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Ionic-strength-dependent transition of hen egg-white lysozyme at low pH to a compact state and its aggregation on thermal denaturation

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Equilibrium acid-induced unfolding of hen egg-white lysozyme has been investigated by a combination of optical methods, size-exclusion chromatography, and differential scanning calorimetry. The results showed the presence of a partially folded state of hen egg-white lysozyme at pH 1.5, characterized by a substantial secondary structure, a large solvent exposure of non-polar clusters, and significantly disrupted tertiary structure. A large enthalpy was also associated with the conversion of the acid-unfolded state to a fully unfolded state. Size-exclusion chromatography and 8-anilino-1-naphthalenesulphonic acid-binding studies showed an ionic-strength-induced transition of the partially folded state to a compact conformation. Furthermore, an ionic-strength-dependent aggregation on thermal unfolding of the partially folded intermediate was also observed. These observations provide insights into the possible features responsible for the stabilization of intermediates in the folding of hen egg-white lysozyme.

Keywords: hen egg-white lysozyme; low pH; ionic strength; compact state; thermal denaturation.

The extent of unfolding of denatured states of proteins under different conditions has long been of interest because of the possible relevance of their conformations to the protein folding pathways. Studies have been focussed on the conditions that disrupt the cooperative folding of the protein but allow substructures to persist. Recently, it has been demonstrated that residual structural preferences, which range from local clusters of side chains to highly ordered side chains to highly ordered subdomains, persist in denatured states of proteins (Kim and Baldwin, 1990; Dobson, 1991, 1994; Redfield et al., 1994; Ptitsyn, 1995; Arcus et al., 1995; Shortel, 1996). Hence, there has been a growing recognition of the importance of the compact denatured and partially folded states of proteins, as determination of their structure and thermodynamic properties may provide critical insight into the mechanisms of protein folding. The characterization of these structures and the factors involved in their stability would provide important insight into the interactions responsible for their formation as well as their role in protein folding.

The importance of electrostatic interactions in determining the stability of a protein has long been recognized (Tanford, 1970). pH is known to influence the stability of a protein by altering the net charge on the protein, and many proteins denature at extreme pH because of the presence of destabilizing repulsive interactions between like charges in the native protein (Creighton, 1993; Tan et al., 1995). Extremes of pH do not always completely denature the proteins but result in denatured states that are partially unfolded as compared to the fully unfolded ones observed in the presence of high concentrations of guanidine hydrochloride (Goto et al., 1990; Fink et al., 1994). This is maybe due to the fact that the driving force for unfolding of proteins in acid is repulsive forces between ionized residues in the protein, which may fail to overcome interactions such as hydrophobic forces, salt bridges, and other factors that favor folding. The exact behavior of a given protein at low pH is a complex interplay between a variety of stabilizing and destabilizing forces, some of which are sensitive to the environment. Salt-dependent conformational transitions at acidic pH from a largely unfolded state to an intermediate conformational state have been reported for several proteins (Goto and Fink, 1989; Goto et al., 1990; Ohgushi and Wada, 1983; Arakawa et al., 1987), which suggests that salt-dependent conformational transitions at acidic or alkaline pH regions may be a general property of many proteins.

Denaturation of c-type lysozyme occurs without any thermodynamically stable intermediate forms (Tanford, 1970; Khechinashvili et al., 1973; Cooper et al., 1992). The thermodynamic and folding behavior of c-type lysozymes under equilibrium conditions agree well with a cooperative two-state model (Radford et al., 1992). However, under conditions far from equilibrium, the existence of a transient species has been suggested for hen egg lysozyme on the basis of refolding studies (Miranker et al., 1991). Furthermore, at least one, and possibly more, intermediate states with characteristics of molten globules, notably ordered secondary structure without specific tertiary interactions as monitored by near- and far-ultraviolet circular dichroism, were reported to be formed during folding of c-type lysozyme (Radford et al., 1992; Miranker et al., 1991). Recently, the existence of an equilibrium partially folded state of human lysozyme at low pH has been reported (Haezebrouck et al., 1995). The population of this intermediate state was found to be strongly dependent on pH and temperature and had at least some characteristics of the molten globule state of homologous α-lactalbumin and of kinetic intermediates observed in the folding of c-type lysozymes. No such partially folded intermediate at low pH has been reported for hen egg lysozyme (Haezebrouck et al., 1995; Cooper et al., 1992). This prompted us to look into the structural details of hen egg lysozyme at low pH.

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Abbreviation. DSC; differential scanning calorimetry.

Enzyme. Lysozyme (EC 3.2.1.17).

In the present work, we have carried out a comparative study on the structural as well as thermodynamic properties of hen egg lysozyme at pH 7.0 and pH 1.5. At pH 1.5, hen egg lysozyme has a substantial secondary structure, a large solvent exposure of non-polar clusters, and a significantly disrupted tertiary structure. Furthermore, a large enthalpy is associated with the conversion of acid denatured lysozyme to a fully unfolded state. These observations suggest the presence of an equilibrium partially folded intermediate of hen egg lysozyme at low pH and this intermediate seems to be more ordered than most other molten globules discussed in the literature. Studies on the effect of ionic strength on the partially folded intermediate showed its ionic-strength-dependent transition to a compact conformation and aggregation on thermal denaturation.

EXPERIMENTAL PROCEDURES

Materials. All the chemicals used were purchased from Sigma and were of the highest purity grade. Hen egg-white lysozyme was purchased from Sigma and its purity was checked by SDS/PAGE followed by silver staining. It was found to be greater than 95% pure.

Circular dichroism. CD measurements were made on a Jasco J-500C spectropolarimeter at 27°C. 0.1-cm and 1-cm path-length cuvettes were used for 190–250 nm and 250–350 nm, respectively. The solvent spectrum was determined and subtracted from the protein spectrum. The concentrations of hen egg lysozyme samples was typically 20–30 μM for the far-ultraviolet and 80–90 μM for the near-ultraviolet regions. Molar ellipticities, θ (in deg · cm^2 · dmol^{-1}), are reported.

Differential scanning calorimetry. All calorimetric scans were performed with a Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translator DT-2801) for automatic data collection and analysis. The protein concentration of each sample used for the calorimetric experiments was about 2–3 mg/ml, unless specified otherwise; a 1.3-ml sample was introduced into the sample cell and a similar amount of buffer was introduced into the reference cell. Samples were scanned at a rate of 60°C/h, unless otherwise mentioned. The samples were degassed for 15 min at room temperature before being scanned in the calorimeter. Data reduction and analysis were performed with software developed by Prof. Ernesto Freire (Bhakuni et al., 1991).

8-anilino-1-naphthalenesulphonic acid fluorescence measurements. Fluorescence spectra were recorded with a Perkin-Elmer LS5B spectroluminescence meter in a 5-mm path length quartz cell. Aliquots of protein with final concentration 5 μM were equilibrated in the desired buffer for 30 min at 25°C. They were then mixed with a concentrated stock solution of 8-anilino-napthalenesulphonic acid fluorescent probe dissolved in the same buffer. The excitation wavelength was 365 nm and the emission was recorded either in the range 400–560 nm or at a fixed wavelength at 480 nm. The final concentration of the probe was 125 μM. The values were normalized by subtracting the baseline recording for 8-anilino-napthalenesulphonic acid alone.

RESULTS

pH-induced structural perturbations in hen egg-white lysozyme. Secondary and tertiary structure (far- and near-ultraviolet CD). The effect of lowering the pH on the secondary and tertiary structure of native hen egg lysozyme was studied by recording the near- and far-ultraviolet CD spectra at pH 7.0 and pH 1.5. The CD ellipticities at 222 nm and 270 nm are commonly used as a measure of the extent of secondary and tertiary structure, respectively, in proteins. The near- and far-ultraviolet CD spectra of hen egg lysozyme at pH 7 and pH 1.5 are shown in Fig. 1A. For hen egg lysozyme at pH 1.5, a spectrum with significant negative ellipticity was observed in the far-ultraviolet CD, but the observed ellipticity at 222 nm was significantly less as compared to that observed for the native protein. Similarly, in the near-ultraviolet region, a significant lowering of ellipticity for acid-denatured lysozyme as compared to the native lysozyme was also observed. These observations suggest that decreasing the pH to 1.5 results in partial unfolding of hen egg lysozyme, as a decrease and not complete disruption of both secondary and tertiary structures was observed.

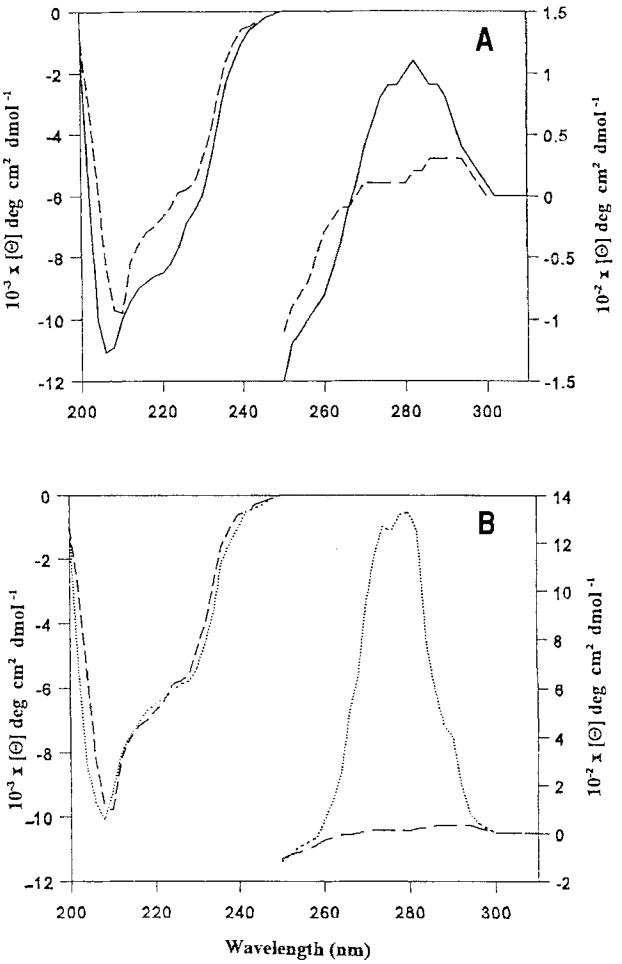

Fig. 1. Effect of low pH and ionic strength on the far- and near-ultraviolet CD spectra of hen egg-white lysozyme. (A) (——) hen egg lysozyme at pH 7.0; (– – –) lysozyme at pH 1.5. (B) (– · –) lysozyme at pH 1.5, (·····) lysozyme at pH 1.5 in the presence of 100 mM NaCl. The spectra were recorded at a protein concentration of 0.35 mg/ml and 1.23 mg/ml for far- and near-ultraviolet regions, respectively, at 27°C. The spectra were collected after substraction of solvent baseline.

Solvent-accessible non-polar clusters (8-anilino-1-naphthalenesulphonic acid binding). The solvent exposure of non-polar clusters in the acid-unfolded hen egg lysozyme at pH 1.5 was studied by 8-anilino-1-naphthalenesulphonic acid binding. This probe binds to solvent-accessible clusters of non-polar groups in proteins. The fluorescence emission of 8-anilino-1-naphthalenesulphonic acid is known to increase on binding to hydrophobic clusters of a protein (Stryer, 1965). The fluorescence spectra

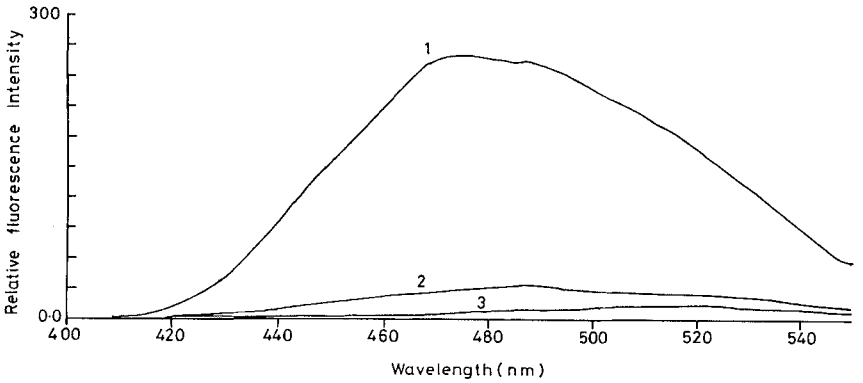

Fig. 2. Effect of sodium chloride on the binding of 8-anilino-1-naphthalenesulphonic acid to hen egg white lysozyme at pH 1.5. Curve 1, protein at pH 1.5; curve 2, protein at pH 1.5 in the presence of 100 mM NaCl; curve 3, protein at pH 7.0. The curves were obtained by incubation of 5 μM hen egg-white lysozyme with 125 μM 8-anilino-1-naphthalenesulphonic acid under different conditions at 27 °C. The excitation wavelength was 365 nm and the emission were recorded in the range 400–560 nm.

on binding of this probe to native and acid-denatured hen egg lysozyme are shown in Fig. 2. At pH 7.0, no significant probe fluorescence was observed, which suggests a very low binding of 8-anilino-1-naphthalenesulphonic acid molecules to native lysozyme. However, for acid-denatured lysozyme at pH 1.5, a very large increase in probe fluorescence intensity, to about 30-fold, was observed. Furthermore, the emission maximum also shifted from 515 nm to 480 nm, which indicates the burial of 8-anilino-1-naphthalenesulphonic acid in a hydrophobic environment under these conditions (Turner and Brand, 1968). The physical reason for these observed behaviors may be the formation of solvent-accessible non-polar clusters on acid-induced partial unfolding of protein; these clusters are screened from the solvent in the native protein.

Structural perturbations in the partially folded intermediate induced by ionic strength. Ionic-strength-induced perturbations in the secondary and tertiary structure of hen egg-white lysozyme at pH 1.5 were studied by far- and near-ultraviolet CD studies in the presence of increasing concentration of sodium chloride (NaCl). The far- and near- ultraviolet CD spectra of hen egg lysozyme at pH 1.5 in the presence and absence of 100 mM NaCl are shown in Fig. 1 B. No significant changes in the far-ultraviolet-CD spectra characteristic of acid-denatured lysozyme were observed in the presence of 100 mM NaCl. In contrast, in the near-ultraviolet CD spectra, the addition of 100 mM NaCl resulted in a large increase in the ellipticity at 270 nm, which indicates that NaCl induces a significant alteration in the tertiary structure. Further increase in NaCl concentration up to 500 mM showed no significant changes either in the far- or near-ultraviolet CD characteristics (data not shown). These observations suggest that an increase in ionic strength of hen egg lysozyme at pH 1.5 has no significant effect on the secondary structure of the protein, but has a pronounced effect on the tertiary structure.

The effect of ionic strength on the solvent exposure of non-polar clusters present in the acid-denatured lysozyme was also studied. As shown in Fig. 2, the addition of 100 mM NaCl to hen egg lysozyme at pH 1.5 resulted in a significant decrease of about 16-fold, in the 8-anilino-1-naphthalenesulphonic acid fluorescence intensity. No further decrease in the fluorescence intensity of the probe was observed on increasing the NaCl concentration up to 500 mM (Table 1). These observations suggest that addition of NaCl to lysozyme solution at pH 1.5, induces a conformational transition such that the solvent-exposed non-polar clusters present in the acid-denatured lysozyme get buried, as is the case in the native protein.

Table 1. Effect of ionic strength on the binding of 8-anilino-1-naphthalenesulphonic acid to hen egg white lysozyme at pH 1.5. The aliquots of protein with final protein concentration of 5 μM were equilibrated in the desired buffers for 30 min at 27 °C. They were then mixed with concentrated stock solution of fluorescent probe dissolved in the same buffer such that the final 8-anilino-1-naphthalenesulphonic acid concentration was 125 μM. The excitation wavelength was 365 nm and the emission was recorded at a fixed wavelength of 480 nm. The values presented were normalized by subtracting the fluorescence intensity observed for 8-anilino-1-naphthalenesulphonic acid alone under these conditions.

[NaCl]	Fluorescence intensity
mM	
0	350
50	88
100	36
200	26
300	19.3
400	19.4
500	19

Compactness. Recent studies have shown that size-exclusion chromatography permits the characterization of native, unfolded, and intermediate states (Uversky, 1993, 1994). Hence, the effect of ionic strength on the molecular size of acid-denatured hen egg lysozyme was studied by gel filtration chromatography. The retention times observed for hen egg lysozyme at pH 1.5 in the presence of increasing concentrations of NaCl are summarized in Fig. 3. For acid-denatured hen egg lysozyme at pH 1.5, a retention time of 21.40 min was observed. The addition of 100 mM NaCl to this lysozyme solution resulted in an increase of 3 min (to 24.43 min) in retention time, which indicates that addition of NaCl to acid-denatured lysozyme results in a decrease in its molecular size. A further increase in NaCl concentration resulted in a significantly smaller increase in retention time. These observations suggest an ionic-strength-dependent transition of acid-denatured hen egg lysozyme to a compact conformation.

Effect of ionic strength on the thermodynamic properties of the acid-induced partially folded intermediate. The effect of ionic strength on the thermodynamic properties of acid-denatured lysozyme at low pH was studied by differential scanning calorimetry. The excess heat capacity versus temperature curves

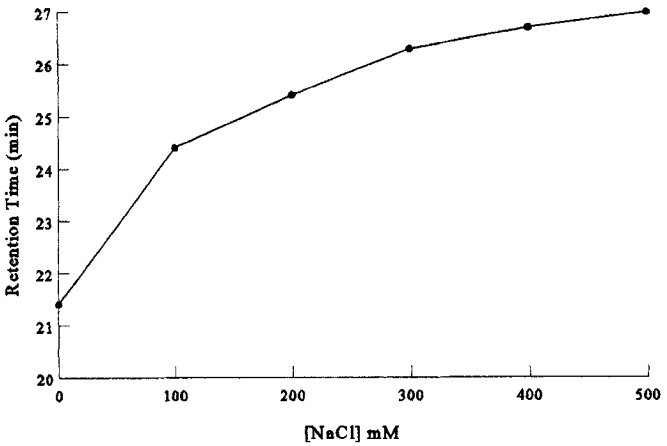

Fig. 3. Dependence of ionic strength on the retention time of hen egg-white lysozyme at pH 1.5 on a TSK G3000 SW column. The stock solutions of proteins were loaded into the column (Pharmacia Ultrapac TSK G3000 SW), which was equilibrated with the same buffer. The flow rate was 30 ml/h and the column was run at 27°C.

Table 2. Dependence of ionic strength on the thermodynamic parameters associated with the melting of hen egg-white lysozyme at pH 1.5. All the data are derived from the analysis shown in Fig. 4. ΔH_{cal} is the transition enthalpy, ΔH_{vh} the van't Hoff enthalpy, and T_m the transition temperature. The lysozyme concentration for these studies was 0.407 mM (5.8 mg/ml) and the scan rate was 60°C/h. At 500 mM NaCl, two independent transitions in *Cp* versus temperature scan were observed.

NaCl	T_m	ΔH_{cal}	ΔH_{vh}	$\Delta H_{vh}/\Delta H_{cal}$
mM	°C	kJ mol^{-1}		
0	53.33	383.67	367.77	0.96
100	50.03	332.63	346.01	1.04
200	49.79	334.72	348.94	1.04
300	50.17	301.25	356.47	1.18
400	50.32	299.57	361.49	1.2
500	50.37	337.23	353.96	1.05
	72.33	68.19	369.86	5.42

for hen egg lysozyme at pH 1.5 in the presence and absence of NaCl are shown in Fig. 4. Lysozyme at pH 1.53 undergoes a reversible two-state transition centered at 53.3°C. This suggests that a substantial enthalpy (383.67 kJ mol^{-1}) is associated with the thermal unfolding of acid-denatured hen egg lysozyme. In the presence of 100 mM NaCl, a decrease of about 3°C in transition temperature was observed. For a further increase in NaCl concentration up to 400 mM, a similar differential scanning calorimetry (DSC) profile and transition temperature was observed (Fig. 4). But, in the presence of 500 mM NaCl, two independent transitions centered at 50°C (corresponding to the thermal denaturation of acid-denatured lysozyme under these conditions) and 72°C, respectively, were observed. The thermodynamic parameters obtained from DSC studies are summarized in Table 2. All the transitions except for the one at higher temperature observed in the presence of 500 mM NaCl, were found to be following the two-state process as indicated by the van't Hoff/calorimetric enthalpy ratio; unity or near-unity values for this ratio provide a necessary and sufficient criterion for a two-state process (Bhakuni et al., 1991). For the higher temperature transition observed in the presence of 500 mM NaCl, the $\Delta H_{vh}/\Delta H_{cal}$ ratio was found to be 5.1, which suggests the presence of an aggregated state.

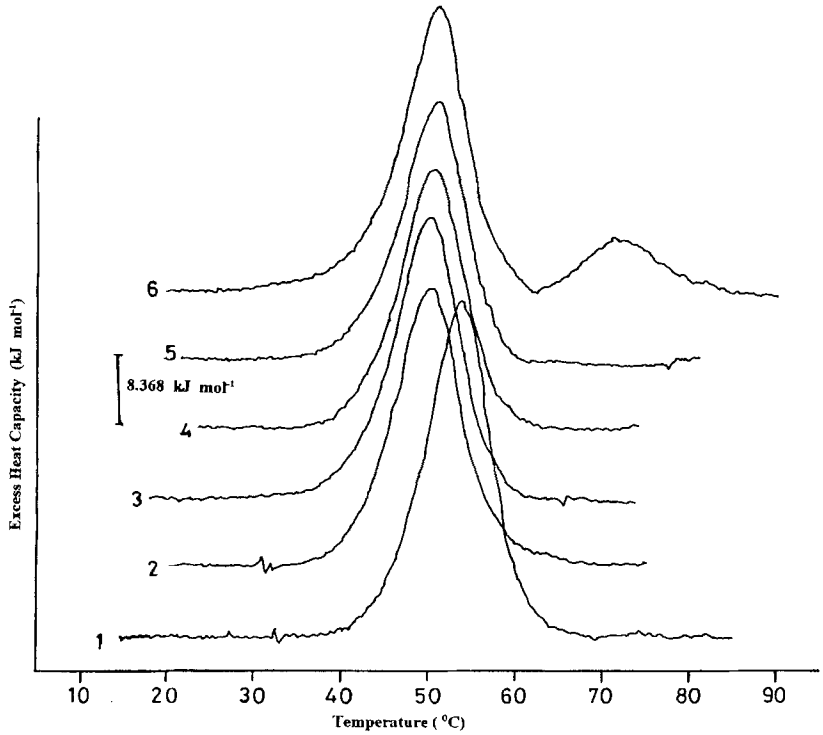

Fig. 4. Excess heat capacity versus temperature curves for hen egg-white lysozyme at pH 1.5 as a function of NaCl concentration. Curve 1, hen egg-white lysozyme at pH 1.5; curves 2–6, pH 1.5 in the presence of 100, 200, 300, 400 and 500 mM NaCl, respectively. The calorimetric scans were performed with a protein concentration of 5.8 mg/ml at a scan rate of 60°C/h. The reversibility of all the scans, except that in the presence of 500 mM NaCl, was better than 95%. The excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

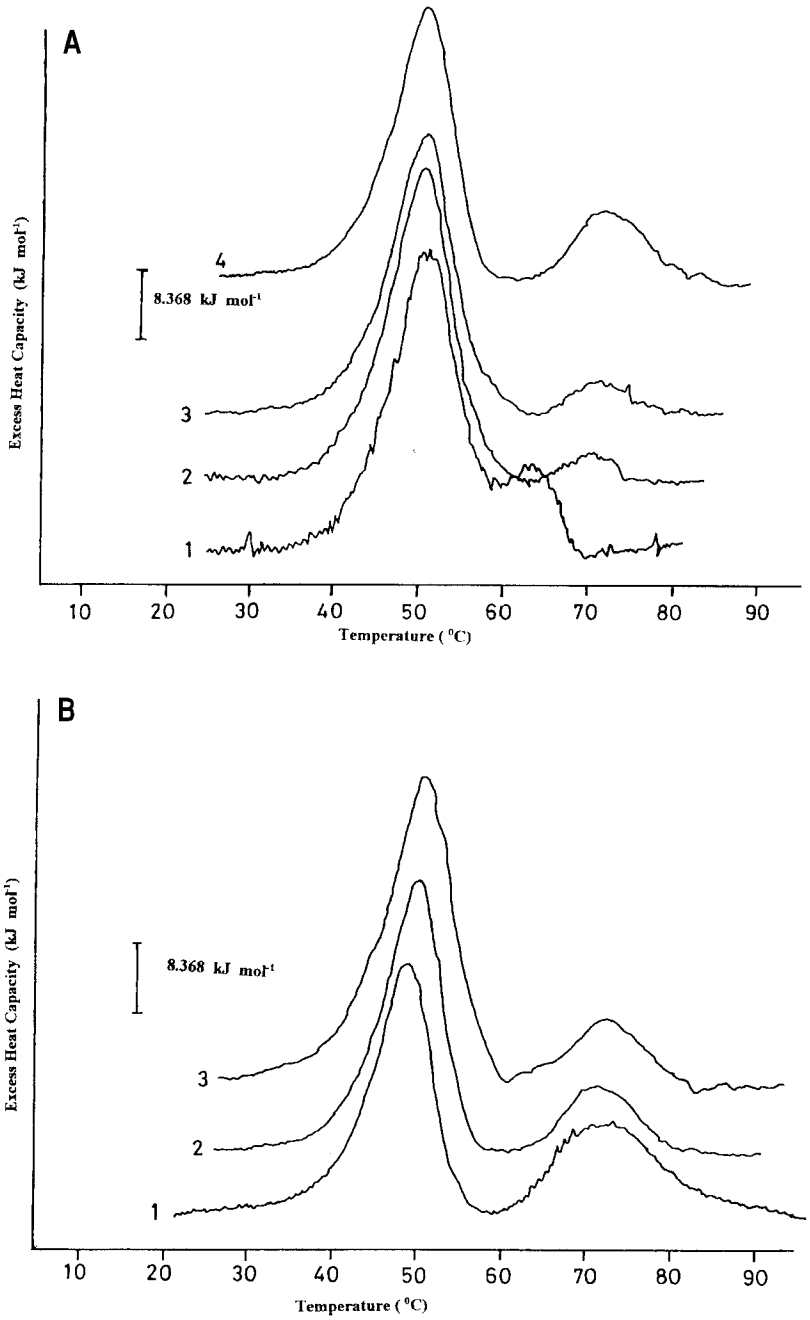

Fig. 5. Dependence of protein concentration and scan rate on the excess heat capacity versus temperature curves for hen egg-white lysozyme at pH 1.5 in the presence of 500 mM NaCl. (A) Different concentrations of hen egg-white lysozyme at pH 1.5 in the presence of 500 mM NaCl were scanned at a fixed scan rate of 60°C/h. The various concentrations of lysozyme shown are as follows: curve 1, 0.112 mM; curve 2, 0.24 mM; curve 3, 0.358 mM; curve 4, 0.479 mM.(B) Hen egg-white lysozyme at a concentration of 0.479 mM, pH 1.5, in the presence of 500 mM NaCl was scanned at different scan rates: curve 1, 30°C/h; curve 2, 60°C/h; curve 3, 90°C/h. Excess heat capacity function was obtained after subtracting the baseline from the heat capacity function.

Ionic-strength-dependent aggregation of the acid-induced partially folded intermediate. As mentioned above, the higher temperature transition observed in the DSC scan of lysozyme at pH 1.5 in the presence of 500 mM NaCl suggested the presence of an aggregated state of lysozyme solution on thermal unfolding. To confirm this, scan-rate- and protein-concentration-dependent studies under these conditions were carried out. The DSC scans of lysozyme at pH 1.5 in the presence of 500 mM salt at varying concentrations of protein are shown in Fig 5A. As seen in the figure, the area under the transition centered at about 71°C, increased with increasing protein concentration. Furthermore, at low protein concentration, a shift in the transition temperature was also observed. Similar changes were observed for studies of the scan rate dependence under these conditions (Fig. 5B). These observations suggest that acid-denatured lysozyme on thermal denaturation undergoes self association leading to aggregation in the presence of 500 mM NaCl.

The ionic-strength-dependent aggregation of the thermally denatured acid-induced partially folded intermediate of hen egg lysozyme was further confirmed by absorbance measurements and proton nuclear magnetic resonance spectroscopic studies. The aggregation monitored by absorbance measurements at 340 nm for hen egg lysozyme at pH 1.5 in the presence of increasing NaCl concentration at 50°C is shown in Fig. 6. As

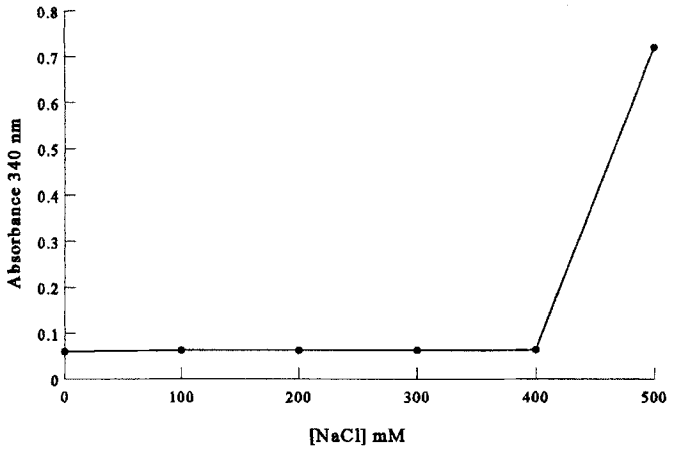

Fig. 6. Thermally denatured hen egg-white lysozyme at low pH shows an ionic-strength-dependent aggregation. For absorbance measurements, hen egg-white lysozyme at pH 1.5 in the presence and absence of varying sodium chloride concentrations was incubated at 50°C for 10 min. These protein samples were then transferred to a cuvette, and the absorbance at 340 nm was monitored in a double beam spectrophotometer equilibrated at 50°C. No absorbing species are present at this wavelength, and the light beam is therefore attenuated by scattering from the aggregated protein particles.

shown in this figure, no change in the absorbance of the protein solution was observed for acid-denatured lysozyme in the presence of NaCl up to 400 mM under these conditions. However, in the presence of 500 mM NaCl, a large increase in absorbance was observed. These observations suggest that acid-denatured lysozyme in the presence of 500 mM NaCl at about 50°C undergoes aggregation. The temperature-dependent aggregation of lysozyme in the presence of 500 mM NaCl at pH 1.5 was further confirmed by the thermal unfolding studies using NMR. The NMR spectra of hen egg lysozyme at low pH and in the presence of 100 mM and 500 mM NaCl at 25°C and 50°C are shown in Fig. 7. At 25°C, a very good dispersion of the aromatic and the amide protons was observed, both in the presence and the absence of NaCl suggesting the presence of a well structured protein. The addition of 100 mM or 500 mM NaCl showed no major changes in the spectra observed for acid-denatured lysozyme. However, several changes in chemical shifts were observed that can be attributed to local changes in the environment associated with the ionization of individual acidic residues in lysozyme. Similar observations have been reported earlier for lysozyme at pH 3.8 in the presence of 500 mM NaCl (Abe et al., 1995). Even at 50°C, lysozyme at pH 1.5 showed a good dispersion of aromatic and amide signals, which suggests that the protein does not undergo denaturation up to this temperature. In the presence of 100 mM NaCl, a spectrum corresponding to a denatured state as observed by the loss of several well defined signals was observed. However, in the presence of 500 mM NaCl at 50°C a significant broadening, loss of signals, and a very high noise-to-signal ratio were observed, which suggests the presence of an aggregated species under these conditions and supports the DSC data presented earlier.

DISCUSSION

Structural properties of the equilibrium partially folded intermediate of hen egg lysozyme and its similarity with the kinetic folding intermediate. The far-ultraviolet-CD spectrum of hen egg lysozyme at pH 1.5 was characterized by a significant negative ellipticity at 222 nm, although this was significantly lower compared to that observed for the native protein. Ellipticity at this wavelength reflects predominantly the α-helical structural content. Hence the above observations indicate that lowering the pH of hen egg lysozyme solution results in a partial loss of α-helical content of the native protein. Similarly, a significant loss in the ellipticity was observed at 270 nm for acid-denatured lysozyme, indicative of a large disruption of tertiary structure on acid denaturation at pH 1.5. These observations suggest that lowering the pH of hen egg lysozyme results in partial unfolding of the lysozyme structure, as a significant secondary and partial tertiary structure were observed for acid-denatured lysozyme.

Semisotnov et al. (1987, 1991) have shown that a specific tool for characterization of intermediate states in protein folding is the binding of 8-anilinonaphthalene-1-sulphonate. The binding of this probe to the equilibrium and kinetic intermediates is usually much stronger, resulting in a strong increase in fluorescence maxima, as compared to that in the native or fully unfolded states. The physical reason for this behavior has been attributed to the formation of solvent-accessible non-polar clusters in the intermediate state that remain screened from the solvent in the native state and which do not exist in the unfolded state. Fluorescence-probe-binding studies reported in this study showed that 8-anilino-1-naphthalenesulphonic acid does not bind to native lysozyme, as a very low fluorescence intensity was observed under these conditions. In contrast, a very strong binding of the probe, as indicated by an approximately 30 times increase in 8-anilino-1-naphthalenesulphonic acid fluorescence intensity for lysozyme at pH 1.5 was observed. These observations suggest that acid denaturation of hen egg lysozyme results in a significant solvent exposure of non-polar clusters that are shielded in the native lysozyme.

The above reported observations suggest the presence of a partially folded intermediate of hen egg lysozyme at low pH. This state is characterized by a significant secondary structure, a significant exposure of non-polar clusters, and a disrupted tertiary structure, which suggests that this state is more ordered than most other molten globules discussed in the literature. Furthermore, a large enthalpy is associated for its conversion to a fully unfolded state (i.e. thermally denatured state), as observed by the DSC studies. Similar properties have been reported for the equine lysozyme at low pH (Morozova et al., 1995), which suggests the structural similarity between the partially folded intermediate of hen egg and equine lysozyme at low pH.

Kinetic refolding of hen egg lysozyme has shown that the protein does not become organized in a single cooperative event but various parts become stabilized with very different kinetics. At least one, and possibly more, intermediate states with characteristics of a molten globule (notably ordered secondary structure without specific tertiary interactions as monitored by near- and far-ultraviolet CD and exposure of non-polar clusters) have been reported to be formed during the folding of hen egg lysozyme (Radford et al., 1992; Miranker et al., 1991). The equilibrium partially folded intermediate at low pH reported in this paper also has similar structural characteristics, which implies the possibility of structural similarity between the kinetic and the equilibrium intermediates.

Structural and thermodynamic characterization of salt-dependent compact conformation. Studies on the effect of ionic strength on the secondary and tertiary structure of hen egg lysozyme at low pH were carried out by far- and near-ultraviolet CD spectroscopy. An increase in ionic strength did not show any significant effect on the secondary structure of acid-denatured lysozyme as observed by the far-ultraviolet CD spectra, but a

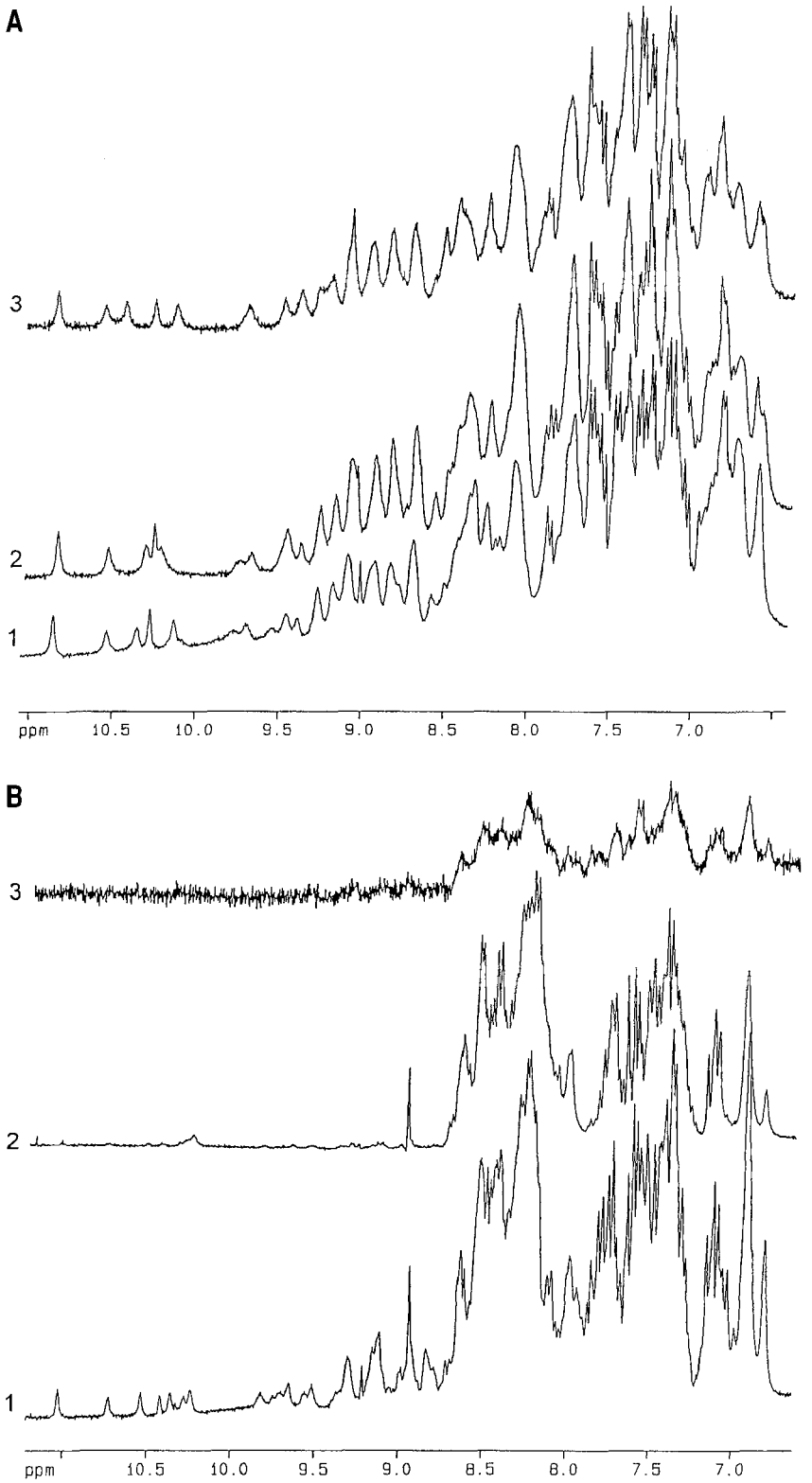

Fig. 7. Temperature- and ionic-strength-dependent aggregation of hen egg-white lysozyme at low pH. One-dimensional ^{1}H-NMR spectra of hen egg lysozyme in the amide and aromatic region at pH 1.5, and in presence of 100 mM and 500 mM NaCl at (A) 25 °C and (B) 50 °C. In both A and B, curve 1 is lysozyme at pH 1.5; curve 2 is lysozyme at pH 1.5 in the presence of 100 mM NaCl and curve 3 is lysozyme at pH 1.5 in the presence of 500 mM NaCl. Spectra were recorded on a Bruker Avance DRX 300 spectrometer operating at 300 MHz, using a digital resolution of 0.182 Hz/point. The concentration of protein used was 30 mg/ml in D_2O buffer at pH 1.5. All spectra are referenced to an internal dioxane standard at 3.743 ppm.

significant enhancement in the elipticity at 270 nm was observed, which suggests that the presence of NaCl affects the tertiary structure of acid-denatured lysozyme. The other significant effect of NaCl on the structural property of acid-denatured lysozyme that was observed was a substantial decrease in the solvent-accessible non-polar clusters. The solvent-accessible non-polar clusters observed in the presence of 100 mM NaCl were similar to those observed for native lysozyme, which sug-

gests that binding of chloride (Cl^-) ions to the acid-denatured lysozyme leads to a conformation in which the hydrophobic portion of the protein is buried inside, as is the case in native protein.

Regarding the thermodynamic properties, no significant effect on cooperativity or enthalpy of the heat-induced transition was observed in the presence of 100–500 mM NaCl, although a slight decrease in T_m, by about 3 °C, compared to the acid-denatured state was observed. These results suggest that an increase in ionic strength of hen egg lysozyme solution at pH 1.5 does not effect the secondary structure or the thermodynamic parameters of acid-denatured lysozyme, but affects the hydrophobic exposure and the tertiary structure significantly.

The main forces that are important for protein structures are the hydrophobic interactions, the valence forces, the dispersion forces, and the repulsive forces (Baldwin and Eisenberg, 1987). Under conditions of extreme pH, the main forces to unfold the protein are the repulsions between charged groups on the protein molecule. The stabilizing or destabilizing effects of salts on proteins will thus arise either by effects on hydrophobic interactions, or by interactions with charged groups. The net effect of salt on protein stability depends critically on the charge distribution on the native protein and on salt effects on its denatured state. The results presented in this paper suggest that increasing the ionic strength of the pH 1.5 buffer by addition of NaCl results in a transition of partially unfolded protein to a compact conformation as observed by a decrease in the 8-anilino-1-naphthalenesulphonic acid fluorescence and an increase of retention time in HPLC studies. Hen egg lysozyme has a net charge of about 6–7 at pH 4–7 (Parson and Raftery, 1972). Furthermore, it has also been suggested that for hen egg lysozyme, an average of nearly one Cl^- ion binds for each proton bound below pH 6 over a wide range of ionic strength (Parson and Raftery, 1972). Hence, it seems that addition of salt to the acid-unfolded state of hen egg lysozyme would decrease the electrostatic free energy of the acid denatured state due to the fact that in the presence of NaCl the positively charged sites of the acid-denatured protein would attract the counter-ion Cl^- from both NaCl and HCl, and consequently the repulsion is reduced, resulting in a decrease of electrostatic free energy that may result in an increased compactness of this conformation.

Salt-dependent conformational transitions at acidic pH from a largely unfolded state to an intermediate conformational state has been reported for a number of proteins (Goto and Fink, 1989; Ohgushi and Wada, 1983; Arakawa et al., 1987). These reports along with the results presented here suggest that salt-dependent conformational transition at acidic or alkaline pH regions may be a general property of many proteins, however the mid-salt concentration of the transition will depend on the protein studied and other conditions.

Ionic-strength-dependent aggregation of the thermally denatured acid-induced partially folded intermediate. Based on several *in vitro* studies, it has been concluded by some investigators that folding intermediates rather than native or denatured proteins are generally involved in aggregation. However, other investigators believe that intermediates, although possibly important for the aggregation of some proteins under certain solvent conditions, do not generally have to be invoked to explain protein aggregation. For proteins consisting of a single domain, there are only a few cases where intermediates have been shown to be involved in the aggregation of the protein (Cleveland and Wang, 1990; Havel et al., 1986). The observations presented in this paper suggest that an increase in the ionic strength of lysozyme at pH 1.5 does not result in any signs of aggregation. Furthermore, removal of NaCl from protein samples containing 500 mM NaCl at pH 1.5 showed ^{1}H-NMR spectra identical with those before NaCl addition. Temperature-dependent studies suggest that the thermally unfolded form undergoes self association leading to aggregation in a salt-dependent manner, favoring higher salt concentration, greater than 400 mM NaCl. These studies prove that the thermally denatured state of lysozyme is involved in the ionic-strength-dependent aggregation and supports a close relation between aggregation and conformational change of the protein with salt.

Taken together, the results presented in this paper demonstrate the presence of an equilibrium partially folded state of hen egg lysozyme at low pH. The presence of an equilibrium intermediate state in a protein supposed to be a prototype of cooperative two-state unfolding is of great significance as it indicates the general occurrence of folding intermediates in the pathways of protein folding.

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REFERENCES

Abe, Y., Ueda, T., Iwashita, H., Hashimoto, Y., Motoshima, H., Tanaka, Y. & Imoto, T. (1995) Effect of salt concentration on the pK_a of acidic residues in lysozyme, *J. Biochem. (Tokyo) 118*, 946–952.

Arakawa, T., Hsu, Y.-R. & Yphanits, D. A. (1987) Acid unfolding and self-association of recombinant *Escherichia coli* derived human interferon γ, *Biochemistry 26*, 5428–5432.

Arcus, V. L., Vuilleumier, S., Freund, S. M. V., Bycroft, M. & Fersht, A. R. (1995) A comparison of the pH, urea and temperature denatured states of barnase by heteronuclear NMR: implications for the initiation of protein folding, *J. Mol. Biol. 254*, 305–321.

Baldwin, R. L. & Eisenberg, D. (1987) Protein stability, in *Protein engineering* (Alan, R., ed.) pp. 127–148, Liss Inc.

Bhakuni, V., Xie, D. & Freire, E. (1991) Thermodynamic identification of stable folding intermediates in the B-subunit of cholera toxin, *Biochemistry 30*, 5055–5060.

Cleveland, J. C. & Wang, D. (1990) Refolding and aggregation of bovine carbonic anhydrase: Quasielastic scattering analysis, *Biochemistry 29*, 11072–11078.

Cooper, A., Eyles, S. J., Radford, S. E. & Dobson, C. M. (1992) Thermodynamic consequences of the removal of a disulfide bridge from hen egg lysozyme, *J. Mol. Biol. 225*, 939–943.

Creighton, T. E. (1993) Chemical properties of polypeptides, in *Proteins: structure and molecular properties* (Creighton, T. E., ed.) pp. 1–48, W. H. Freeman and Company, New York.

Dobson, C. M. (1991) Characterization of protein folding intermediates, *Curr. Opin. Struct. Biol. 1*, 22–27.

Dobson, C. M. (1994) Solid evidence for molten globule, *Curr. Biol. 4*, 636–640.

Dobson, C. M., Evans, P. A. & Radford, S. E. (1994) Understanding how protein folds: the lysozyme story so far, *Trends Biol. Sci. 19*, 31–37.

Fink, A. L., Calciano, L. J., Goto, Y., Kurotsu, T. & Palleros, D. R. (1994) Classification of acid denaturation of proteins: intermediates and unfolded states, *Biochemistry 33*, 12504–12511.

Goto, Y. & Fink, A. L. (1989) Conformational states of β-lactamase: molten globule states at acidic and alkaline pH with high salt, *Biochemistry 28*, 945–952.

Goto, Y., Calciano, L. J. & Fink, A. L. (1990) Acid induced folding of proteins, *Proc. Natl Acad. Sci. USA 87*, 573–577.

Goto, Y., Takahashi, N. L. J. & Fink, A. L. (1990) Mechanism of acid-induced folding of proteins, *Biochemistry 29*, 3480–3488.

Haezebrouck, P., Joniau, M., Van Dael, H., Hooke, S. D., Woodruff, N. D. & Dobson, C. D. (1995) An equilibrium partially folded state of human lysozyme at low pH, *J. Mol. Biol. 246*, 382–387.

Havel, H., Kaufman, E., Plaisted, S. & Brems, D. (1986) Reversible self-aggregation of bovine growth hormone during equilibrium unfolding, *Biochemistry 25*, 6533–6538.

Khechinashvili, N. N., Privalov, P. L. & Tiktopulo, E. I. (1973) Calorimetric investigation of lysozyme thermal denaturation, *FEBS Lett. 30*, 57–60.

Kim, P. S. & Baldwin, R. L. (1990) Intermediates in the folding reactions of small proteins, *Annu. Rev. Biochem. 59*, 631–660.

Miranker, A., Radford, S. E., Karplus, M. & Dobson, C. M. (1991) Demonstration by NMR of folding domains in lysozyme, *Nature 349*, 633–636.

Morozova, L. A., Haynie, D. T., Arico-Muendel, C., Van Dael, H. & Dobson, C. M. (1995) Structural basis of the stability of a lysozyme molten globule, *Nature Struct. Biol. 2*, 871–875.

Ohgushi, M. & Wada, A. (1983) Molten-globule state: a compact form of globular proteins with mobile side chains, *FEBS Lett. 164*, 21–24.

Parson, S. M. & Raftery, M. A. (1972) Ionization behavior of the catalytic carboxyls of lysozyme. Effects of ionic strength, *Biochemistry 11*, 1623–1629.

Ptitsyn, O. B. (1995) Molten globule and protein folding, *Adv. Protein Chem. 47*, 83–229.

Radford, S. E., Dobson, C. M. & Evans, P. A. (1992) The folding of hen lysozyme involves partially structured intermediates and multiple pathways, *Nature 258*, 302–307.

Redfield, C., Smith, R. A. G. & Dobson, C. M. (1994) Structural characterization of a highly-ordered molten globule at low pH, *Nature Struct. Biol. 1*, 23–29.

Semisotnov, G. V., Rodionova, N. A., Kutyshenko, V. P., Ebert, B., Blank, J. & Ptitsyn, O B. (1987) Sequential mechanism of refolding of carbonic anhydrase B, *FEBS Lett. 224*, 9–13.

Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F. & Gilmanshin, R. I. (1991) Study of the molten globule intermediate state by a hydrophobic fluorescent probe, *Biopolymers 31*, 119–128.

Shortle, D. (1996) The denatured state (the other half of the folding equation) and its role in protein stability, *FASEB J. 10*, 27–34.

Stryer, L. (1965) Interaction of naphthalene dye with apohemoglobin. A fluorescent probe of non-polar binding site, *J. Mol. Biol. 13*, 482–495.

Tan, Y.-J., Oliverberg, M., Davis, B. & Fersht, A. R. (1995) Perturbed pK_A-values in the denatured states of proteins, *J. Mol. Biol. 254*, 980–992.

Tanford, C. (1970) Protein denaturation (part c), *Adv. Protein Chem. 24*, 1–95.

Turner, D. C. & Brand, L. (1968) Quantitative estimation of protein binding site polarity. Fluorescence of *N*-arylaminonaphthalene sulfonate, *Biochemistry 7*, 3381–3390.

Uversky, V. N. (1993) Use of fast protein size-exclusion liquid chromatography to study the unfolding of proteins which denature through the molten globule, *Biochemistry 32*, 13288–13298.

Uversky, V. N. (1994) Gel-permeation chromatography as a unique instrument for quantitative and qualitative analysis of protein denaturation and unfolding, *Int. J. Biochromatogr. 1*, 103–114.