Monitoring Protein Folding at Atomic Resolution

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ABSTRACT

Elucidation of the mechanisms by which proteins fold from disordered conformations to their unique native conformations is one of the most challenging tasks facing structural biologists. Understanding the mechanism(s) of protein folding involves the characterization of all structural species that occur in the protein-folding reaction. Nuclear magnetic resonance (NMR) spectroscopy is a powerful and versatile technique that provides an avenue to investigate the structures of the various conformational states at the residue level along the protein-folding reaction coordinate. In this Account, we provide a comprehensive review of the recent progress on the applications of NMR to monitor equilibrium and kinetic conformational states of the protein-folding reaction.

Introduction

The mechanism by which proteins fold to their unique native conformations is still a central problem in structural biology. Understanding protein folding represents a unique opportunity to unravel the relationship between protein sequence, structure, stability, and ultimately function.[1,2] The folding process is now coupled to a wide array of biological processes including protein trafficking and regulation of the cell cycle.[3] In addition, there is accumulating evidence that diseases because of amyloidosis, such as Crefzfeld disease, Alzheimer's disease, and Parkinson's disease are directly related to the presence of unfolded or misfolded states.[3,4] The final goal of the study of protein folding is to develop a model(s) that could accurately predict the folding mechanism and the final structure of a protein based on its sequence.[5,6] However, development of such a model requires in-depth knowledge of the structural and thermodynamical features of various conformational states of a protein, as it navigates from its denatured state(s) to its native state.

Several biophysical techniques have been employed to monitor structural events that occur during protein folding/unfolding (Figure 1).[5] In this Account, we discuss recent applications of nuclear magnetic resonance (NMR) spectroscopy to monitor protein folding/unfolding reactions at atomic resolution.

Characterization of Equilibrium Intermediate States

Investigations of the structure and dynamics of nonnative, partially folded states provide important insight into the nature of protein-folding intermediates and the protein-folding process itself.[7] The folding/unfolding intermediates range from near-native well-structured intermediates to mostly unstructured intermediates resembling the denatured state(s) (Figure 1). One of the conformations frequently observed under mildly denaturing conditions is a compact denatured state called the "molten globule (MG)" state(s).[8] These partially folded species are compact, have extensive nativelike secondary structure, but contain little or no tertiary structural interactions (Figure 1).[8] Several studies suggest that there is a close resemblance between the MG states observed at equilibrium under mild denaturing conditions and those formed in the early stages of refolding in a number of proteins.[8−11] Therefore, structural characterization of such equilibrium MG or MG-like partially folded intermediates will help in the elucidation of structures of transient MG states that occur in the kinetic refolding pathways.

Partially folded proteins do not crystallize, because they do not adopt unique three-dimensional structures in solution but fluctuate over an ensemble of conformations. Characterization of structures of such partially folded states of the protein in atomic detail can only be achieved using NMR spectroscopy. Availability of high-field NMR spectrometers and new experimental methodologies have enhanced the capability of NMR to structurally characterize almost all types of equilibrium intermediates realized under a variety of experimental conditions.

Monitoring Chemical-Shift Perturbation. A ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectrum is a fingerprint of the overall three-dimensional structure of a protein.[12] Each ^{1}H-^{15}N cross peak in the spectrum represents a amino acid residue in the protein; therefore, effects of addition of the denaturant (leading to unfolding) on the protein conformation could be followed by monitoring the ^{1}H-^{15}N chemical-shift perturbation of the cross peaks in the spectrum. Complete equilibrium unfolding curves could be effectively traced based on the change in the cross-peak volume or peak intensities in the ^{1}H-^{15}N HSQC spectra (Figure 2).[9] This strategy has been successfully used to identify and characterize equilibrium intermediate states in several small

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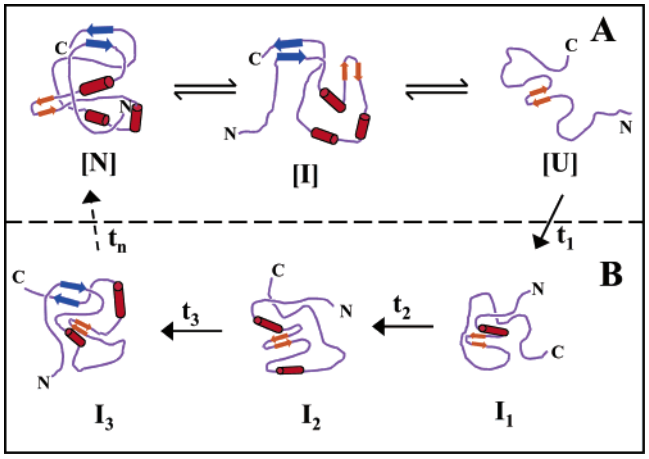

FIGURE 1. Graphical representation of the various conformational states of a protein that can be potentially characterized by NMR spectroscopy. (A) Equilibrium unfolding/folding of a protein. [N], [I], and [U] represent the native, MG-like intermediate, and the unfolded states, respectively. In the MG-like intermediate, most of the tertiary structural interactions are disrupted but the native secondary structures are mostly intact. Residual β-turn-like structures (indicated as orange arrows) are shown to be persistent in the unfolded state ensemble. (B) Kinetic intermediates (I_1, I_2, and I_3) formed during the refolding of the protein. The residual structure (β-hairpin-like structure) in the unfolded state(s) is shown to be the nucleation site for refolding of the protein. The native secondary structural elements are shown to be sequentially formed during refolding. t_1, t_2, t_3, and t_n represent the time constants of formation of the various kinetic intermediates (I_1, I_2, and I_3). The letters N and C indicate the N- and C-terminal ends of the protein. Helical and β-sheet structures in the protein are indicated by red cylinders and blue arrows.

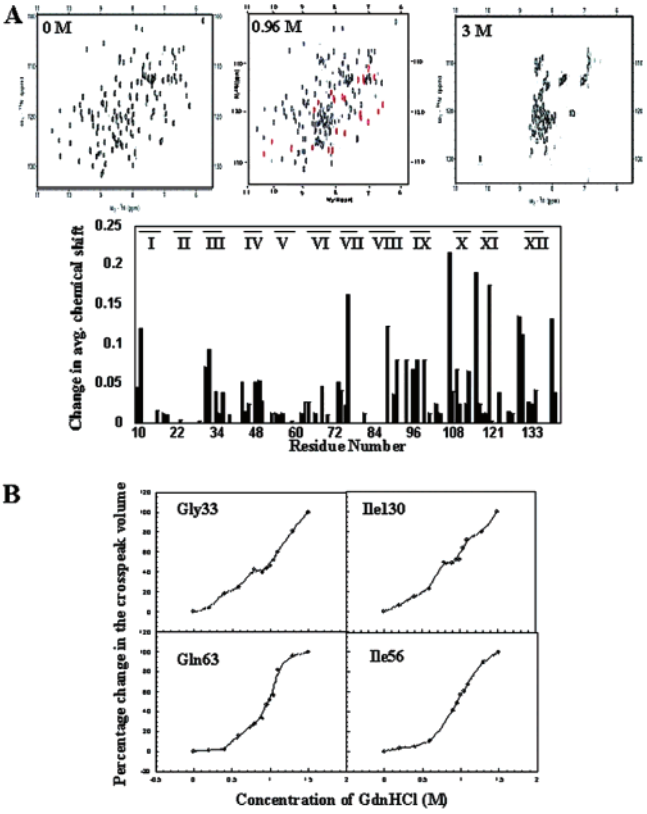

FIGURE 2. Equilibrium unfolding of hFGF-1 monitored by ^{1}H-^{15}N chemical-shift perturbation. (A) ^{1}H-^{15}N HSQC spectra of hFGF-1 at various concentrations of GdnHCl. The cross peaks, which undergo maximal chemical-shift perturbation, are indicated in red. The lower subpanel in A shows the ^{1}H-^{15}N chemical-shift perturbation of hFGF-1 in the intermediate state (in 0.96 M GdnHCl). The most prominent chemical-shift perturbation occurs in the C-terminal heparin-binding region, such as the β-strands VIII, IX, and XI, respectively. (B) GdnHCl-induced unfolding profiles of hFGF-1 (at the individual amino acid level) monitored by changes in the ^{1}H-^{15}N cross-peak volume. Unfolding profiles of residues such as Gly-33 and Ile-130 show a clear three-stage transition with a plateau between 0.5 and 1.0. In contrast, the unfolding curves of residues such as Ile-56 and Gln-63 appear to represent a two-state (native−unfolded state) transition. This figure is reproduced from ref 13.

proteins (M_r < 20 kDa) under a variety of denaturant conditions.[10−12] In addition, the most stable core, possibly representing the nucleation center, has been assigned in many proteins based on the denaturant-induced ^{1}H-^{15}N chemical-shift perturbation data. Schulman et al. traced the unfolding events in α lactalbumin at residue level by monitoring the ^{1}H-^{15}N chemical-shift perturbation (at various concentrations of the denaturant) and convincingly demonstrated that the transition from the molten globule state to the unfolded state(s) is not cooperative.[11] Specific regions of the protein appeared to form a stable core of the structure even at high concentrations of the denaturant, whereas the rest of the polypeptide chain is highly denatured. The stable core was proposed to provide a template for refolding of the protein to its native conformation.[11]

Partially structured intermediate states of proteins are highly dynamical and consist of a wide range of interconverting conformers.[8] Therefore, understanding the inherent high flexibility of partially structured folding intermediates at equilibrium is crucial for their complete characterization. NMR spin relaxation experiments, which are sensitive to nanosecond and subnanosecond motions have shown that partially folded intermediates are substantially more flexible than the folded states.[9,12] Backbone dynamics of stable partially structured intermediates populated in equilibrium unfolding pathways of several small proteins (M_r < 20 kDa) have been investigated by T_1, T_2, and nuclear Overhauser effect (NOE) measurements using 2D NMR methods.[9,12,13]

Characterization Using Hydrogen Exchange. Hydrogen−deuterium (H/D) exchange monitored by NMR spectroscopy is a powerful tool because it can provide direct information on the protein structure change, interactions, dynamics, and folding resolved to the level of individual amino acids.[13−15] The kinetic and thermodynamic relationships that relate structural unfolding reactions with measured hydrogen exchange rates were described by Linderstrom-Lang and co-workers.[15] Exchange occurs by the EX2 (second-order reaction) limit, if refolding of the transient structural opening is fast compared with the intrinsic chemical exchange rate. Exchange by the EX1 (first-order reaction) limit is observed in special cases when the exchange is attributed to one-way unfolding above the unfolding transition or to transient global unfolding in destabilized proteins.[15] The two extreme cases of the two-step exchange model are limited local fluctua-

tions and complete global unfolding. The slowest exchanging amide protons are generally believed to exchange by the global unfolding pathway.[15] In addition, for these residues, the free energy of exchange ($-G_{ex} = -RT \ln P$, where G_{ex} is the free energy of the exchange and P is the protection factor) estimated from the hydrogen-exchange experiments is expected to be similar to the free energy of unfolding (ΔG_u) calculated from the equilibrium unfolding experiments monitored by the optical methods.[16] On the other hand, the faster exchanging amides are assumed to exchange through local fluctuatuions. The ΔG_{ex} values for these residues are predicted to be lower than the average free energy of unfolding (ΔG_u) because the exchange that would occur from an intermediate is not necessarily dependent on global stability.[15]

In general, amide proton H/D exchange in the protein could be conveniently investigated by NMR or mass spectrometry. H/D exchange could be monitored by acquiring ^{1}H TOCSY or ^{1}H-^{15}N HSQC at various time periods of the exchange. Amide protons that are hydrogen-bonded or involved in secondary structure formation are relatively more resistant to exchange as compared to those that are solvent-exposed. Amide proton exchange rates of individual residues are characterized in terms of protection factors.[15] Protection factors are estimated from the peak intensities or peak volumes (at each time point) of cross peaks representing the amide protons. The H/D exchange experiment provides the relative contribution of each amino acid toward the global stability of the protein.[16] The slowest exchanging amino protons are said to comprise the stability core of the protein. Amide proton exchange kinetics have been reported for a number of proteins.[14,17,18] Woodward and co-workers proposed that the stability core characterized by H/D experiments constitutes the folding nucleus of the protein, "first in, last out".[19] This proposal was found valid in the case of the bovine pancreatic trypsin inhibitor ($M_r \sim 6$ kDa)[19] and snake venom cardiotoxin analogue III ($M_r \sim 7$ kDa),[20] but experimental data from many other proteins failed to validate this proposal.[21]

Detection of Partially Unfolded States (PUFs) by Native-State Hydrogen Exchange. The lack of evidence for a detectable intermediate in the folding/unfolding process is generally assumed to imply that the process is cooperative or two states (native ↔ unfolded states). Observation of the same single-exponential kinetics using two different spectroscopic probes, such as CD and fluorescence, is the usual criteria for a two-state process. However, it is well-known that the conventional optical probes used are inadequate to detect small populations of intermediate states that exist at higher energy levels, which are swamped by signals from the abundant native state.[22,23] In contrast, H/D exchange measurements monitored by NMR spectroscopy can identify some of those nativelike conformers because it has the advantage that the native state does not always contribute to the measurement because it is a H/D-exchange-incompetent state with regard to the majority of the slowly exchanging amide protons. Hence, H/D exchange experiments could provide valuable clues about the ruggedness of the energy landscape and are useful to characterize PUFs. Native-state H/D exchange monitored by ^{1}H-^{15}N HSQC has been successfully used to detect PUFs within the native state of several small proteins ($M_r < 20$ kDa).[22−24] Similarly, Chu et al. demonstrated the existence of two PUFs in a redesigned four-helix bundle protein, Rd-apocyst b_{262}, which was previously shown to fold kinetically by an apparent two-state mechanism.[25] The power of the native-state exchange technique is clearly elucidated in barnase ($M_r \sim 12.4$ kDa), which is believed to fold in a two-state manner.[25] On the basis of the hydrogen exchange data, Bai and co-workers predicted accumulation of a hidden intermediate (PUF) during the folding/unfolding of barnase.[26] Their prediction is supported by Fersht, who proposed that the folding of barnase proceeds with the occurrence of a kinetically significant intermediate in the initial rate-limiting step.[27] Recently, Chi et al. showed that the β-trefoil architecture of the human acidic fibroblast growth factor does not behave as a single cooperative unit and at least two structurally independent units were found to exist in the protein (Figure 3).[24] The subglobal folding isotherm in the protein was shown to be constituted by a broad continuum of stabilities among four β strands.[24]

Characterization of Denatured States of Proteins

Characterization of structures of native and denatured states is important to understand the mechanism by which proteins fold into their unique three-dimensional structures because the stability of a protein is essentially determined by a subtle balance of the free energies between the folded and unfolded states.[28] Elucidation of the structural features of the denatured states are expected to throw light on the role of preferential conformations in the initiation of folding or on the events than precede the formation of secondary and tertiary structures.[29] In addition, denatured states are implicated in a variety of key cellular processes such as protein translocation across membranes and vesicle fusion. Unfolded and partially structured states are also proposed to play crucial roles in a number of disease states such as amyloidosis and cancer.[3]

It is generally assumed that denatured states of proteins are random-coil ensembles wherever the conformational averaging is independent of their neighboring environment.[29] However, it is increasingly clear that denatured states are distinct from unstructured random coils.[30] They can be highly compact with significant amounts of residual/secondary structure(s). These persistent structures in the denatured state(s) are suggested to play important role(s) as potential initiation sites at an early stage of protein folding.[29] Therefore, characterization of the structure(s) and dynamics of the denatured state(s) are of profound importance to understand protein folding and stability.

Utility of NMR Techniques in the Characterization of Residual Structures in Unfolded States. NMR is probably

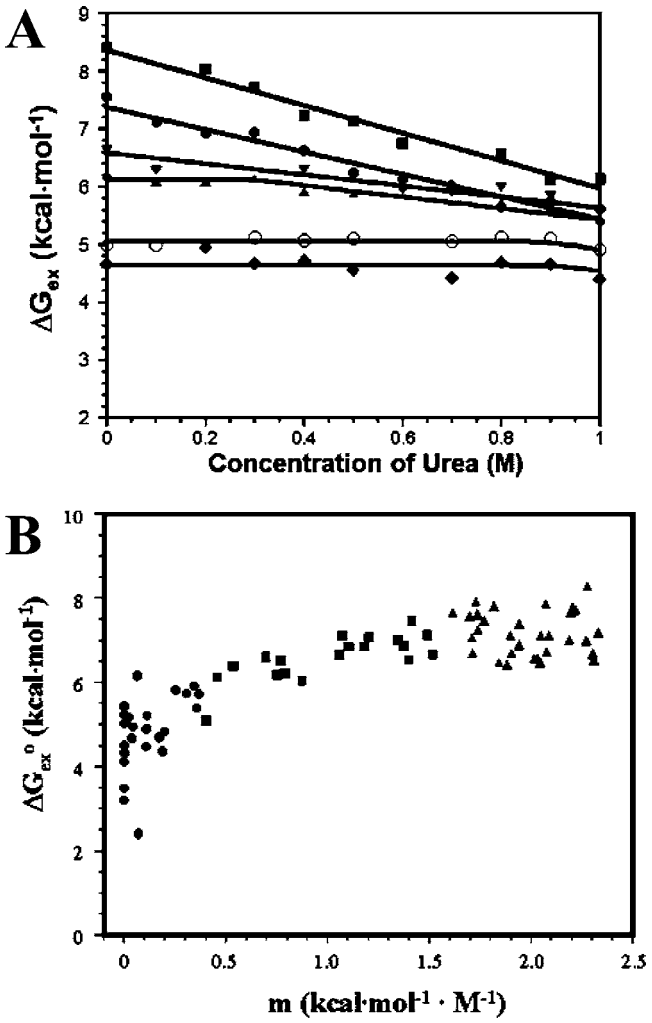

FIGURE 3. Native-state hydrogen exchange in hFGF-1. (A) Urea concentration dependence of free energy of H/D exchange (ΔG_{ex}) illustrated for selected amide protons. ΔG_{ex} values of Leu-40 (●) and Ala-80 (■) show a linear relationship with the denaturant concentration, implying that these residues exchange by the global unfolding isotherm. Leu-87 (○) and Glu-101 (◆) show very little change in the ΔG_{ex} values up to 1.0 M urea, indicating that these residues exchange by local unfolding. In contrast, the *m* (the slope) values of residues Leu-147 (▼) and Lys-114 (▲) are similar (0.78 ± 0.08 kcal mol^{-1} M^{-1}) and are intermediate to those of residues that exchange by global and local unfolding mechanisms. These residues represent the subglobal unfolding isotherm and suggest the presence of PUFs in the equilibrium unfolding pathway of hFGF-1. (B) Plot of change in free energy of exchange (ΔG_{ex}) versus the cooperativity factor (*m* value). Residues in hFGF-1 exchange by three distinct isotherms, namely, local (●), subglobal (■), and global (▲) isotherms. This figure is reproduced from ref 24.

the best technique available for characterizing denatured states, because it is capable of defining specific contacts in the ensemble of denatured conformations.[29] Recent developments in NMR techniques have improved spectral resolution and have led to significant progress in the structural characterizations of denatured states.[29] Evidence from a nonrandom structure in denatured states is generally inferred from the macroscopic properties of proteins. This has resulted in direct evidence for the nature of interactions persisting in denatured states. In general, assignment of resonances (^{1}H, ^{15}N, and ^{13}C) are accomplished using a combination of triple resonance and magnetization transfer techniques. The evidence of the residual structure in denatured states of proteins is provided by a consistent series of weak correlations in chemical shift, NOE, and coupling constant data.[29] In principle, $^3J_{HNH\alpha}$ coupling constants yield fair clues of residual structures in unfolded states of proteins. A comparison of the predicted statistical random-coil $^3J_{HNH\alpha}$ coupling constant values and the experimentally observed $^3J_{HNH\alpha}$ coupling constant values provides information on the nonrandom structures in the unfolded state(s) of interest. Similarly, measurements of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts show a significant dependence on backbone torsion angles and can be reliably used to locate residual structures in unfolded state(s) of proteins.[31] In many cases, residual structures in many proteins have been characterized by the presence of long- and medium-range ^{1}H-^{15}N NOE constraints.[29,32] However, because of the transient nature of the long- and medium-range interactions, NOEs exist in only a subset of molecules in the unfolded ensemble. To overcome this problem, Crowhurst and Kay selectively deuterated the sample [the N-terminal domain of *Drosphila drk* (drkN SH3, $M_r \sim 7$ kDa)] to eliminate the nonproductive ^{1}H and ^{13}C relaxation pathways and to obtain data with decreased line widths and an enhanced signal−noise ratio.[31] In addition, the decrease in the spin-diffusion pathways permitted them to employ higher mixing times (in their NOESY experiments) to successfully detect weak long-range NOEs.[31]

Dipolar couplings contain information on the orientation of heteronuclear vectors and have become an important adjunct of traditional structural constraints in refinement of NMR structures of globular proteins.[33] Residual dipolar coupling (between ^{15}N and ^{1}H in backbone amide groups) measurements in strained polyacrylamide gels have been successfully used to obtain short- and long-range interactions in denatured states of many small proteins ($M_r < 20$ kDa).[33−35]

Recently, use of selective paramagnetic spin labeling in conjunction with NMR revealed long-range structural contacts in highly unfolded proteins. Spin labels are known to cause substantial broadening of resonances of spins located within a spatial distance of 15 Å and are valuable probes to characterize long-range interactions. Lietzow et al.[36] and Gillespie and Shortle[37] independently demonstrated the usefulness of spin labels in the determination of nonlocal interactions in unfolded states of proteins. Instead of extracting for quantitative distances (which could be inaccurate because of global backbone fluctuations), Lietzow et al. monitored the trends in the paramagnetic enhancement of nuclear relaxation as a function of the primary sequence of the protein.[36] For a typical random coil, the paramagnetic broadening is expected to decrease as the distance from the site of insertion of the spin label increases along the primary sequence. Any deviation from this trend is an indication of the presence of transient long-range interactions in the vicinity of the spin label. Using the spin-labeling technique

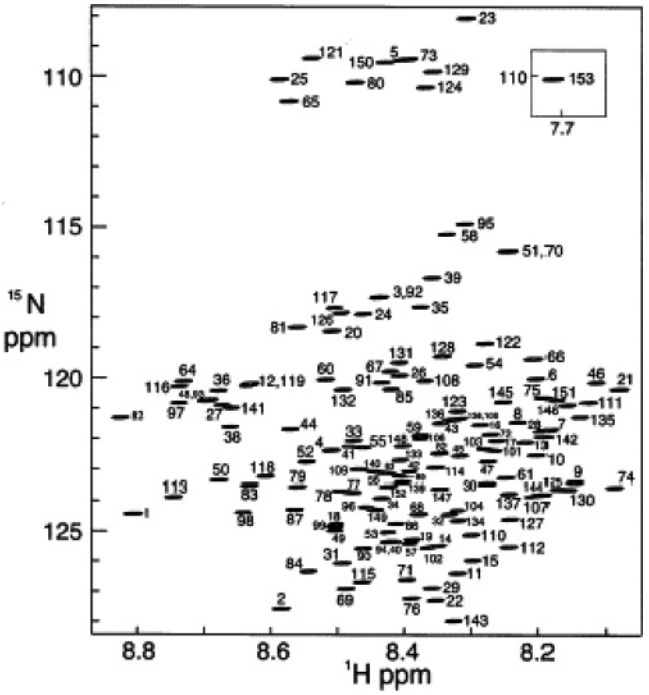

FIGURE 4. ^{1}H-^{15}N HSQC spectrum (750 MHz) of apomyoglobin in 8 M urea at pH 2.3 and 20 °C. Assignments are shown. Inset: cross peak for G153, shifted from its normal position on the far right spectrum. This figure is reproduced from ref 28 with the permission of the American Chemical Society.

in conjunction with ^{1}H-^{15}N HSQC spectra, long-range contacts between residues at the N- and C-terminal ends were observed in the highly denatured state of apomyoglobin (Figure 4).[28] A disordered ensemble cannot be completely characterized without knowledge of its dynamical features.[38] In general, ^{15}N chemical shifts are well-dispersed in unfolded proteins and hence ^{15}N relaxation measurements are commonly employed to probe backbone dynamics on a per residue basis in unfolded states of proteins.[29] Recently, Kay and co-workers have developed techniques to measure ^{2}H spin-relaxation rates at side-chain $^{13}CH_2D$ methyl positions in ^{15}N- and ^{13}C-labeled and fractionally deuterated samples of unfolded proteins.[38] These methods are especially useful to detect and characterize compact hydrophobic structures in highly denatured states. It should be mentioned that, although the levels of internal motions within unfolded states are found to be correlated to the nature and extent of the residual structure within the protein, a clear-cut relationship between the residual structure and the internal dynamics of unfolded states of proteins remains poorly understood, principally because of a small number of systems that have currently been characterized.

Monitoring Kinetics of Protein Folding

Kinetics of protein folding/unfolding has been conventionally studied by stopped-flow optical techniques. Although the information gained using optical probes is of considerable value, these techniques provide little structural resolution of the events of protein folding/unfolding. On the other hand, technical advancements in NMR have provided an opportunity to investigate the chronology of structural events occurring during the protein folding/unfolding in the milli- and submillisecond time scale.

Quenched Flow (QF) Hydrogen Exchange. QF-H/D exchange detected using two-dimensional NMR techniques have been successfully used to gain information on the refolding events in the millisecond time regime.[39−44] In QF-H/D, the distributions of proton and deuterium at various labile sites (amide hydrogen) in proteins are measured as a function of the refolding times. Protein is denatured in high concentrations of the denaturant dissolved in D_2O. Under these conditions, all of the amide hydrogens in the protein are substituted by deuterons. Refolding is then initiated at lower pH (3.0−5.0) to minimize H/D exchange. After variable refolding times, the pH of the solution is raised to about 9.0 using a buffer in H_2O. During this phase, all of the amide deuterons that could not forge hydrogen bonds (during refolding) are exchanged with protons. The distributions of protons and deuterons at the labile sites (after various times of refolding) are followed in a site-specific manner by 2D NMR methods to trace the structural events during protein refolding. The refolding pathways of several proteins including staphylococcal nuclease ($M_r \sim 16.8$ kDa),[39] cytochrome *c* ($M_r \sim 11.4$ kDa),[40] RNase A ($M_r \sim 13.7$ kDa),[41] lysozyme ($M_r \sim 14.3$ kDa),[42] hFGF-1 ($M_r \sim 15.8$ kDa)[43] (Figures 5 and 6), and snake venom cardiotoxin analogue III (CTX III, $M_r \sim 7$ kDa)[44] have been investigated using QF-H/D exchange.

Laurents et al.[45] devised a modified version of the conventional QF (called the pulse-chase-competition experiment) to determine if the intermediates observed along the course of folding of proteins are on- or off-pathway. An important property of this method is that it does not yield false-positive results. However, there are some criteria for the application of this method. First, the kinetics of formation of the intermediate must be measurable, and second, the pH of the second step must not be high enough to the ^{1}H label in the intermediate state to exchange before the intermediate forms the native state. The intermediate is on-pathway only if a significant difference is observed between the retained label in the control and test sample. Using the pulse-chase-competition experiment, Laurents et al. convincingly demonstrated that a nativelike intermediate of RNase A is an on-pathway intermediate.[45] Similarly, Bai and co-workers proved the existence of a hidden on-pathway intermediate in barnase using pulse-chase-H/D-exchange experiments.[26]

There are two major limitations for the application of the pulse-labeling method. First, pulse-labeling methods provide information only on the amide protons that become protected from exchange during refolding and hence only yield indirect information on the nature of the structure that gives rise to protection. Second, these experiments do not provide any information on the folding of portions of the structure that are not stable to protect amide protons from exchange in the native conformation.

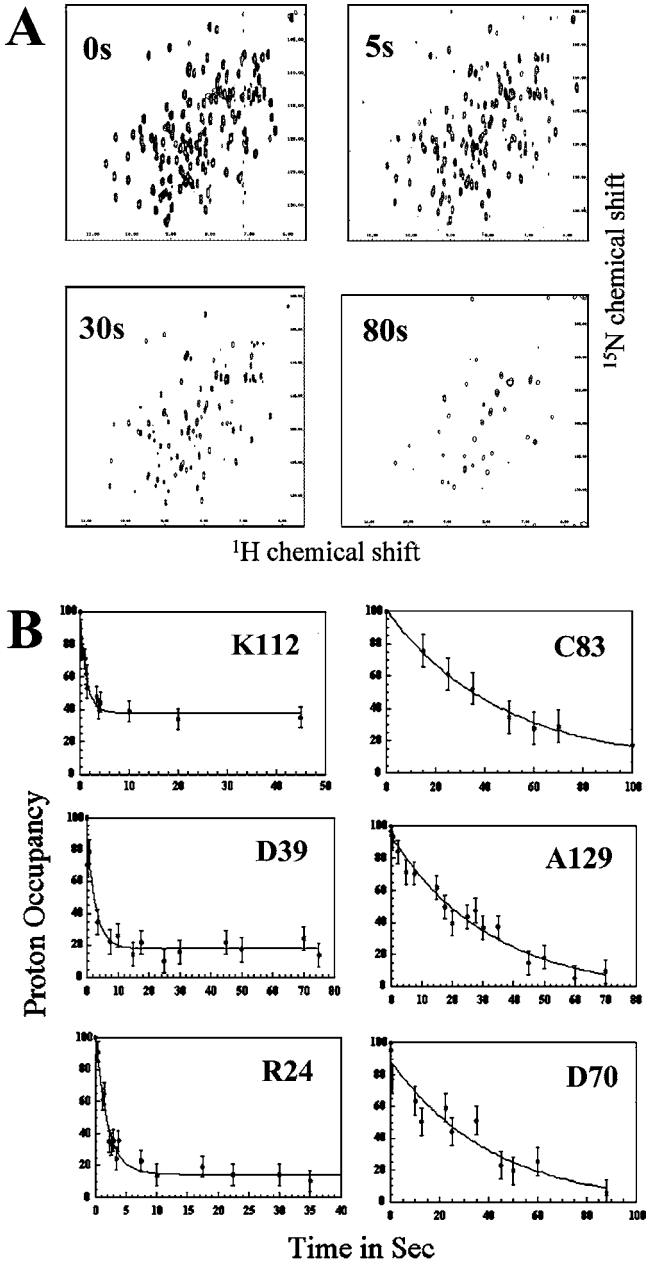

FIGURE 5. Structural events in the refolding pathway of hFGF-1 monitored by QF-H/D exchange. (A) ^{1}H-^{15}N HSQC spectra of hFGF-1 samples prepared by QF hydrogen exchange experiments at various refolding time periods. (B) Comparison of the observed time courses of selected residues as a function of the length of the refolding time. All residues were fit to a first-order exponential decay. (A) Percentage of the proton occupancy of residues located in the various β strands. (B) Percentage of the proton occupancy of residues located in the unstructured region. This figure has been reproduced from ref 43.

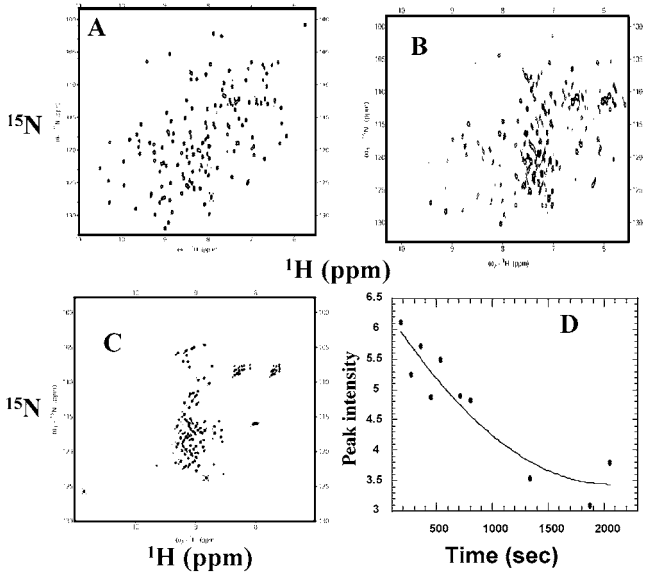

FIGURE 6. Real-time unfolding of hFGF-1 monitored by ^{1}H-^{15}N HSQC. (A−C) Spectra obtained after 0, 270, and 2500 s of initiation of unfolding. (D) Unfolding decay curve for Arg-102 located in β-strand VIII.

Real-Time NMR and Protein Folding. NMR has been successfully used to investigate the kinetics of protein-folding reactions in real time in the milli-to second time regime.[46−49] The simplest experiments involve investigation of protein unfolding reactions, which can take minutes or hours.[46] The reactions are basically initiated by dilution from or into denaturant solutions and simultaneously acquiring a series of proton 1D spectra during the course of the folding/unfolding reaction. The kinetic curves are obtained by following the intensity changes in chosen aliphatic, aromatic, or amide proton peaks. Folding/unfolding of a number of proteins has been followed in real time using 1D NMR spectroscopy.[46−49]

A major drawback of the 1D experiment is its limited resolution, and hence recent work is focused on the use of ^{1}H-^{15}N HSQC experiments to follow the folding of proteins in real time.[46] This approach provides an opportunity to apply high-resolution NMR spectroscopy to investigate folding reactions whose rates are too rapid for conventional repetitive-accumulation procedures. The folding of several proteins including lactalbumin ($M_r \sim$ 14 kDa),[46] interleukin 1 ($M_r \sim 17.4$ kDa),[47] apo-nFGF-1 ($M_r \sim 16$ kDa),[48] and plastocyanin ($M_r \sim 10.4$ kDa)[49] (Figure 5) have been studied in real time using 2D NMR methods. In several cases, real-time NMR experiments revealed an accumulation of kinetic intermediates in proteins that were believed to fold/unfold cooperatively by stopped-flow optical spectroscopy techniques.

Frieden and co-workers pioneered the use of fluorine (^{19}F) NMR to investigate both protein unfolding and folding reactions.[50] In ^{19}F NMR of proteins, specific residues (particularly, aromatic amino acids) are replaced by fluorine analogues. When the number of environments is dependent upon the fluorine nuclei, the resonances are well-resolved, facilitating the use of 1D NMR to monitor equilibrium or kinetic folding processes. This feature also enables data to be collected within 100 ms of mixing. This technique has been successfully employed to investigate the folding events in dihydrofolate reductase ($M_r \sim 18.2$ kDa). Baum and colleagues have extended this strategy to follow the slow folding and assembly of peptide fragments of collagen.[51]

Photo-CIDNP (chemically induced dynamic nuclear polarization) NMR is a recently developed technique to study protein folding kinetics in real time.[52] The CIDNP

technique probes the accessibility of aromatic side chains as the folding progresses. Typically, in these experiments, a protein solution mixed with a flavin photosensitizer is irradiated inside the NMR probe by a laser flash.[52] As a consequence, the excited photosensitive dye reacts with surface-exposed amino acid residues to yield transient radical pairs whose magnetic interactions lead to nuclear polarization in the protein. The advantage of this technique is the time resolution and its selectivity limited to aromatic residues, which decreases spectral crowding. Real-time photo-CIDNP NMR have been used to detect relatively disorganized collapsed states in the initial stages of folding of proteins including lysozyme, α-lactalbumin A ($M_r \sim 14$ kDa), and histidine containing phosphocarrier protein HPr ($M_r \sim 18.1$ kDa).[52]

Conclusion

In this Account, we have attempted to summarize the recent contributions toward the application of NMR spectroscopy to investigate protein folding/unfolding reactions. We anticipate that NMR would continue to play a pivotal role in the characterization of dynamic systems such as partially structured folding intermediates and denatured states of proteins that are not amenable to X-ray crystallography. NMR is expected to be the technique of choice to investigate the kinetics of protein folding/unfolding at the residue level. Availability of spectrometers equipped with cryoprobes in the future will significantly improve the sensitivity of NMR experiments and consequently decrease the dead time of detection (using NMR) of kinetic events of protein folding. In general, spectacular new developments in the hardware, pulse sequences, and isotope-labeling strategies are expected to further enhance the sensitivity and versatility of NMR to probe complex folding reactions.

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