Real-Time NMR Studies of Protein Folding

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Introduction

The question of how proteins fold to their unique, compact, and highly organized functional states is a central issue in structural and cellular biology. The extreme starting point for protein folding is the "random coil" state of a polypeptide chain, an ensemble of conformers with a wide distribution of global and local structures.[1] The folding process involves the conversion of all of these conformers to the single global minimum (native) state, and until this is achieved any intermediate stage of folding is characterized by a distribution of structures, often interconverting rapidly. This heterogeneity, coupled with the generally rapid nature of the folding process, makes its study by structural methods an extreme challenge. Any description of an intermediate stage of folding has to involve a definition of an ensemble of structures, a major conceptual as well as practical problem. The existence of such distributions is, however, an intrinsic feature of the "new view" of folding, where individual polypeptide chains are envisaged to move over an energy surface or landscape describing the folding reaction.[2−4] The ultimate objective of an experimental description of the folding process is to define in molecular detail the ensembles of structure that exist at different stages of the reaction, i.e., to map by experiment the folding surface or landscape.

A major approach toward this end has been to use a battery of biophysical techniques, in particular a combination of stopped flow optical techniques (e.g., circular dichroism, fluorescence) and quenched flow hydrogen exchange methods, each able to probe a specific aspect of the native character.[5,6] Such approaches have produced extremely valuable information, in particular identifying the degree of cooperativity of the reaction and the existence and nature of any populated intermediate states. In addition, protein engineering methods have turned out to be a key link between kinetic and equilibrium experimental approaches, particularly as they offer a unique opportunity to probe interactions within the transition states of folding reactions.[7] Nuclear magnetic resonance (NMR) spectroscopy has played an important part in these studies in a variety of ways.[6,8,9] Of particular importance has been its role in analyzing the distribution of deuterium in specific sites resulting from hydrogen exchange pulse labeling procedures.[10,11] This has also allowed links to be made between species formed in kinetic refolding processes and stable analogues of such species formed under milder conditions where more complete characterization is possible.[12,13] In this Account, however, we discuss the more direct use of NMR spectroscopy to study folding as it takes place in "real time".

Developments in NMR Spectroscopy

Advances in NMR spectroscopy over the last 20 years have revolutionized its application to studies of proteins.[14,15] It is now possible to use NMR to determine the complete 3-D solution structures of proteins of molecular weight in excess of 25 000, as well as probing many aspects of their structures including their dynamics and solvation properties.[16,17] More recently, substantial advances have

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been made in studying nonnative states of proteins.[1,9,18] Such studies have revealed a wealth of information about these states, including detailed evidence about local structural propensities and residual structure, such as clusters of hydrophobic residues, as well as information about the global dynamics of the polypeptide chains. One particularly exciting development has been the use of statistical distributions to describe nonnative states, which has very direct analogies with the new view of folding through its emphasis on the existence of ensembles of conformations.[1,19] Such approaches are the key to being able to interpret experimental data relating to the distributions of structures associated with the folding reaction.

Early NMR studies of protein folding involved investigation of the equilibrium conversion between native and denatured states resulting from a protein being subjected to heat, extremes of pH, or chemical denaturants. Experiments of this type have provided much valuable information about the thermodynamics and degree of cooperativity of folding. Because of the ability of NMR to probe the interconversion of species existing at equilibrium, however, this approach has also enabled studies of kinetic events in the folding reaction to be initiated.[20,21] Interestingly, these techniques have also helped in the development of NMR spectroscopy of nonnative states themselves by allowing cross-correlation of resonances with the well-resolved native state spectrum.[22,23] The potential of this type of experiment has expanded considerably in recent years particularly through the development of methods to probe extremely fast processes through chemical exchange effects on the NMR line shapes.[8,24,25]

In parallel with such developments, however, have been nonequilibrium studies in which reactions have been followed directly within the NMR probe. Early studies focused on enzyme kinetics,[26−28] but more recently this approach has been used to investigate protein folding and unfolding. The simplest experiments involve the sequential accumulation of spectra following initiation of the reaction. Because of the time limitation of this approach, even a single one-dimensional (1-D) experiment takes on the order of 1 s to record, relatively slow reactions have been particular targets. These have included unfolding reactions,[29] which can take minutes or hours, and folding reactions where the rate is limited by the need to isomerize peptidyl−prolyl bonds.[30−33] In these studies, which have provided important information about the nature of folding intermediates, the reactions were initiated by dilution from or into denaturant solutions,[29−31] or by quenching of thermally denatured samples.[32,33] Recently, such approaches have been extended to utilize two-dimensional (2-D) techniques, notably in studies of the folding kinetics of trimeric peptides related to collagen.[34]

To monitor rapid reactions, it is necessary to initiate the reactions within the NMR probe, and to attain high magnetic field homogeneity in the resulting solutions. A number of approaches for this purpose have been developed including continuous flow recycling methods[35] and temperature jump procedures using gas flow methods.[36]

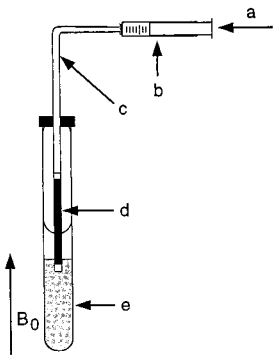

FIGURE 1. Schematic representation of stopped flow NMR. The NMR tube containing refolding buffer (e) is inserted in the NMR probe in a magnetic field B_0. The refolding buffer is separated from the denatured protein solution (d) in the Teflon transfer line (c) by an air bubble precluding a liquid−liquid interface that would allow diffusion and mixing of materials before injection. The remainder of the transfer line is filled with D_2O. During the recording of a series of NMR spectra, the injection piston (b) located outside the magnet is pneumatically triggered (a) from within the NMR pulse sequence and results in the rapid transfer of the denatured protein solution into the NMR tube.

Stopped flow procedures, utilizing rapid mixing of solutions, have however been particularly effective in enabling short experimental dead times to be obtained.[37−39] Of particular note have been studies using ^{19}F NMR, in which data collection could be started within 100 ms of the initiation of the mixing. By incorporation of ^{19}F-labeled tryptophan residues into dihydrofolate reductase, for example, the resolution of the spectra is sufficient to follow folding and unfolding reactions at the level of individual residues. With such approaches, distinct steps in the reaction have begun to be resolved and characterized.[38,39]

Following the Folding Process in Real Time

The primary objective of our own work in the area of kinetic NMR spectroscopy is to develop an overall strategy to bring together different experimental procedures to build up a comprehensive description of the structural and dynamical changes occurring during the folding of a range of proteins. A relatively simple injection mixing device is adequate for many of these experiments, and a schematic representation is shown in Figure 1. In subsequent sections of this paper a range of different experiments will be described in the context of this diagram. The experimental strategy will be illustrated largely with results from bovine α-lactalbumin (BLA), a well-defined system for studying many aspects of protein folding. Particularly important characteristics of this protein are that its folding kinetics can be varied over several orders of magnitude by control of the Ca^{2+} concentration of the solution,[40] and that it forms under mild denaturation conditions a partially folded molten globule state that has been subjected to detailed study as a model system for understanding compact nonnative states of proteins.[41]

Figure 2 shows a series of 1-D NMR spectra recorded after initiation of the refolding reaction of BLA in the absence of Ca^{2+}. The initial spectra show broad reso-

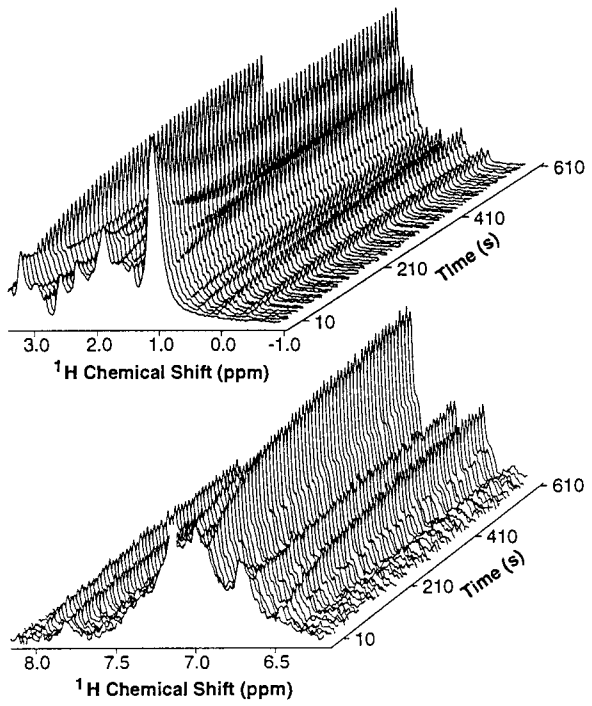

FIGURE 2. Stack plot of 1-D NMR spectra of BLA.[42] For the refolding experiment, BLA in 6 M GdnDCl (d, Figure 1) was diluted into a refolding buffer containing EDTA (to sequester the Ca^{2+} ions; e, Figure 1), and ^{1}H NMR spectra were recorded at 20 °C at incremented time points (between 1.2 s and 10.3 min) after initiation of refolding. The region shown at the top contains resonances from methyl and methylene groups, and that at the bottom from aromatic residues.

nances with little chemical shift dispersion, resembling closely the spectrum of the compact molten globule (or A-state) state of the protein formed at low pH.[42] This broadening arises from the heterogeneity of the conformations of molecules present early in the folding reaction, and the slow interconversion of such conformations in compact states.[22] The emergence of the relatively narrow signals of the unique native state can readily be detected at longer refolding times. The kinetics of the appearance of the well-resolved resonances of aromatic and methyl group side chains characteristic of the native protein were found to be closely similar; indeed all spectra recorded during the refolding reaction can be reconstructed to a good approximation as linear combinations of the first and last spectra of the series recorded during folding. This experiment, therefore, demonstrates the cooperativity of the formation of the close-packed native structure in different regions of the protein.[42]

This simple experiment is widely applicable. In our laboratory it has been used to characterize the folding of several small proteins.[43−45] Of particular value in such studies is the ability of NMR to detect differences from the fully native state at the level of individual residues with much greater reliability and generality than any other technique. It is crucial, however, to choose experimental conditions in which the protein under study remains soluble and monomeric during the refolding process; to ensure this is the case requires investigation of the concentration dependence of the spectra and comparison of the kinetic parameters obtained by NMR with those derived from other techniques, for example, stopped flow optical methods. It is essential, in addition, to ensure that appropriate account is taken of any time-dependent effects of the mixing process. These include inhomogeneities associated with the mixing of the solutions, the recovery of the field-frequency lock signal (if used), and temperature, NMR polarization, and relaxation effects resulting from the transfer of the solutions from one region of the magnetic field to another. Particular care is needed in the interpretation of line width and intensity changes in the initial spectra recorded after the injection process. The magnitude of these various effects can at least partly be assessed by use of internal standards (e.g., by inclusion of reference molecules whose properties are not altered significantly by the change of solution conditions). Interestingly, if an experiment detects signal in only a limited volume of the NMR sample, then the effective dead time of the experiment can be much reduced. Such a situation applies to the chemically induced dynamic nuclear polarization (photo-CIDNP) reaction described below where measurements can be made on a time scale of a few tens of milliseconds.[46]

A major limitation of 1-D experiments is of course that the spectra have limited resolution, and recent work has focused on the extension of these experiments to utilize the power of multidimensional NMR in kinetic experiments. One approach we have mentioned above is to record 2-D spectra in a sequential manner.[34] This, however, exacerbates the time-resolution problem. We have begun to explore another approach in which kinetic events are detected during the accumulation of single 2-D spectra, by exploiting the time-dependent information inherent in such experiments.[47] This information arises because of the fundamental design of these experiments in which a time delay between the preparation and observe pulses is incremented during the course of the experiment while a series of free induction decays (FIDs) is recorded. If a chemical reaction occurs during the accumulation of data in the experiment, it influences the amplitude of the different signals recorded at the various incrementation times, and determines both the line shapes and intensities of the cross-peaks in the resulting 2-D spectrum. The single 2-D spectrum therefore reflects the kinetic history of the reaction, allowing strategies to be developed to reconstruct the events that occur during its accumulation.[47]

The principle and effectiveness of this strategy have been demonstrated for BLA using a ^{1}H−^{15}N HSQC experiment[47] and are illustrated in Figure 3. The initially formed species in the refolding reaction, as in the 1-D experiment illustrated in Figure 2, is broad and poorly dispersed. The product, however, is the native state with a well-resolved spectrum. In the spectrum recorded during the refolding reaction, resonances of the latter state are prominent, but their intensities are less than those in a spectrum recorded after refolding is complete. This reflects the fact that the native state is not present at its final concentration in the FIDs recorded in the initial time increments, Figure 4. In

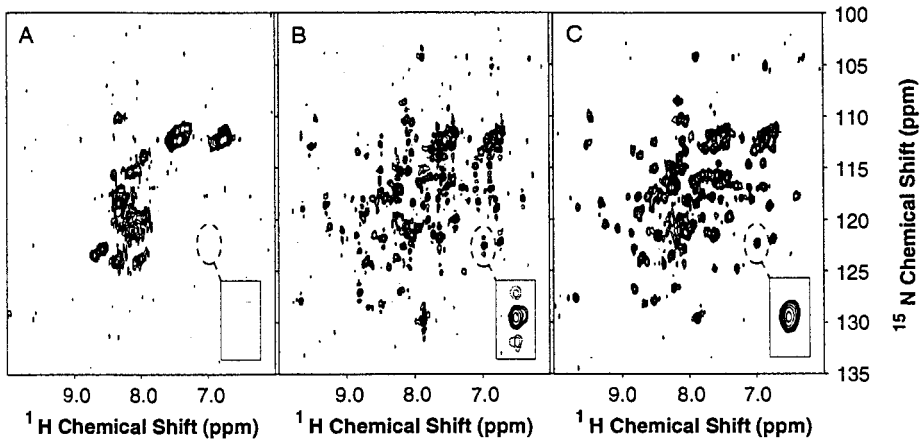

FIGURE 3. $^1H-^{15}N$ HSQC spectra of BLA during folding.[47] Refolding was initiated by a pH jump in the absence of Ca^{2+} by injecting a solution at pH 8.8 containing EDTA (to sequester the Ca^{2+} ions; d, Figure 1) into a protein solution at pH 2.0 (e, Figure 1). (A) Poorly resolved HSQC spectrum of the A-state at pH 2.0, recorded prior to initiation of refolding. (B) Kinetic HSQC spectrum accumulated during the folding reaction (30 min) and (C) the well-resolved, HSQC spectrum of the native state at pH 7.0 recorded after the refolding reaction. All the resonances in this spectrum are assigned to individual amide NH groups. The insets show representative enlargements of the indicated regions.

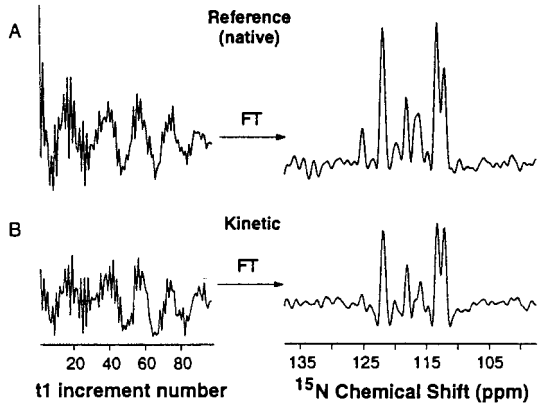

FIGURE 4. Cross sections from $^1H-^{15}N$ HSQC spectra of BLA.[47] F-1 cross section at a ^1H chemical shift of 7.6 ppm (A) from the reference HSQC data set for the native state data shown in Figure 3C and (B) from the kinetic HSQC data set recorded during refolding (Figure 3B). The time-domain spectrum (left) shows the magnetization as a function of the t_1 increment steps. The corresponding frequency-domain spectra (right) show the different ^{15}N resonances correlated with the ^1H resonances at this frequency. Note the difference in magnitude of the time-domain signal in (B), reflecting the appearance of the native state with time. After Fourier transformation (FT) this results in a frequency-domain spectrum containing resonances with lower intensity and negative components in the wings of the peaks (right, panel B).

addition, the resulting spectrum after Fourier transformation shows spectral line shapes that are markedly different from those characteristic of the native state recorded after the reaction is complete. This is a consequence of the same phenomenon; the time domain of the spectrum is convoluted by a function reflecting the kinetic development of the native state. These two effects of a reaction occurring during the accumulation of the 2-D spectrum result in the ability to analyze kinetic data associated with the folding process.[47]

The key aspect of this experiment is that the intensities and line shapes of resolved cross-peaks enable the folding kinetics to be monitored at individual residues within the protein. In the case of BLA this has enabled the conclusion to be drawn that the close-packed native environment of the main chain amide groups of the different residues is achieved cooperatively, and with the same kinetics as observed for the side chains.[47] In this case, no well-structured intermediates exist; indeed the extremely broad signals of all species present until the native structure is formed indicate that the conformational heterogeneity characteristic of the initially formed state persists throughout the reaction. In other cases, however, such a situation may not arise and resonances corresponding to intermediates may be resolved. One example is found in the case of ribonuclease T_1, where a partly folded species accumulates because of a barrier associated with *cis*−*trans* proline isomerism.[56] This species is sufficiently well structured that the kinetics of both its formation and disappearance could be monitored from the intensities and line shapes of the individual resonances in single 2-D spectra; local and long-range effects of the nonnative proline residue could be identified by recording a single 2-D nuclear Overhauser effect spectroscopy (NOESY) spectrum. This experiment illustrates the power of this approach to characterize such species under appropriate conditions. The success of experiments of this type is such that one can envisage the application of a range of multinuclear and multidimensional NMR experiments in kinetic mode to characterize protein folding and unfolding, and indeed other complex chemical reactions.[47]

Monitoring Structure Directly during Folding

The combination of kinetic NMR experiments with photo-CIDNP methods in which nuclear polarization is generated by a laser flash (Figure 5) represents an approach to characterizing the structure of a protein directly during folding. An example of this approach is shown in Figure 5 using lysozyme, a protein homologous to α-lactalbumin which folds on a time scale of ca. 1 s under the conditions used here.[46] The first spectrum shown is of the protein after 30 ms of folding and is markedly different from that

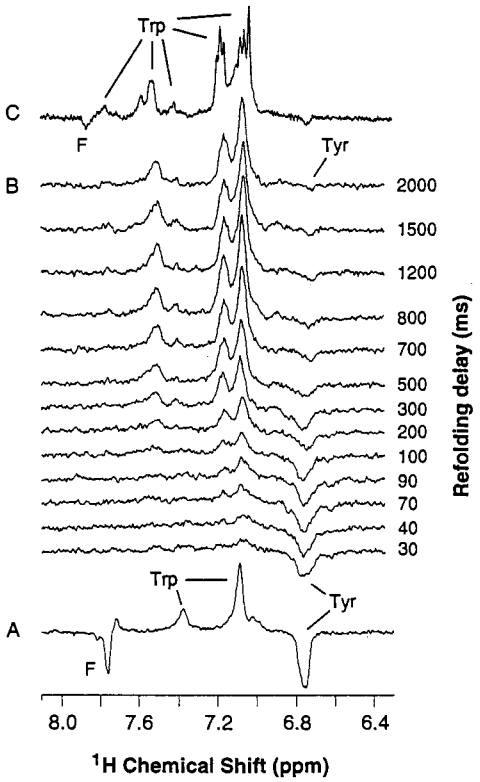

FIGURE 5. Photo-CIDNP spectra of hen lysozyme.[46] Refolding was initiated by a simultaneous jump in pH and denaturant concentration generated by injecting denatured protein in 10 M urea (d, Figure 1) into buffer (e, Figure 1) to a final pH of 5.2. Both solutions contained 0.5 mM of a light sensitive dye, flavin mononucleotide (F). A 50 ms light pulse was applied at incremented times after the 30 ms injection pulse, immediately followed by an rf pulse and acquisition of the FID. The detectable region of the NMR tube is determined by the width of the laser pulse and thus reduced to the center of the sample (~50 μL). (A) Spectrum of the denatured state in 10 M urea, (B) spectra at various times after initiation of refolding, and (C) spectrum of the native state in the absence of urea.

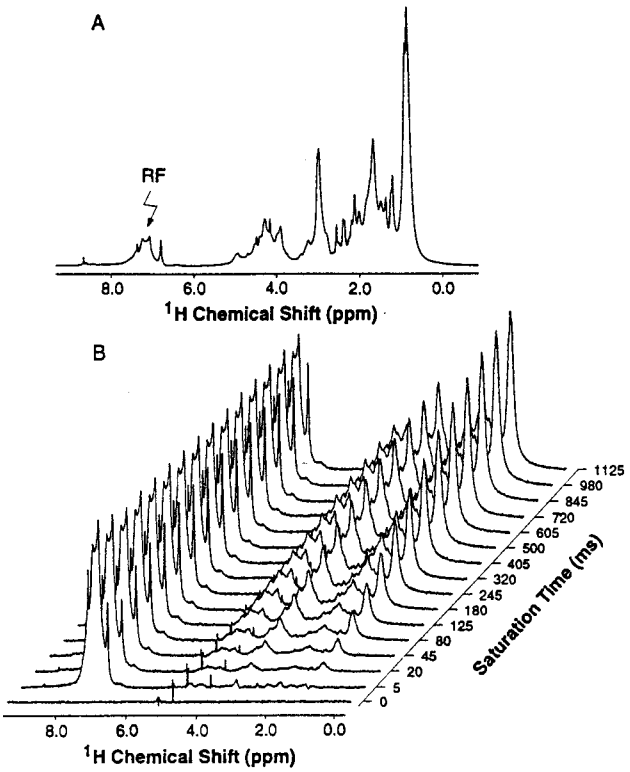

FIGURE 6. 1-D NOE spectra of BLA.[49] (A) 1-D ^{1}H NMR spectrum of BLA. (B) NOE difference spectra obtained from BLA following saturation of the aromatic resonances (7.0−8.0 ppm) by high-power continuous wave irradiation at the position labeled by an arrow in (A). The difference spectra were obtained by subtracting reference spectra acquired after saturation at a frequency 2 kHz downfield of the aromatic region.

of the denatured state in 10 M urea. Moreover, significant additional spectral changes can be observed in spectra recorded at times well below 100 ms. The strength of the photo-CIDNP approach is, however, not only in its time resolution but also in its spectral resolution; the latter is improved significantly because the polarization is generated in only a limited number of residues (tyrosine, tryptophan, and, under some conditions, histidine). Moreover, polarization occurs only when these residues are accessible to the photoexcited dye molecules in the solution, enabling the environment of individual residues to be probed directly in real time during folding. The results of the experiments shown in Figure 5 suggest that a relatively disorganized collapsed state forms initially during the folding of lysozyme, and that reorganization then occurs to generate the native structure.[46] Recent experiments indicate that a similar process occurs for BLA.[48] Experiments of this type are likely to be extremely valuable in studying folding, although care is needed to ensure that intensity effects are correctly interpreted in such complex systems. Extension to 2-D and to greater sensitivity using more efficient polarization methods is viable and will further enhance the value of this approach.[46]

Despite the detailed information that such experiments may be able to provide, the ultimate objective of structural studies of folding is to define the interactions between residues at different stages of the folding process, and to map out the distributions of structures that relate to the energy surface which determines the progress of the folding reaction.[49] The strength of NMR in this context is that it is able to detect residue contacts through NOE effects that result from the close proximity of nuclear spins. In the case of relatively well defined and long lived intermediates it is clear that such experiments are possible. In recent experiments, however, we have investigated the potential for monitoring NOEs in species formed transiently during folding, again using BLA as our test system.[49] NOEs can be generated in a variety of ways, but the simplest is by saturating the resonances of specific protons using radio frequency (rf) pulses, and monitoring the resulting changes in the intensity of other resonances.

Figure 6 shows the effect of saturating the aromatic resonances of BLA in its A-state at pH 2.0. There is a clear buildup of saturation in the methyl group resonances, indicating that close contacts between aromatic and methyl group protons are present under these conditions. Remarkably, both the rate and magnitude of the NOE

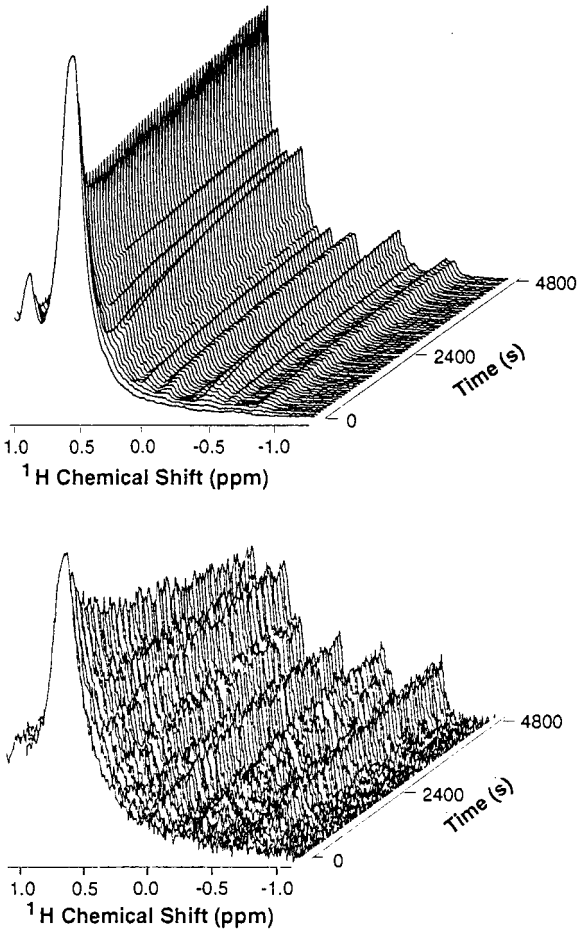

FIGURE 7. Kinetic NOE spectra of BLA. (A) Reference and (B) NOE-difference spectra recorded during the refolding of BLA. Refolding was initiated by a pH jump in the absence of Ca^{2+} generated by injecting an EDTA solution at pH 8.8 (to sequester the Ca^{2+} ions; d, Figure 1) into a protein solution at pH 2.0 (e, Figure 1). During the refolding, the aromatic resonances (7.0−8.0 ppm) were saturated prior to spectral acquisition to obtain the NOE spectra.

buildup are more than 70% of those seen when the same experiment is carried out for the native state, and considerably greater than those for the protein in 6 M guanidine hydrochloride (GdnHCl). This indicates that, on time average, the sum of the close contacts between aromatic and methyl groups is similar in the native state and A-state. Moreover, as the NOE also depends on the overall correlation time of the molecular tumbling, it suggests that the molecular dimensions are also closely similar in the two states.[49] This experiment has recently been extended to study the species present during the kinetic refolding of the protein (Forge et al., unpublished results). Here, the experiment shown in Figure 2 was modified to include a saturation pulse prior to accumulation of spectra in the kinetic refolding experiment, and NOE difference spectra were recorded as a function of time after initiation of folding (Figure 7). The total NOE intensity changes by less than 10% in the various spectra recorded in this experiment. This shows dramatically, therefore, that during the folding process the average compactness and number of contacts do not alter significantly; rather the structure undergoes a search within the collapsed ensemble of states for the native packing of the side chains.[4]

The ability to generate NOEs in this manner shows that in principle the information needed to define the structural characteristics of a folding protein is accessible. Nevertheless, the lack of dispersion and broad nature of the spectra that result from the ensemble of states present during folding of BLA prior to the formation of the native structure makes it impossible to identify individual interactions. We have proposed one approach to identify the NOEs which exploits an idea similar to that behind NMR hydrogen exchange labeling experiments.[49] The idea is to generate information in a nonnative state and then convert the protein into its native state where this information can be read out through the high-resolution assigned spectra of the native protein. The technical difficulties are, however, particularly demanding for the NOE experiment as the nuclear magnetization decays relatively rapidly (typically with a time constant of ca. 1 s) while isotope labels can be trapped in the native state, at least in many sites, for periods of time often in excess of days. These "radio frequency pulse labeling" experiments therefore need to be carried out very rapidly.

In the case of BLA the ability to generate rapid folding by addition of Ca^{2+} enables these experiments to be viable at least in principle. Initial experiments support this contention in practice, as shown in Figure 8 in which NOEs generated in the A-state can be observed in the native state spectrum with high intensity following transfer of magnetization in a refolding step. The initial 1-D experiments suggest that the A-state has a significant proportion of nativelike contacts, although evidence has also been found for some nonnative interactions.[49] This approach is in principle amenable to substantial development, both by using double-jump experiments to study species generated kinetically and by using strategies to improve the selectivity of the experiment, for example, through isotope incorporation or site-directed mutagenesis. Ultimately it may be possible to probe the distribution of contacts sampled by individual protons at a given state of the folding reaction, and to use this information to provide a detailed description of the structural ensembles populated during folding.

Future Prospects

NMR is already established as an important technique in structural studies of protein folding.[6,8,9] We have highlighted here the use of NMR spectroscopy to follow protein folding in a real-time mode where the reaction occurs within the NMR spectrometer. In the case of the α-lactalbumins, on which we have focused in this Account, the kinetic NMR experiments have provided some key information about the folding process. They show that a highly compact ensemble of species is formed early in folding which buries hydrophobic residues such as tryptophan in the globular interior. They show clearly that subsequent events involve the relatively slow rearrangement in the compact state of the various structures contributing

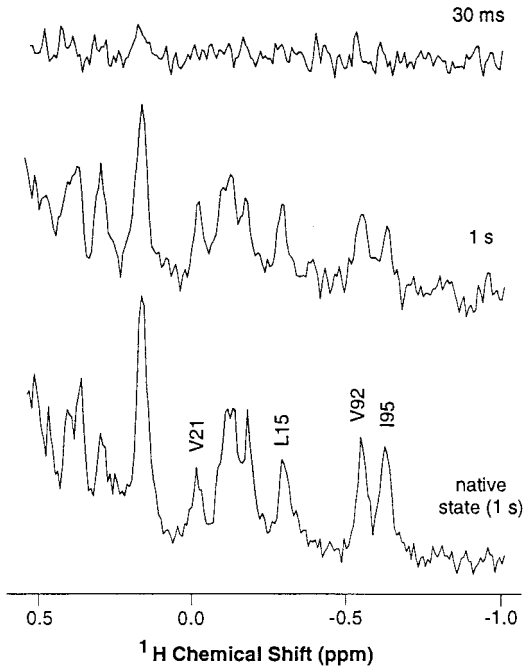

FIGURE 8. Example of "rf pulse labeling" of BLA.[49] Prior to initiation of refolding by means of a pH jump in the presence of Ca^{2+}, an rf pulse was applied at the aromatic ^{1}H resonant frequencies in the A-state. During the NOE generation, the magnetization is transferred to the methyl groups that are in close proximity to these aromatic side chains through NOE effects. The protein refolds rapidly to its native state following injection of a solution at pH 8.9 containing Ca^{2+} (d, Figure 1) into a protein solution at pH 2.0 (e, Figure 1). The NOE effects were then detected in the native state after 800 ms by recording a single scan. Difference spectra were obtained as described in the caption to Figure 6. Spectra are presented after normalization in absolute intensity, and are shown for the native state at equilibrium and for the refolded state after saturation of the A-state aromatic resonances for 30 ms and 1 s. The substantially reduced intensity changes in the spectra recorded following a 30 ms pulse indicate that the observed NOE intensities in the 1 s spectrum predominantly reflect the NOEs generated in the A-state.

to this ensemble. And they reveal that the final step to form the native state involves the highly cooperative close-packing of the amino acid side chains. These findings complement and extend the results of more established techniques such as stopped flow optical spectroscopy as well as NMR studies of stable unfolded and partially folded species under equilibrium conditions.[18,22,23,55] Most importantly, however, they begin for the first time to enable us to glimpse directly the folding process at the level of individual residues.

Although much has already been learned from such studies, they are at a very early stage of development and there are many possibilities for future advances. Improved mixing procedures will undoubtedly reduce the dead time of the experiments; indeed in studies of simple chemical reactions by NMR dead times of less than 10−20 ms have been reported.[50,51] Folding reactions can be initiated in situ by rapid redox and photochemical means that avoid the need for mixing solutions altogether.[6,52−54] It is possible under some circumstances to record NMR data very rapidly; fast reactions can, for example, be monitored by nonequilibrium events occurring within individual free induction decays.[51] Novel methodologies, notably in 2-D experiments, should increase the versatility of these experiments and open up new approaches to probe, for example, the dynamics and solvation of species present during the folding process. Kinetic NMR experiments promise, therefore, to play a key role in the quest to understand how the amino acid sequence of a protein defines its overall three-dimensional fold.

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