decoupling pulse introduces a constant phase shift into the C^{α} chemical shift evolution due to Bloch–Siegert effects (Grzesiek and Bax, 1992), which are compensated for by the second CO 180° pulse applied immediately prior to the ^{13}C trim pulse.

The shared time evolution is also implemented in the ^{15}N dimension of the H(C)(CO)NH- and the (H)C(CO)NH-TOCSY experiments, where ^{15}N chemical shift evolves during $(\varepsilon_8 - \varepsilon_9 - \tau_7 - \varepsilon_{10} - \tau_8)$. For the initial τ_2 increment, $\varepsilon_8 = (\tau_7 + \tau_8$ + 180° CO pulse + 180° C^{α} pulse + 90° ^{1}H pulse) = 11.3 ms, and the initial t_2 delay, ($\varepsilon_9 + \varepsilon_{10}$ + 180° C^{α} pulse) = dwell/2, with $\varepsilon_9 = \varepsilon_{10}$. ^{15}N chemical shift evolution is obtained by incrementing ε_{10} by dwell/2 and ε_9 by (dwell/2 − ε_8 (initial)/k_N) while decrementing ε_8 by (ε_8 (initial)/k_N) in subsequent t_2.

Our strategy for assigning the resonances of denatured proteins using these methods is illustrated in NMR studies of the FK506 binding protein (FKBP) denatured in 6.3 M urea at pH 6.3 (Egan et al., 1993). FKBP is fully denatured under these conditions. The first step in the sequential resonance assignment process is to identify the resonances constituting the individual spin

systems (Wüthrich, 1986). For denatured proteins, this is best accomplished by correlating the non-labile ^1H signals in each spin system with the intraresidue backbone amide ^{15}N and $^1H^N$ through scalar interactions using the 3D ^{15}N-edited $^1H,^1H$-TOCSY-HSQC (Marion et al., 1989a; Fesik and Zuiderweg, 1990). Figure 2 shows $^1H(\omega_1)$, $^1H^N(\omega_3)$ strips taken from an ^{15}N-edited 3D TOCSY-HSQC experiment at the amide ^{15}N and $^1H^N$ chemical shifts of FKBP residues E102 to E107 (strips labeled as residue i). The favorable relaxation properties of denatured proteins allow correlation of the entire ^1H spin systems with the amide. From the characteristic ^1H chemical shifts, the individual spin systems are identified and assigned by residue type. In the next step of the assignment procedure, adjacent amino acid spin systems are linked together by correlating the amide nitrogen and proton of residue i with the ^1H resonances of the i–1 residue, using the 3D H(C)(CO)NH-TOCSY experiments as shown in Fig. 2 (strips labeled as residue i–1). Using complete spin systems to link together the individual residues minimizes ambiguity and allows immediate sequential assignment of unique dipeptide fragments within the protein sequence.

In the last step of the assignment procedure, sequential ^{13}C chemical shift assignments are obtained using the 3D (H)C(CO)NH-TOCSY experiment. As shown in Fig. 3, complete ^{13}C spin

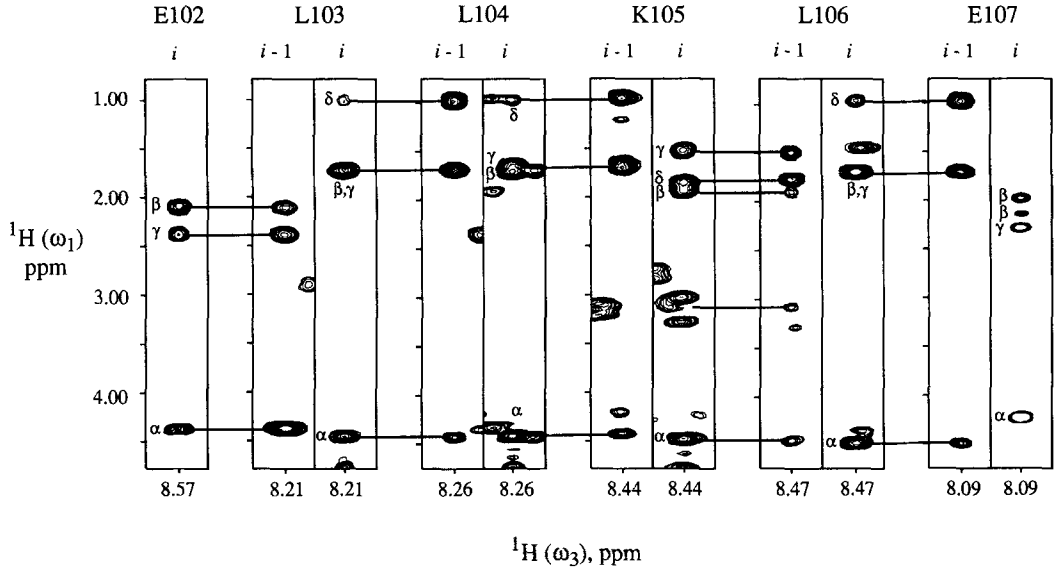

Fig. 2. $^1H(\omega_1)$, $^1H^N(\omega_3)$ strips taken from a ^{15}N-resolved 3D TOCSY-HSQC experiment (51 ms mixing time) acquired on [U-^{15}N]FKBP (strips labeled as i) and from a 3D H(C)(CO)NH-TOCSY experiment (18.1 ms mixing time) performed on [U-^{15}N,^{13}C]FKBP (strips labeled as i–1) at the amide ^{15}N frequencies of the indicated FKBP residues. Protein samples were 3 mM in a solution of 6.3 M urea, pH 6.3 in 90% H_2O. Both experiments were collected at 25°C on a Bruker AMX500 NMR spectrometer equipped with a home-built fourth channel in 8 scans per t_1 increment. Indirect ^1H and ^{15}N sweep widths were 5681.8 Hz and 1115.1 Hz, respectively, collected as 96 ($^1H,t_1$) × 64 ($^{15}N,t_2$) × 1024 ($^1H,t_3$) complex points. Total acquisition times were 4 and 2.5 days for the TOCSY-HSQC and H(C)(CO)NH-TOCSY experiments, respectively. The data sets were extended by linear prediction of the complex data in both indirect dimensions (Olejniczak and Eaton, 1990) and zero-filled to 512 ($^1H,\omega_1$) × 256 (^{15}N, ω_2) × 2048 (^1H, ω_3) real points. Spectra were processed using in-house written software on Silicon Graphics computers.

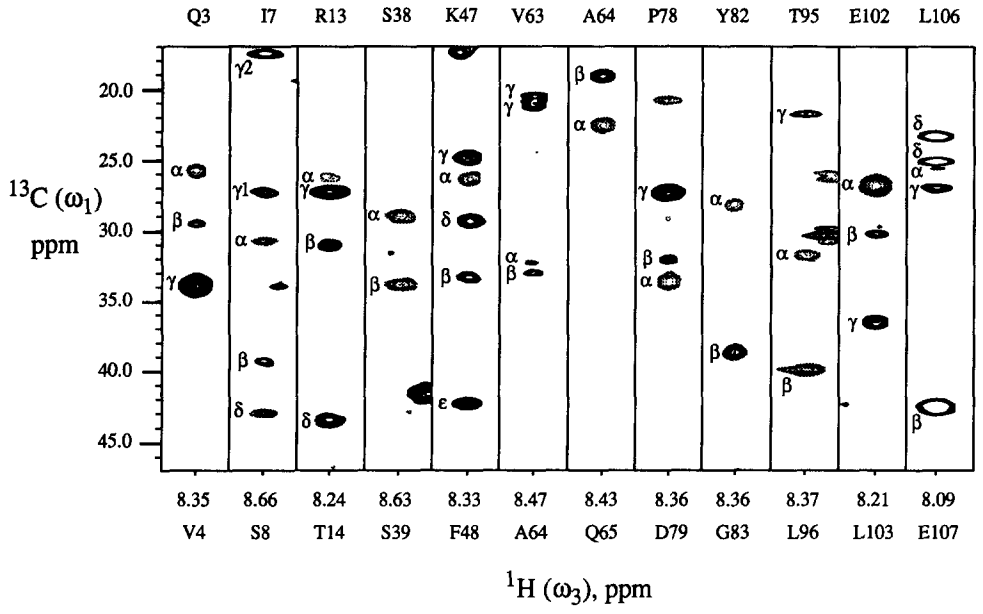

Fig. 3. $^{13}C(\omega_1)$, $^{1}H^{N}(\omega_3)$ strips taken from a 3D (H)C(CO)NH-TOCSY experiment showing the ^{13}C resonances for the residues indicated at the top of the strips detected at the amide ^{15}N and $^{1}H^{N}$ frequency of the indicated (bottom) FKBP residues. The ^{13}C-^{13}C TOCSY mixing times were 18.1 ms (S8, T14, F48, A64, D79, E107) and 9.1 ms. The (H)C(CO)NH-TOCSY experiment was collected in 8 scans per t_1 increment as 64(^{13}C,t_1) × 64 (^{15}N,t_2) × 1024 (^{1}H, t_3) complex points over 3773.6 Hz and 1115.1 Hz in ω_1 and ω_2, respectively. Total acquisition time was 2.5 days for each experiment. The data sets were extended by linear prediction of the complex data in both indirect dimensions (Olejniczak and Eaton, 1990) and zero-filled to 256 (^{13}C, ω_1) × 256 (^{15}N, ω_2) × 2048 (^{1}H, ω_3) real points. Spectra were processed using in-house written software on Silicon Graphics computers. The true chemical shifts of folded (gray) peaks can be obtained by adding or subtracting 30.0 ppm from the observed chemical shift.

systems of the i–1 residue are detected on the ^{15}N and $^{1}H^{N}$ of residue i using this experiment. Observation of the entire ^{13}C spin systems facilitates assignment by residue type (Richarz and Wüthrich, 1978; Oh et al., 1988; Olejniczak et al., 1992) and is used to confirm the ^{1}H assignments. ^{13}C resonance assignments for the C-terminal residue and residues having proline as the i + 1 residue are obtained using the 3D (H)CNH-TOCSY experiment (Logan et al., 1992; Lyons and Montelione, 1993). Any ambiguity in assigning specific ^{1}H and ^{13}C resonances to a particular site in a spin system can be resolved by acquiring a 4D HC(CO)NH-TOCSY experiment (Logan et al., 1992).

Using the assignment procedure outlined above, complete sequential ^{1}H, ^{13}C, and ^{15}N chemical shift assignments were obtained for denatured FKBP. These assignments are currently being used for detailed structural characterization of denatured FKBP and its interactions with solvent (manuscript in preparation). Our initial attempts to assign the resonances of denatured FKBP using the established chemical exchange methods failed because conditions could not be found to generate suitable exchange rates between the native and denatured states. The new assignment strategy is quite general and should be applicable to the study of other non-native states of proteins.

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