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Real-time NMR studies on folding of mutants of barnase and chymotrypsin inhibitor 2

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Abstract The folding and unfolding of proteins is generally assumed to be so co-operative that the overall process may be followed by a single probe, such as tryptophan fluorescence. Folding kinetics of three mutants of barnase and chymotrypsin inhibitor 2 (CI2) were studied by real-time NMR. Rate constants for changes in individual residues during the unfolding or refolding of the mutants studied by real-time NMR are all within experimental error of the overall process of folding/unfolding measured by stopped-flow measurements of tryptophan fluorescence. Folding of these mutants is thus highly co-operative. Changes in the tryptophan fluorescence give accurate measurements of the protein folding process.

Key words: Protein folding; Nuclear magnetic resonance

1. Introduction

Nuclear magnetic resonance (NMR) is the method of choice for studying the structure and dynamics of proteins under both native and denatured conditions in solution [1]. In theory, NMR can give information about the environment of every residue in a protein. Recently, using rapid mixing methods that allow mixing inside the magnetic field, it has been possible to use NMR to observe the folding process directly on a time scale of seconds [2,3]. This paper demonstrates the applicability of real-time NMR spectroscopy by applying the technique to proteins in H_2O, using several unfolding and refolding protocols. Two proteins, barnase and CI2, were used as test systems. The folding of these proteins has been well characterised [4–6] and mutants that fold and unfold in the appropriate time scales can be engineered.

2. Materials and methods

The preparation of barnase mutants has been described previously [4]. CI2 was a kind gift from Andreas Ladurner. Barnase A43C/S80C was donated by Jane Clarke. Homonuclear real-time NMR experiments were acquired on a Bruker AMX 500 spectrometer equipped with *z*-gradients. Water suppression used the Watergate sequence [8]. Signals from urea were suppressed via low-power presaturation. A modified NMR tube was used as a stopped-flow device to achieve mixing inside the magnet. The dead time was estimated at 7 s from bench top mixing experiments. To allow for pre-equilibration, 16 dummy scans were taken at the start of the experiment.

Different mixing protocols were used for each of the three protein systems. Unfolding of 1 mM A43C/S80C barnase was achieved by injecting 60 μl of 1 M HCl into 400 μl protein solution in 90% H_2O/10% D_2O. The experiment was run at 298 K with two scans acquired for each spectrum.

For urea-induced unfolding, 225 μl of 10 M urea was injected into 325 μl of 0.9 mM barnase to give 500 μl of 0.6 mM barnase in 4 M urea (90% H_2O/10% D_2O). The experiments were acquired at 288 K using eight scans for each spectrum.

To initiate refolding from acid of A16G/L49A CI2, 100 μl of 500 mM potassium phosphate buffer in 1.9 M urea was injected into 400 μl of 2.25 mM A16G/L49A CI2 in 1.9 M urea, 20 mM HCl to give 500 μl of CI2 in 90% H_2O/10% D_2O, pH 6.3, 100 mM phosphate and 1.9 M urea. Spectra were recorded at 288 K. 2D spectra were recorded with 1024 1D increments of 16 scans (~8 s per time point) each or 512 increments of four scans (~2 s per time point). A wider spectral width of 8 kHz was used to detect shifted signals from tryptophan residues.

The data were processed on UXNMR (Bruker, Karlsruhe) and on FELIX95 (Biosym Technologies, San Diego, CA). Fluorescence measurements were carried out with a Perkin Elmer MPF-44B spectrophotometer using a T-jet mixer. Kinetic data were analysed using Kaleidagraph (Abelbeck Software) and fitted to exponential equations.

3. Results

The three mutants studied were chosen to test different ways of initiating folding and unfolding. Real-time NMR studies of refolding by dilution of urea solutions have been previously reported [2].

The barnase mutant A43C/S80C was chosen to test unfolding by pH jump. The disulphide bond stabilises barnase A43C/S80C so that it is 50% denatured at pH 2 [7]. The observed unfolding rate constant of 0.05 s^{-1} is fast for the real-time NMR time scale, assuming a dead time of ~7 s and recording a data point every 1.2 s. The time resolution is limited because 0.6 s per scan is needed for relaxation of the NMR sample and two scans are used for each point to take advantage of a minimal phase cycling. Higher quality data, with lower signal/noise, can be obtained for slower processes.

Barnase L98T unfolds slowly in 4 M urea with a rate constant of 0.010 s^{-1}. A stack plot of successive NMR experiments is shown in Fig. 1. Peaks from the native protein can be seen to decay and peaks from the denatured state appear throughout the time course of the experiment. The intensities of several peaks, for both native and denatured signals, throughout the spectra were recorded and analysed. The most interesting peaks were the folded signals for the three methyl signals of Ile-76 at −0.25 ppm, Ile-51 at −0.40 ppm, Leu-63 at −1.00 ppm and the signal corresponding to the unfolded Trp at 10.01 ppm. These are well separated peaks, which can be readily assigned from the 1D spectra. Ile-76, Leu-63 and Ile-51 are in the three separate hydrophobic cores of barnase. Trp-94 makes an excellent control for the real-time NMR, as this Trp residue is observed in fluorescence studies [4,5]. A typical real-time NMR kinetic curve is shown

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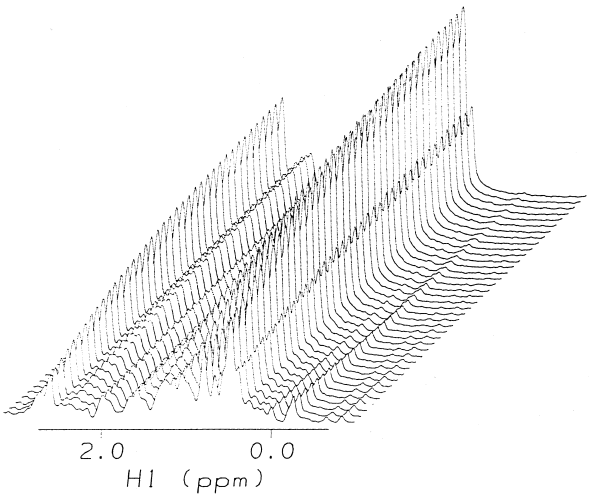

Fig. 1. Real-time NMR stack plot of upfield region of barnase L89T during unfolding in 4 M urea. Upfield shifted methyls, characteristic of folded proteins, can be seen disappearing.

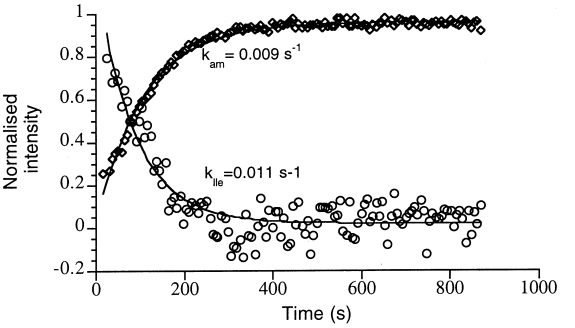

Fig. 2. Kinetic rate curves for the unfolding of a Barnase L89T in 4 M urea. k_{am} is the rate of appearance of a native amide signal at 8.14 ppm. k_{Ile} is the rate of disappearance of the denatured signal for Ile-76. The NMR measured unfolding rate corresponds with the fluorescence measured unfolding rate, $k = 0.009$ s^{-1}.

in Fig. 2. The fact that signals from all three hydrophobic cores display the same kinetics is strong evidence for the co-operative nature of the barnase folding.

The A16G/L49A mutant of CI2 was refolded from acid by in the presence of 1.9 M urea at 288 K. This mutant was chosen for its slow refolding rate. A number of peaks throughout the spectrum, including upfield shifted methyls, were followed for 2000 s. All signals display the same kinetics, which are within experimental error of the kinetics measured by fluorescence. Despite the extremely slow nature of the refolding, the system remains two-state and there is no evidence of any intermediate species.

4. Discussion

The three systems used in this study are in H_2O, allowing use of all the NH signals as structural probes as well as aliphatic signals. Rates obtained from fluorescence were compared with those from real-time NMR. Representative results are shown in Table 1. Signals for residues in different parts of the proteins all show the same kinetics. For all three protocols the kinetics observed are within experimental error of those measured by fluorescence studies. This confirms the validity of using a single tryptophan as a probe for the structure of the whole protein. This is because of the co-operative nature of the protein folding process.

Real-time NMR provides evidence for the two state co-operative nature of the folding process, under the chosen experimental conditions. In each experiment, only two sets of peaks, corresponding to native and denatured states, are observed. The members of each set of peaks all grow/decay at the same rate. When the normalised intensities for native and denatured state peaks are added the results is constant over the time course of the experiment. There are no observable peaks in the spectrum attributable to intermediate species. This is in agreement with previous observations; CI2 is an ideal two-state system [6], it refolds without any intermediates. Barnase is known to unfold by a two-state process [5].

This study has demonstrated the validity of using real-time NMR as a spectroscopic technique to study protein folding under various denaturing conditions. The potential strength of the real-time NMR technique is giving not only kinetic data but also structural information about the environment of all of the residues. Changes in chemical shift as well as line shapes can provide useful insight into the protein folding

Table 1
Rate constants determined by real-time NMR measurement and stopped-flow fluorescence for the unfolding of barnase mutant L89T in urea, the unfolding of barnase A43C/S80C in acid and the folding from acid of CI2 mutant A16G/L49A

Native peaks		Denatured peaks		Fluorescence
Shift (ppm)	k_{obs} (s^{-1})	Shift (ppm)	k_{obs} (s^{-1})	k_{obs} (s^{-1})
Barnase L89T				0.009 ± 1e−3
Leu-63 at −1.00	0.012 ± 2e−3	Trp at 9.94	0.0099 ± 1e−3	
Ile-51 at −0.40	0.010 ± 1e−3	amine at 8.14	0.0090 ± 5e−4	
Ile-76 at −0.25	0.0011 ± 1e−3	amine at 7.25	0.011 ± 1e−3	
amine at 8.75	0.012 ± 1e−3	amine at 7.14	0.010 ± 5e−4	
Barnase A43C/S80C				0.045 ± 5e−3
Leu-63 at −1.00	0.054 ± 5e−2			
CI2 A16G/L49A				0.0016 ± 5e−5
−0.28	0.0019 ± 5e−5	8.16	0.0019 ± 5e−5	
0.45	0.0018 ± 1e−4	8.64	0.0015 ± 2e−4	
6.55	0.0017 ± 1e−4	9.08	0.0016 ± 2e−4	

The same rate is observed, by real-time NMR, for different residues over each system, implying two-state folding. The real-time NMR measured rate constants are within experimental error of those measured by fluorescence.

process. Although this structural information might be obscured by limited resolution in 1D spectra approaches such as selective labelling can be used to overcome this problem.

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