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Protein Folding Kinetics from Magnetization Transfer Nuclear Magnetic Resonance†

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ABSTRACT: The rates of folding and unfolding of lysozyme in the region of the thermal denaturation transition have been measured directly by saturation transfer ^{1}H NMR. Rates measured for three residues in quite different regions of the molecule were closely similar, demonstrating that the transition is highly cooperative under these conditions. The rates were measured as a function of temperature and were entirely consistent with the thermodynamics of denaturation. Both folding and unfolding rates were strongly temperature dependent, the apparent activation energies being positive for unfolding and negative for folding. The significance of these results for studies of the nature of the unfolded state and of protein folding is discussed.

Nuclear magnetic resonance studies of reversible denaturation have been carried out for a number of proteins (McDonald et al., 1971; Benz & Roberts, 1975; Jardetzky & Roberts, 1981). NMR is a particularly attractive technique for such studies because it can provide specific information about the behavior of individual groups within the protein. In many cases distinct spectra of folded and unfolded states have been observed and denaturation equilibria have been characterized in terms of their relative intensities. In general, however, no information about the kinetics of the folding and unfolding processes is obtainable in these experiments. A method that provides quantitative kinetic data would clearly extend considerably the utility of NMR in protein folding studies.

It has been shown by ^{1}H NMR that, in favorable cases, magnetization transfer can be observed between interconverting folded and unfolded states of proteins (Wüthrich et al., 1978, 1980; Dobson et al., 1984). Quantitative analysis of magnetization transfer effects can permit the determination of the rates of chemical exchange reactions (Forsén & Hoffman, 1963; Campbell et al., 1978). In this paper we explore the application of this technique to kinetic studies of the reversible unfolding of one protein, hen egg-white lysozyme. This protein has been the subject of intensive NMR studies, and resonances of more than 40 of its 129 amino acid residues have been assigned in the ^{1}H NMR spectrum of the native state (Poulsen et al., 1980; Delepierre et al., 1982; Redfield et al., 1982). Lysozyme is therefore a particularly favorable system with which to investigate the potential of the technique.

Experimental Procedures

Hen egg-white lysozyme (EC 3.2.1.17) was obtained from Sigma and dialyzed extensively at low pH before use. Labile protons were exchanged for deuterons by warming to 80 °C in D_2O at pH 3.8 and lyophilizing. Solutions for NMR studies were 6 mM in protein at pH 3.8 in D_2O.

NMR spectra were recorded at 300 MHz with a Brüker WH300 spectrometer. Probe temperatures were measured with ethylene glycol (Van Geet, 1968). Saturation transfer experiments were carried out by irradiating selectively individual resonances prior to the observation pulse. The effects of this perturbation, which in general were measured by difference spectroscopy, included intramolecular nuclear Overhauser effects (Noggle & Schirmer, 1971; Poulsen et al., 1980), as well as chemical exchange effects. The former were distinguishable because they were also detectable in temperature ranges well outside that of the unfolding transition, in which chemical exchange effects would be absent.

The denaturation curve was determined by following intensity changes of a number of resolved native resonances as a function of temperature (McDonald et al., 1971). It was confirmed that each showed essentially the same temperature dependence. In the saturation transfer experiments the intensity of one proton in the native protein was determined directly by measuring the area of a resolved native peak. The

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intensity of one proton in the unfolded state was then calculated from the experimental denaturation curve.

Two approaches were used for the determination of the time dependence of saturation transfer. One method was to perform a set of experiments in which the duration of the presaturation pulse was varied (Campbell et al., 1978). In the other method a selective inversion pulse was applied to a resolved resonance and its rate of recovery determined in the presence of a saturation pulse applied to the other resonance, from which saturation transfer was observed. The latter method was only applicable to resolved resonances but had the advantage of increased dynamic range of the magnetization. A DANTE pulse was used to achieve the selective inversion (Morris & Freeman, 1978). An alternative experimental approach would be to apply a transient perturbation such as a selective inversion pulse to a resonance, rather than the saturation pulse (Led & Gesmar, 1982). This method was not used here, principally because a complete analysis would require both native and denatured resonances to be resolved, which is seldom the case in protein spectra.

The analysis of these experiments is based on a model of two spins in chemical exchange (McConnell, 1958; Forsén & Hoffman, 1963). If A and B are protons in chemical exchange

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

where k_1 and k_2 are first-order rate constants, it can be shown that saturation of the B resonance will cause the intensity of A to fall from its unperturbed value, I^0, toward a new limiting value, I^∞, given by

$$I^0/I^\infty = 1 + k_1 T_{1,A} \quad (1)$$

where $T_{1,A}$ is the apparent spin–lattice relaxation time of spin A. The time development of this effect is given by

$$\frac{\mathrm{d} \ln |I^\infty - I|}{\mathrm{d}t} = -(k_1 + 1/T_{1,A}) \quad (2)$$

The recovery of resonance A from selective inversion is also determined by this equation when B is saturated. Simultaneous solution of these equations leads to values for k_1 and $T_{1,A}$. The complementary experiment in which A is saturated leads to values for k_2 and $T_{1,B}$. In our experiments we thus measured the rates of exchange between folded and unfolded states for individual sites within the lysozyme molecule.

Results

The effect of unfolding on the ^{1}H NMR spectrum of lysozyme is shown in Figure 1. It is apparent that, under conditions where native and denatured states coexist, exchange between them is slow, so that protons in the different states give rise to distinct signals. This is particularly clearly seen for the resonances of His-15 $H^{\epsilon 1}$; the two resolved peaks to low field in Figure 1b are from this proton in the native (8.72 ppm) and denatured (8.58 ppm) states (Meadows et al., 1967). In no other cases are the resonances of a given proton in both forms of the protein fully resolved at 300 MHz.

Rate constants for unfolding (k_1) and folding (k_2) were determined at a number of temperatures by measuring saturation transfer between the His-15 $H^{\epsilon 1}$ resonances. An example of the determination of k_1 by the two methods described above is shown in Figure 2. The kinetic parameters determined in this experiment and in similar experiments at other temperatures show good agreement between these two methods, which gives confidence in the validity of the analysis used. The results are presented in Figure 3 as Arrhenius plots. These

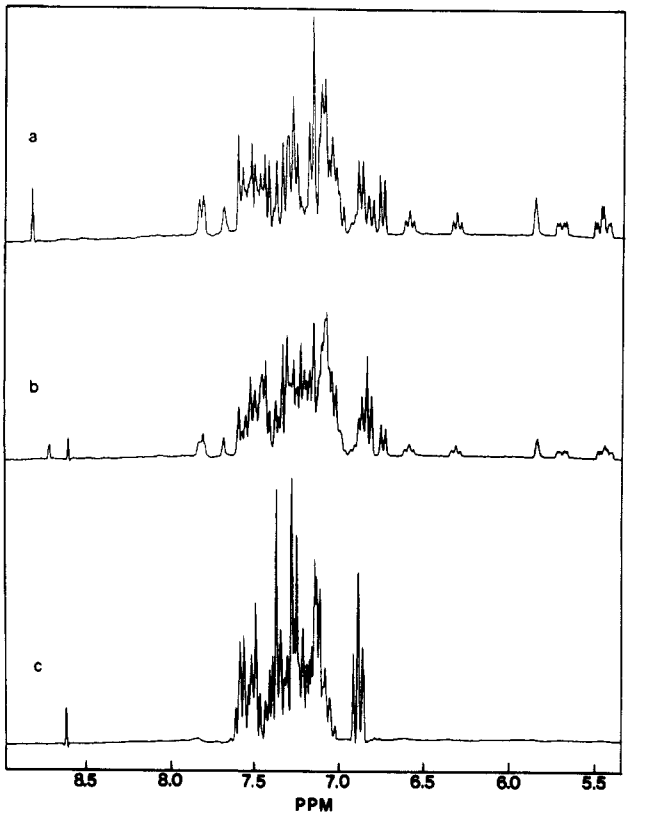

FIGURE 1: Low-field part of the 300-MHz proton NMR spectra of lysozyme, pH 3.8, in D_2O at various temperatures. Labile protons have been exchanged for deuterons, and resolution has been enhanced by Gaussian multiplication. (a) 68 °C, protein fully native; (b) 77 °C, protein close to the midpoint of unfolding; (c) 83 °C, protein almost all unfolded.

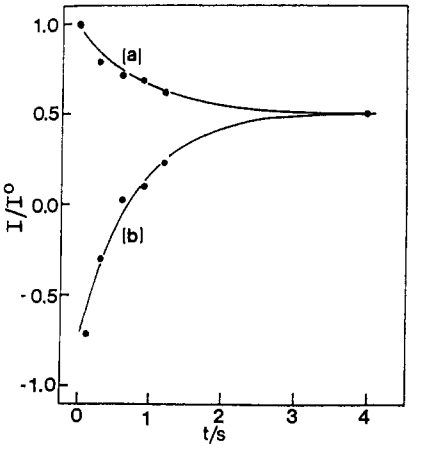

FIGURE 2: Determination of the unfolding rate of lysozyme at 77 °C. (a) Time development of saturation transfer to the resonance of His-15 $H^{\epsilon 1}$ in the native state (N) on irradiating the corresponding denatured resonance (D). (b) Inversion–recovery of resonance N while D is saturated. The limiting saturation transfer effect $I^0/I^\infty = 1.96 \pm 0.10$. The curves are calculated from best-fit straight lines given by eq 2. The kinetic parameters derived are (a) $k_1 = 0.60 \pm 0.10$ s^{-1}, $T_{1,N} = 1.61 \pm 0.21$ s and (b) $k_1 = 0.63 \pm 0.10$ s^{-1}, $T_{1,N} = 1.52 \pm 0.17$ s.

are approximately linear in this temperature range and show apparent activation energies of $+61 \pm 2$ kJ mol^{-1} for the unfolding and -40 ± 4 kJ mol^{-1} for the folding process. The T_1 values obtained were independent of temperature within experimental error, their values being 1.5 ± 0.2 s for the native and 1.7 ± 0.3 s for the denatured resonance.

In a two-state system the equilibrium constant for unfolding, K, would be equal to the ratio of rate constants k_1/k_2. To test this, K was determined independently as a function of tem-

Table I: Rates of Folding and Unfolding for Three Residues of Lysozyme

temp (°C)		k_1 (s^{-1})	k_2 (s^{-1})	$T_{1,N}$ (s)	$T_{1,D}$ (s)
72.0 ± 0.5	His-15 $H^{\epsilon 1}$	0.14 ± 0.05		1.39 ± 0.20	
	Cys-64 H^{α}	0.19 ± 0.11		0.39 ± 0.05	
	Met-105 H^{ϵ}	0.13 ± 0.07		0.47 ± 0.03	
77.0 ± 0.5	His-15 $H^{\epsilon 1}$	0.60 ± 0.10	0.73 ± 0.14	1.61 ± 0.17	1.58 ± 0.24
	Cys-64 H^{α}	0.59 ± 0.12		0.36 ± 0.03	
	Met-105 H^{ϵ}	0.64 ± 0.09	0.77 ± 0.14	0.41 ± 0.05	0.82 ± 0.12

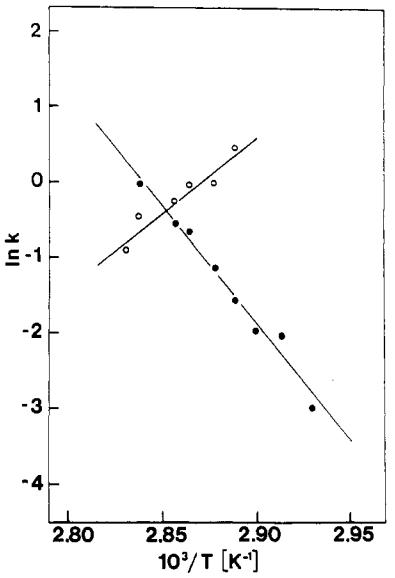

FIGURE 3: Temperature dependences of the unfolding (●) and folding (○) rates of lysozyme, pH 3.8. The values were derived from determination of the time development of saturation transfer between the resonances of His-15 $H^{\epsilon 1}$ in the folded and unfolded states.

perature by measuring intensity changes of the resolved native resonances of Cys-64 H^{α} (5.82 ppm) and Leu-17 $H^{\delta 2}$ (−0.63 ppm). These data gave a van't Hoff plot to which a straight line could reasonably be fitted over this temperature range. From this analysis, the unfolding enthalpy was determined to be +110 ± 6 kJ mol^{-1} and the midpoint of denaturation 77.5 ± 1.0 °C. These results accord well with the kinetic data: the best-fit lines of Figure 3 indicate that $k_1 = k_2$ at about 77.5 °C, in good agreement with T_m, and that the combined activation energies $\Delta H_1^{\ddagger} - \Delta H_2^{\ddagger} = 101 \pm 5$ kJ mol^{-1}, which is consistent with the thermodynamic unfolding enthalpy.

Saturation transfer studies were also made with other resonances of lysozyme. Table I presents results that were obtained with the resonances of Met-105 H^{ϵ} and Cys-64 H^{α}. Met-105 H^{ϵ} gives rise to singlet resonances at 0.03 ppm in the native protein (Campbell et al., 1975) and 2.01 ppm in the unfolded state. Neither resonance is completely resolved, but rates of folding and unfolding were determined from the time development of saturation transfer between them by means of difference spectroscopy. Cys-64 H^{α} gives rise to a resolved resonance at 5.82 ppm in the native state (Delepierre et al., 1982). The rate of unfolding was determined from the inversion recovery of this resonance while the denatured resonance, at 4.73 ppm, was saturated. In these cases, as with His-15 $H^{\epsilon 1}$, any NOE's observed did not interfere with the saturation transfer effects. It is apparent that the rates of folding and of unfolding measured were essentially the same for each residue.

Discussion

The results in this paper demonstrate that measurements of the time dependence of saturation transfer between folded and unfolded states can be used to determine the rates of folding and unfolding of lysozyme at equilibrium. The approach should be applicable to other proteins, provided that conditions can be chosen such that the interconversion is fast enough to compete with spin–lattice relaxation and that individual resonances can be observed, directly or indirectly, in the spectrum.

The analysis of the time dependence applied in this paper is based on a model of two-site exchange between the native and denatured states. It is now well established that in many proteins there is significant kinetic heterogeneity in the refolding process associated, it is believed, with the effects of proline isomerization as a slow step in refolding (Brandts et al., 1975; Kim & Baldwin, 1982). In the case of lysozyme, biphasic refolding kinetics were observed in a recent study, with a slow folding population constituting about 10% of the total (Kato et al., 1981, 1982). These studies were, however, made in conditions very different from those of the experiments described in this paper. Serious deviation from the two-site model would have been expected to result in nonexponential time dependence of the magnetization and differences in the results of the experiments with and without selective inversion. Further, errors in the analysis would have produced disparity between the apparent rate constants and the independently determined equilibrium constant. The results provided in this paper thus indicate that the assumption of kinetic homogeneity is a reasonable one for both the folded and the unfolded lysozyme populations in these conditions.

Kinetic measurements are reported for three residues in quite different regions of the lysozyme molecule. The crystal structure shows that the native conformation is divided into two parts by a deep cleft (Blake et al., 1967). One part includes a region of β-sheet structure in which one of the residues studied, Cys-64, is situated. The other part includes a hydrophobic core in which Met-105 is buried. His-15 is in a helical region, close to the surface of the molecule. The equality of the folding and unfolding rates measured for these three residues reflects the highly cooperative nature of the processes under these conditions. This result also gives further confidence in the validity of the approach used.

The temperature dependences of the folding and unfolding rates provide the interesting observation that the apparent activation energy for the former is substantial and negative. In earlier studies of the folding kinetics of lysozyme, which were made in the presence of chemical denaturants, the folding rate was similarly found to decrease with increasing temperature in the transition zone (Segawa et al., 1973; Kato et al., 1982). Over a wider temperature range the Arrhenius plots obtained in these studies were found to be curved so that the folding rate passes through a maximum at a temperature below the transition zone. This behavior is not general for globular proteins, however. In the case of ribonuclease A, for example, the direct folding rate passes through a minimum at the midpoint of denaturation (Hagerman & Baldwin, 1976). These temperature dependences cannot straightforwardly be interpreted, since folding must involve a sequence of many individual rearrangements and cannot be considered as a single reaction.

The saturation transfer experiment measures an overall rate of transition between folded and unfolded populations. Neither state is defined by a single static conformation, however, and the NMR spectrum in each case represents an average spectrum of many rapidly interconverting conformations. Although the dynamics of the native protein are dominated by limited fast fluctuations about a well-defined average structure (Karplus & McCammon, 1981), the unfolded state is much more disordered. It undoubtedly comprises a large number of significantly different conformations in rapid equilibrium (Tanford, 1968). The conformational distribution need not, however, be a random one. Indeed, NMR spectra of denatured lysozyme suggest that it is not and that there are inherent conformational preferences in local regions of the unfolded protein (Dobson et al., 1984; Howarth & Lian, 1984). This behavior is likely to be of major importance in determining the pathways and kinetics of folding.

One approach to investigating the properties of the unfolded state would be to attempt to use the NMR methods that are used in the study of the conformations of native proteins. Individual resonances could be observed and assigned by correlation with those of the native state via the saturation transfer experiment (Dobson et al., 1984). The major difficulty with this method would be attempting to interpret the average spectrum of a wide range of conformational states. Another approach is provided by the observation that at temperatures close to the unfolding transition labile hydrogens buried in the native structure may exchange with solvent via the unfolded state (Wedin et al., 1982). Since the folding and unfolding rates can be measured directly as described in this paper, experimental hydrogen exchange rates can provide information about the lability of hydrogens in the unfolded state. Available data on the exchange kinetics of individual labile hydrogens suggest that the exchange behavior of the unfolded state could differ significantly from that expected for a random coil (Delepierre, 1983), indicating that this approach could be a useful method of characterizing nonrandom behavior of the unfolded protein.

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