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Review Article

The Molten Globule State as a Clue for Understanding the Folding and Cooperativity of Globular-Protein Structure

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INTRODUCTION

For simple globular proteins, all the information necessary for the formation of the native three-dimensional structure is contained in the amino acid sequence.[1] As yet, however, the detailed mechanism by which this "programming" of the tertiary by the primary structure is achieved during the folding process is not fully understood, and the problem has been a major challenge in biophysical chemistry.

Because an immense number of possible conformations are accessible to a polypeptide chain of even moderate length, the random search mechanism of folding must be unlikely,[2–5] and most researchers agree on the presence of nonrandom pathways of folding (see also ref. 6). Major interests in protein folding studies are thus concerned with what types of intermediate conformations between the native and the fully unfolded states are present on the pathway of folding and how they are accessible to real experiments. However, there has been some controversy and confusion as to the nature of the intermediate species present, particularly concerning its stability at an early stage of the folding reaction. The classical two-state theory of the protein unfolding transition seemed to be well established[7,8] and seriously influenced the general trend in formulation of a model of protein folding. As a result, some researchers made the assumption that the incipient structures of folding must be very unstable, i.e., essentially unfolded on the average, but stable enough to restrict a conformational space available in a late stage of folding.[3–6] The assumption of instability of the intermediates was a logical solution of the requisite of the two-state theory of protein folding or unfolding. On the other hand, other researchers demonstrated that the two-state theory is no longer applicable to the kinetics of folding in native conditions and assumed that the protein folding must occur in a stepwise manner with well-populated structural intermediates.[9] Therefore, what was important at the time was to investigate the possible occurrence of stable intermediates between the native and the fully unfolded states, i.e., to find out exceptions to the two-state rule; the two-state rule itself does not tell us anything about the pathway of folding. In fact, more than two decades ago, Tanford and other researchers already reported that some proteins can assume stable intermediate conformations,[10,11] but these had been thought to be no more than exceptions to the two-state rule and had not been taken seriously until recently, when it was recognized that the intermediates might relate to a more general problem of possible physical states of protein molecules and hence to the problem of protein folding.

At present, the presence of the stable intermediates during the denaturant-induced equilibrium unfolding reactions, i.e., deviations from the two-state theory, has been well established in at least three different proteins, α-lactalbumin, carbonic anhydrase B, and bovine growth hormone.[12–23] Similar intermediate conformations have also been observed in a number of other globular proteins when the native structure is disrupted in a relatively mild way by changing pH or temperature in the absence of strong denaturants, by inorganic-salt denaturants, or by substitutions of amino acids or modifications of the primary structures (see the next section). The intermediate states of different proteins have some common characteristics, the compact globularity of the molecule with native-like secondary structure but unfolded tertiary structure, and the term "molten globule" was introduced to emphasize the possible occurrence of such an intermediate as a general physical state of globular proteins.[17–20,24] More importantly, the state identical to the

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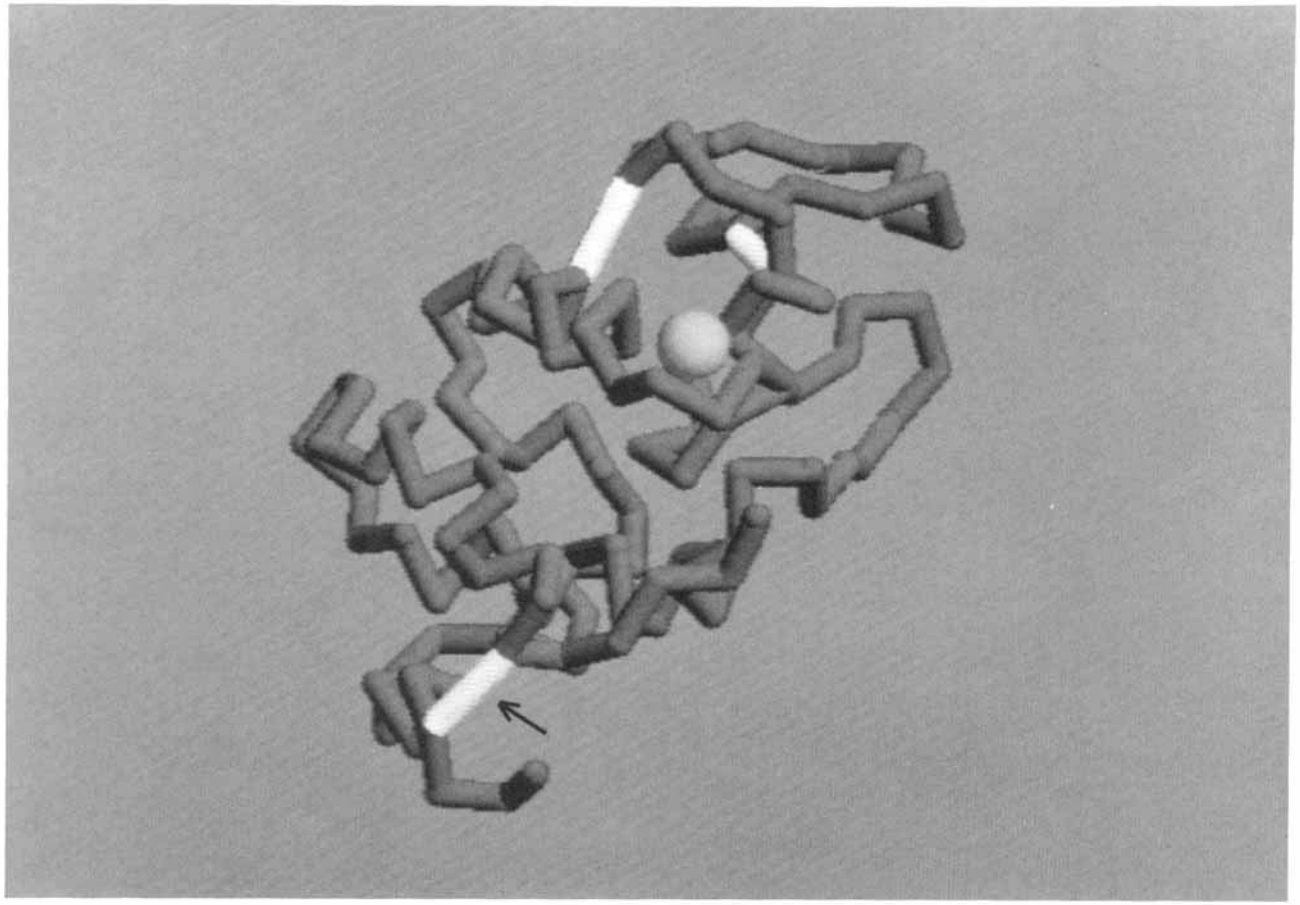


Fig. 1. The backbone structure of bovine α-lactalbumin viewed from the side of the Ca^{2+}-binding site. The structure is based on the model of Warme et al. in the T3 tail conformation,[37] and the bound Ca^{2+} shown by a sphere is placed at the appropriate position based on the X-ray result of Stuart et al.[38] The Cys-6–Cys-120 disulfide bond is indicated by a black arrow. The figure was made by means of a computer graphics program elaborated by Dr. Nakano of Himeji Institute of Technology.

molten globule has been shown to be accumulated at an early stage of kinetic refolding starting from the fully unfolded state, in α-lactalbumin and other proteins.[13–20] Although the deviations from the two-state theory of equilibrium unfolding in the above proteins are apparently exceptional, more important aspects are clearly involved in such exceptional behavior, and the concept of the molten globule state is now rather widely accepted by protein chemists. This review thus summarizes our current knowledge of the molten globule state and discusses its relationships to the hierarchical nature of the protein structure, the classical view of the cooperative two-state unfolding of a protein, and finally the mechanism of protein folding. In the third section, I also discuss the nature of the critical activated state of folding, which may exist between the molten globule intermediate and the native state, as the study of the activated state is required for a full description of the folding reaction.[16] However, because of its nature, this is not a comprehensive review of the protein folding problem and it is confined to small monomeric globular proteins that are known to have single-domain characteristics unless otherwise specified, mainly focusing on the author's own results and the related results by other researchers. More comprehensive and excellent reviews of protein folding, including multidomain proteins and subunit assembly, are currently available in the literature.[1,5,8–10,20,25–32] Recently, the possible biological significance of the molten globule state has been recognized by some researchers, especially in relation to the translocation of proteins across biomembranes.[33] However, this is also outside the scope of this review, and only physicochemical aspects of the protein structure and folding will be described.

CHARACTERISTICS OF THE MOLTEN GLOBULE STATE

α-Lactalbumin

To the present, the molten globule state has been best characterized in α-lactalbumin, so that it may

be helpful to describe in detail various conformational transitions undergone by this molecule. α-Lactalbumin is a small globular protein contained in mammalian milk whey and has a molecular weight of 14,200 (Fig. 1). Its amino acid sequence is known to be highly homologous to that of well-known hen lysozyme.[34,35] The structural similarity of the two proteins was previously inferred from model-building[36] and energy-refinement studies[37] and has recently been confirmed by the X-ray crystallographic analysis of baboon α-lactalbumin.[38,39]

α-Lactalbumin assumes partially unfolded states, which are characteristic of the molten globule, under a variety of conditions, e.g., at extreme pHs or at a moderate concentration of a denaturant such as guanidine hydrochloride (GdnHCl).[12–17] In spite of its structural similarity to α-lactalbumin, lysozyme does not show the state like the molten globule state at equilibrium under most conditions where α-lactalbumin is partially unfolded.[7,8,14] Careful comparison between the two proteins, in equilibrium unfolding and kinetic folding reactions, will thus provide a clue to the understanding of the physicochemical origin that brings about the presence of the molten globule state.[14,15]

α-Lactalbumin is also known to be a Ca^{2+}-binding protein that binds one Ca^{2+} per molecule with an apparent affinity constant as large as $10^6 \sim 10^9$ M^{-1}.[38–44] Removal of the bound Ca^{2+} destabilizes the native structure of this protein, so that the presence or absence of Ca^{2+} may influence the apparent behavior of the unfolding transitions.[14]

Acid and alkaline transitions

α-Lactalbumin undergoes the acid conformational transition in a pH range of 4.2–3 and the alkaline transition above pH 9.5.[44–47] The disruption of specific interactions involving ionizable groups, such as abnormal carboxyl and tyrosyl residues in the native molecule, caused by changing pH is known to be responsible for these transitions,[45] and both the transitions lead to essentially the same conformational state; this state is termed the A state in conformity with previous studies.[12] The conformational properties of the A state were first extensively studied by Kronman and his co-workers[44,46–52] and more recently by Ptitsyn's group[53–57] and also by us[58–69] by a variety of techniques. Their results and related studies by other groups are summarized as follows.

1. The molecule is not fully unfolded and has the native-like backbone secondary structure as indicated by the peptide CD, ORD, and IR spectra in the A state, while the NMR spectra and the aromatic difference absorption, fluorescence, and CD spectra indicate a denaturation-like alteration of the environment of side chains including tryptophan residues.[12,17,45–50,53,58–61]

2. Two to three of the four tryptophan residues are inaccessible to long-range perturbants such as sucrose and glycerol in solvent-perturbation experiments, so that there may be little or only a small change in exposure of tryptophans during the denaturation-like transition from the native (N) to the A state (25°C).[51] However, the ionization of abnormal tyrosyl residues buried in the interior of the molecule in the N state is normalized in the A state.[62]

3. The molecule is somewhat expanded but retains a compact shape with a hydrodynamic radius of 21 ~ 23 Å in the A state instead of 19 Å for native α-lactalbumin as indicated by ultracentrifuge measurements.[47] Compactness of the molecule in the A state has been demonstrated using electrophoretic and gel-chromatographic analyses.[58,70] The recent analyses of diffuse X-ray scattering and quasielastic light scattering and other data, mainly by Ptitsyn's group, also demonstrate that the effective linear dimensions for the N and A states of the protein do not differ by more than 10% (18 Å and 20 Å for the hydrodynamic radii in the N and A states, respectively),[53–55,63,71] suggesting that both forms are similar in compactness.

4. The molecule in the A state is susceptible to aggregation at acid pH.[46,47] Exposure of hydrophobic surface in the A state may be responsible for the self-association and aggregation. Hydrophobic probes made of naphthalene dyes are known to be bound by the protein more strongly in the A state than in the N state.[52] Interaction of the protein with phospholipid vesicles is known to increase in the acidic A state.[72,73]

5. The temperature dependence of the molecular properties in the A state shows diffuse thermal unfolding without any significant cooperativity. The CD spectral ellipticity in the peptide region decreases monotonically with increasing temperature above 20°C,[61] and there is no detectable heat absorption during the thermal transition of the A state as indicated by scanning calorimetry.[53,56,74] The cooperative network of long-range specific interactions may be absent or largely disrupted in the A state. However, the unfolding transition of the A state induced by treatment with GdnHCl shows some cooperativity.[12–15]

6. There are slow but extensive intramolecular structural fluctuations in the A state. The rotational relaxation time of tryptophan movement in the A state does not differ significantly from that in the N state (~20 nsec) as indicated by polarization of tryptophan fluorescence.[17,53] This fact, together with the absence of the aromatic CD signals, demonstrates the existence of slow intramolecular structural fluctuations in the A state in a time range longer than 10^2 nsec. It is suggested that these fluctuations may lead to the time-averaged symmetrization of environment of aromatic residues and hence the practical absence of their CD signals. The

TABLE I. Physical Properties of Different Forms of Bovine and Human α-Lactalbumins*

Form	Conditions	Sample	Native-like CD spectra		$[\eta]^{\dagger}$	$\tau_{Trp}^{\ddagger}$	Cooperative
			Aromatic	Peptide	(cm^3/g)	(nsec)	thermal transition
Native	pH~7, 20°C	Bovine	+	+	3.4	16	+
		Human	+	+	3.1	20	+
Acid	pH~2, 20°C	Bovine	−	+	3.1	19	−
		Human	−	+	4.2	33	−
Partially unfolded in GdnHCl	~2 M GdnHCl pH~7, 20°C	Human	−	+	—	22	−
Thermally unfolded	pH~7, 90°C	Bovine	−	+	3.0¶	—	
Partially unfolded by removal of Ca^{2+}	pH~7, 20°C 10 mM EDTA	Human	−	+	3.6	17	−
Fully unfolded in concentrated GdnHCl	~6 M GdnHCl pH~7, 20°C	Bovine	−	−	6.1	8**	
		Human	−	−	6.6	9	

*From ref. 17, by permission of the authors.
†Intrinsic viscosity.
‡Rotational relaxation times of tryptophans measured by the fluorescence polarization.
¶Measured at 50°C in the presence of 10 mM EDTA that shifts the transition temperature from 65 to 37°C.
**Measured in 8 M urea.

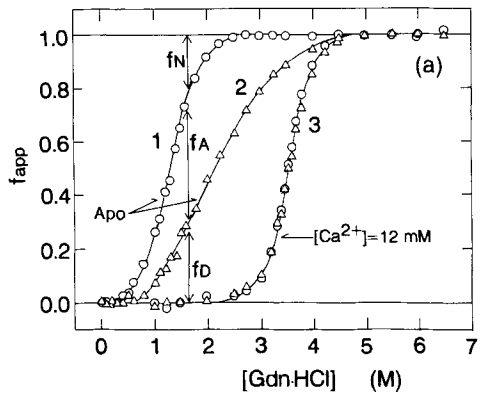
dynamic accessibility of the peptide NH-protons measured by the hydrogen-exchange kinetics is also known to be enhanced extensively in the A state.[53,69]

These characters of the A state of α-lactalbumin led Ptitsyn and his co-workers to introduce the concept of the "molten globule": that is, the compact globule with native-like secondary structure and with slowly fluctuating tertiary structure.[17–20] Some representative properties of the A state are shown in Table I and compared with the properties in the N state and in other unfolded states.

All the lines cited above as the characters of the A state reflect more or less overall or averaged properties of the molecule in the molten globule state, and, at first sight, they may give an impression that the molten globule might correspond to a nonspecific globule without any specific (supersecondary or tertiary) arrangement of the secondary structure segments. However, the question may be raised, to what extent the arrangement of the structured segments is nonspecific, because some segments of folded structure may be specifically organized if the segments are sufficiently localized within the molecule. This problem has recently been studied by Dobson and his co-workers using 500 MHz ^{1}H-NMR spectra of guinea pig α-lactalbumin.[75] Their careful analysis of the NMR spectra has given a clear indication that some resonances in the A state are markedly shifted both from the resonances in the N state and from those in the fully unfolded states, although overall features of the spectrum in the A state are consistent with global unfolding of the molecule. The perturbed residues that have been identified in the A state are all localized in the "hydrophobic box" region in the native structure, suggesting that hydrophoblic clustering of aromatic residues persists in the molten globule state. Furthermore, the hydrogen–deuterium exchange of peptide NH protons has demonstrated a localized arrangement of structured segments in the A state. After a definite time of exchange-out of nonprotected protons in the A state, the pH was jumped to a neutral pH to take the NMR spectrum. This technique allowed them to investigate the exchange behavior in the A state of individual NH protons assigned in the spectrum in the N state. Most of the NH protons within the α-helix encompassing residues 89 to 96 in the N state are shown to be highly protected in the A state and there are also other stretches of neighboring NH protons that are slowly exchanging. Considering the chemical exchange rate of the NH protons in the A state ($\tau_{1/2}$ = 3.6 minutes at pH 2.0 and 20°C) and the exchange time reported (6 ~ 10 hours), the degree of protection of these protons may be larger than 50 in the A state. As only 8 ~ 10 NH protons were found to be slowly exchanging, in spite of a helical content of 30 ~ 40% (36 ~ 48 NH protons) in the A state estimated by CD and IR spectra,[53,61] the secondary structure does not uniformly protect NH protons, but rather there are specific regions of particular stability, with other regions being more labile. These specific regions of higher stability will be termed the "kernel" within the structure of the molten globule. It is highly conceivable that such a kernel acts as a folding initiation site at an early stage of refolding of the protein (see below).

Three-state unfolding transition in GdnHCl

As seen in most globular proteins, the fully unfolded state (D state) of α-lactalbumin with cross-linked random-coil characters is brought about by concentrated GdnHCl;[12–14] α-lactalbumin has four disulfide cross-links. However, the apparent equilibrium unfolding behavior of this protein is remarkably different from other globular proteins, includ-



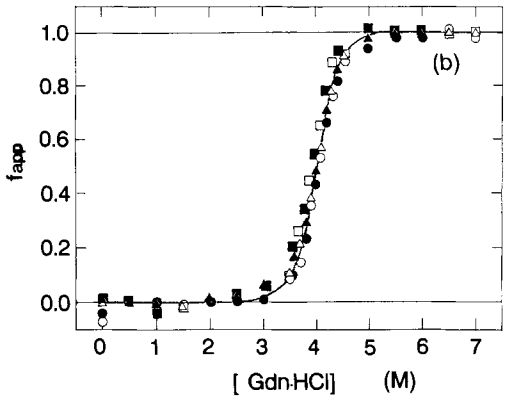


Fig. 2. GdnHCl-induced transition curves of α-lactalbumin (**a**) and lysozyme (**b**) (pH 7.0 and 25°C). The apparent fractional extent of unfolding, f_{app}, is plotted as a function of GdnHCl concentration. The solutions contained 50 mM NaCl and 50 mM sodium cacodylate. (a) Lines 1 and 2 refer to apo-α-lactalbumin in the absence of Ca^{2+}, line 3 refers to the holoprotein at 12 mM excess Ca^{2+}, the f_{app} values were calculated from the ellipticities at 270(○)and 222 nm (△), and f_N, f_A, and f_D denote the fractions of the N, A, and D states, respectively [Eq. (2)]. (**b**) The f_{app} values were from the ellipticities at 289 (□, ■), 255 (○, ●), and 222 nm (△, ▲), and open symbols refer to the data in the absence of Ca^{s+} and filled symbols to those at 12 mM $CaCl_2$. The figures were taken from ref. 14.

ing homologous lysozyme, for which the unfolding reactions conform rather closely to the classical two-state theory of the cooperative transition between the N and D states:[7,8,10,14]

$$N \rightleftharpoons D \qquad (1)$$

The unfolding reaction of α-lactalbumin when treated with GdnHCl shows noncoincident changes in the CD spectra at different wavelengths, unless the protein is stabilized by the presence of a large excess of Ca^{2+} (Fig. 2). The specific tertiary structure, measured by the aromatic CD signals, unfolds first and is followed by disruption of the secondary structure measured by the peptide CD band, indicating the presence of at least one stable equilibrium intermediate. On the basis of a three-state unfolding model, the thermodynamic parameters, the free energy changes of unfolding, and the transition-cooperativity indices for the transitions from the N to the intermediate and from the intermediate to the D state were evaluated and were compared with the corresponding parameters for the GdnHCl-induced unfolding transition of the A state at acid pH.[12-15] The results have demonstrated that the intermediate observed in the GdnHCl-induced unfolding at neutral pH is essentially identical to the A state. Ptitsyn and his co-workers have also studied the molecular properties of the intermediate of human α-lactalbumin at 2 M GdnHCl and neutral pH and compared them with the properties of the A state (Table I);[17,53] for the human protein, the two transitions observed in the GdnHCl-induced unfolding are well separated and most molecules (~90%) are in the intermediate state at 2 M GdnHCl.[59] In all the respects studied, the properties of the intermediate are identical to those in the A state. Therefore, the total unfolding equilibria of α-lactalbumin are represented by

$$N \rightleftharpoons A \rightleftharpoons D \qquad (2)$$

The molten globule state is stably populated at a moderate concentration of GdnHCl at neutral pH. The apparent contradiction in the unfolding behavior between α-lactalbumin and lysozyme has been accounted for in terms of a difference in stability of the N state between the two proteins. Whether or not the equilibrium intermediate occurs during the transition depends sensitively on the stability of the intermediate relative to the N and D states. The relative population of the intermediate at equilibrium may be below a detectable level in lysozyme because of a higher stability of the N state, i.e., a relatively low stability of the intermediate. This proposal has been proven by kinetic CD measurements that detect the intermediate of lysozyme as a transient species during the refolding from the D state, and the stability of the transient intermediate has been investigated as a function of GdnHCl concentration (see below).[15,61] In Figure 3, the stabilization free energies, ΔG, of the N and the intermediate states against the D state are plotted as a function of GdnHCl concentration. For α-lactalbumin, the three states, N, A, and D, have comparable stabilities in the transition zone around 1.5 M GdnHCl.[14,15] The stability of the intermediate in lysozyme, if comparable to or slightly less than that of the A state in α-lactalbumin, is lower than the stability of the N state, and suppresses the equilibrium population of the intermediate species. Furthermore, stabilization of native α-lactalbumin brought about by the presence of sufficient excess Ca^{2+} (≧1 mM) is known to produce an apparent

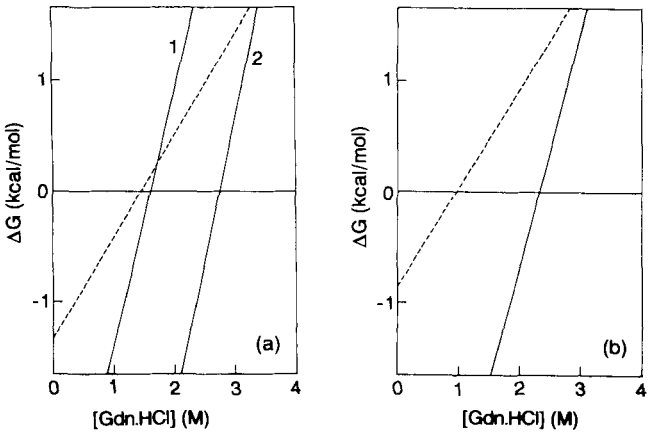


Fig. 3. The stabilization free energy, ΔG, as a function of GdnHCl concentration for the N state (solid line) and for the intermediate (A) state (broken line) of α-lactalbumin (pH 7.0 and 4.5°C) (**a**) and lysozyme (pH 1.5 and 4.5°C) (**b**). The figures were drawn based on the data reported in ref. 15. (**a**) Line 1 refers to the N state of apo-α-lactalbumin and line 2 to that of the holoprotein in the presence of 1 mM $CaCl_2$. (**b**) The broken line shows the stability of the transient folding intermediate of lysozyme, which can be detected by kinetic CD measurements.

two-state transition between N and D (Fig. 2a and line 2 in Fig. 3a); Ca^{2+} does not influence the stability of the A state under the condition.[14] Therefore, the three-state model of protein unfolding [Eq. (2)] is not necessarily an exception to the general case of the protein unfolding transitions induced by GdnHCl, but rather the classical two-state theory is a simplified version of the three-state mechanism.

Other denaturation-like transitions bring about the molten globule state

Conformational states similar to the A state have been observed in other denaturation-like transitions of α-lactalbumin. Complete removal of the bound Ca^{2+} from the protein leads to a partially unfolded state at a very low ionic strength above room temperature, while the protein assumes the native structure at a sufficiently high ionic strength.[41,64,65] Conformational properties of partially unfolded apo-α-lactalbumin have been shown to be identical to those in the A state (Table I). Denaturation by inorganic salts, $NaClO_4$ and $LiClO_4$, and also by heat results in partially unfolded species similar to the A state.[61,65–67] The backbone structure in the heat-denatured protein at a high temperature (70 ~ 80°C) is, however, significantly less ordered than the structure in the A state at room temperature.[61] Some researchers have used the compactness of the molecule in the heat-denatured state to classify this state also as the molten globule state (Table I),[53–55] while another researcher has reported a molecular size in the heat-denatured state rather close to the size in the D state.[70] Full reductive cleavage of the four disulfide bonds in α-lactalbumin also leads to a partially unfolded species.[13,68] Conformational properties of the reduced protein and its carboxymethylated derivative have been studied at pH 2.0.[68] Although the stability of the ordered backbone structure is decreased in the reduced protein, it can assume the structure similar to that in the A state. It is demonstrated that the disulfide crosslinks are not essential for maintenance of the secondary structure in the A state. However, unlike the A state of the disulfide-intact protein, the structure in the reduced protein strongly depends on ionic strength and extensively unfolds without salt, probably owing to electrostatic repulsion between charged groups, and also the folded form of the reduced protein has a stronger tendency to self-aggregation than the A state of the intact protein.[68]

Other Proteins

A number of globular proteins undergo acid and alkaline transitions that produce partially unfolded states reminiscent of the A state of α-lactalbumin.[10,11,25,76–84,153] Best characterized examples include the acid state of bovine and human carbonic anhydrase B[85–87] and the acid and alkaline states of β-lactamase from *Bacillus cereus*.[88] The intermediate conformational states of these proteins may correspond to the molten globule state. The compact intermediate conformation of the β-lactamase is, however, stable only at high ionic strength at acid or alkaline pH, and the protein is close to fully unfolded at extreme pHs when the ionic strength is too low. This behavior is analogous to the salt-induced formation of the secondary structure in disulfide-reduced α-lactalbumin. A salt-dependent conformational transition at acid pH was also reported for cytochrome *c* and at that time some researchers concluded that the high-salt form of ferricytochrome *c* at acid pH was the molten globule,[24] although another group did not confirm this conclusion.[20,53] At least several globular proteins,

including carbonic anhydrase B and bovine growth hormone mentioned above, exhibit a stable equilibrium intermediate during the unfolding transition induced by GdnHCl or urea,[21–23,77,89–94] and the unfolding behavior of these proteins may be analogous to the three-state unfolding of α-lactalbumin. The equilibrium intermediate could be detected by noncoincident transitions monitored by different structural probes such as the peptide and aromatic CD bands or by a biphasic transition curve with a single structural probe, depending on the apparent extent of unfolding of the intermediate expressed by the structural probe employed. The equilibrium intermediates of carbonic anhydrase B and bovine growth hormone during the unfolding by GdnHCl or urea are characterized by preferential loss of the tertiary structure with the stable secondary structure and have many features characteristic of the molten globule state,[18,21–23,93,94] so that the intermediates are similar to the acid states of these proteins. However, the intermediate of β-lactamase from *Staphylococcus aureus* observed at a moderate concentration of urea or GdnHCl has been shown to be largely expanded with a hydrodynamic volume rather close to the volume in the fully unfolded state and hence may not be regarded as the molten globule.[78,89,95] The compact intermediate state of this protein was observed as a transient intermediate in refolding at very low urea concentrations or as conformations of folding-defective mutant proteins, when monitored in urea-gradient gel electrophoresis (see below).[95,96] The GdnHCl- and urea-induced unfolding transitions of tryptophan synthase α subunit show biphasic transition curves when monitored by the peptide CD and the tryptophan absorption bands, and the results have been interpreted in terms of the stepwise unfolding of two structural domains rather than accumulation of the molten globule state;[77,92] the X-ray structure of this molecule recently reported does not show distinct domains, however.[97] The mutations in the amino acid sequence of a protein occasionally lead to a species that is devoid of the ability to form the specific tertiary structure. The Thr-40 to Ile and Asp-146 to Asn mutants of staphylococcal β-lactamase[96] and a large fragment of staphylococcal nuclease composed of residues 1–128[98] are such examples, and the mutant proteins are in the compact intermediate states similar to the molten globule under conditions where the wild-type enzymes are fully folded. In the nuclease fragment, single amino acid substitutions have been shown to alter profoundly the stability of the compact intermediate structure.[98] Mutations in a helix pairing site in apomyoglobin are also known to destabilize the native state and to enhance our ability to detect the equilibrium intermediates.[99] In some cases, a conformational state similar to the molten globule is produced by cleavage of disulfide bonds in disulfide-containing proteins or by removal of the protein-bound cofactors such as a prosthetic group in a conjugated protein and a metal ion in a metalloprotein. Examples of this sort include reduced bovine growth hormone[100] and pancreatic trypsin inhibitor,[101] apomyoglobin,[99,102] and apoparvalbumin.[84] Inorganic-salt denaturants also produce partially unfolded states in proteins. A well-studied example is the $LiClO_4$-induced unfolded state of ribonuclease A.[103,104] It has also been shown, however, that some secondary structure or the whole molecule of the $LiClO_4$-induced state is not fully stable under refolding conditions without $LiClO_4$ (see also ref. 15).

Observation of the similar intermediate conformational states in widely diverse proteins under a variety of conditions clearly demonstrates that the molten globule state is not exceptional but rather has a general significance as a conformational state of globular proteins.

COOPERATIVITY AND HIERARCHY

The close-packed nature of a protein structure stabilized by a cooperative network of various types of interactions might at first give the impression that alterations of a part of the structure may be propagated to all other parts of the molecule, so that the folding or unfolding transition of the protein may occur between the two states, the native and the essentially unfolded states, without any significant population of intermediate conformational states. However, this primitive picture of the protein unfolding transition must be discarded, once more careful examinations have been made of protein structures, which clearly demonstrate that folding must be considered as a hierarchy of various levels of substructure. On the basis of the anatomy of known X-ray structures of globular proteins, various algorithms of hierarchical organization of the protein structure have been presented,[5,26–30,105–109] and the possible compatibility of such an algorithm with the real pathway of protein folding has been demonstrated. Thus, the three-state unfolding transitions observed in α-lactalbumin and several other proteins and also the presence of the molten globule state in a variety of globular proteins may have some relationship with the hierarchical nature of the protein structure. In fact, there is an implication that the secondary structure segments form first and these structured segments assemble to form the specific tertiary structure during the hierarchical organization of the protein structure,[107–109] suggesting that the molten globule may correspond to a folding intermediate in the organization process. On the other hand, it has also been argued that the presumed secondary structure segment at an intermediate stage of the hierarchical organization must be essentially unfolded in reality because of the expectation that a short helical segment itself is very unstable without the tertiary interactions as pre-

dicted by the classical Zimm–Bragg theory of coil-to-helix transition of various synthetic polypeptides.[110] The value of this counterargument has, however, been diminished by recent experimental findings that a number of short peptide fragments isolated from globular proteins or designed de novo have remarkable preferences to form specific secondary structures in aqueous solution in the same regions as found in the native structures or the expected regions in the designed peptides.[111–121] The secondary structure segments are only marginally stable but they must be stabilized further within the compact structure of the molten globule state. Thus, in the following, whether the hypothesis of the hierarchical organization of a protein with such a stable intermediate (the molten globule state) is compatible with known facts of thermodynamics of protein unfolding will be discussed.

It is a well-known hypothesis that the equilibrium unfolding transitions of small globular proteins are represented, to first approximation, as a cooperative two-state reaction. This classical view of the two-state unfolding has recently been substantiated by the calorimetric studies of protein unfolding made by Privalov and his co-workers,[7,8] and they have demonstrated that the heat-, pH-, and GdnHCl-induced unfolded states of a protein are thermodynamically identical to each other. Identity of ΔH_{cal} determined from the calorimetric measurement and ΔH_{vH} from van't Hoff plots obtained from the thermally induced transition curve has been thought to validate the two-state hypothesis of protein unfolding. Nevertheless, the molten globule intermediate is present in the unfolding reactions of a number of globular proteins. For a long time, it has also been known that the thermally unfolded state of a protein is not fully unfolded and that the residual structure in the thermally unfolded state is further unfolded by treatment with GdnHCl.[10,25] Therefore, the question arises: Does the population of such intermediate structural states conflict with the calorimetric criterion of the two-state unfolding? This problem was first studied by Pfeil[122] who examined the thermal transition of α-lactalbumin at various concentrations of GdnHCl, where the A state is sufficiently populated, by scanning calorimetric measurements. In spite of the well-established three-state unfolding of this molecule, the calorimetric results have shown that the unfolding transition of the protein in GdnHCl does not show, surprisingly, any deviation from the two-state behavior, i.e., $\Delta H_{cal}/\Delta H_{vH}$ is close to unity. This dilemma may be solved by more careful examination of the temperature dependence of the three-state unfolding. The temperature dependence of the unfolding transitions of α-lactalbumin in GdnHCl was studied by CD spectra,[13] and the apparent ΔH for the A $\rightleftharpoons$ D transition [Eq. (2)] is close to zero at 2 ~ 3 M GdnHCl where the transition takes place. The nearly zero ΔH of the A $\rightleftharpoons$ D transition in the presence of GdnHCl has been confirmed by direct isothermal calorimetric titration of human α-lactalbumin with GdnHCl.[56] The cooperative transition detected in the calorimetric experiment coincides with the transition from the N to the A state and, after the production of the A state, everything observed calorimetrically is completed. Therefore, the A and D states are calorimetrically indistinguishable, although the two states are very different structurally. As a result, the two-state behavior of protein unfolding demonstrated by the calorimetric studies does not exclude the presence of the molten globule state. Furthermore, according to scanning calorimetric investigations in the absence of GdnHCl, the thermal transition apparently disappears in the A state of α-lactalbumin at acid pH,[53,56] and this is consistent with the diffuse thermal transition of the secondary structure in the molten globule state measured by the CD and IR spectra.[61,86] Thus, the unfolding of the molten globule is accompanied by only a small, if not zero, enthalpy change even in the absence of the denaturant. The molten globule state at a high temperature still has a residual structure,[61] which may be akin to the residual structure found in the thermally unfolded state of the native protein. Accordingly, the thermal transitions of native globular proteins are, in most cases, well approximated as a two-state transition not only calorimetrically but also when measured by various spectral probes, while the GdnHCl- or urea-induced unfolding transitions of proteins occasionally show a hierarchical three-state transition, depending on the relative stability of the molten globule relative to the native and the fully unfolded states. In some cases, there are further implications that even the thermal unfolding might be represented by a number of successive but overlapping stages.[123,124]

There are some indications suggesting the presence of cooperativity in the transition from the molten globule to the fully unfolded state. The GdnHCl-induced unfolding of the molten globule state often shows a sigmoidal transition curve.[12,15] The transitions followed by different structural probes such as peptide CD and aromatic absorption bands and the effective hydrodynamic radius by gel filtration are shown to be approximately coincident with each other,[15,59,98] suggesting a cooperative disruption of the structure. The compact to coil transition of amphiphilic copolymers in aqueous solution is known to occur more or less cooperatively,[125,126] and the compact globularity of the molten globule might be a source of the cooperativity of its transition to the fully unfolded state (see also ref. 5). The noncooperative feature of the thermal transition of the molten globule may be interpreted in terms of a small ΔH of the unfolding transition, but a smooth transition from the molten globule to the unfolded state by gradual expansion of a molecule and gradual destruction of the secondary structure has also been

suggested.[20] In any event, the degree of cooperativity of the transition of the molten globule is much smaller than that of the unfolding transition of the native molecule. The cooperativity index, given by the differential free energy difference of unfolding with respect to GdnHCl concentration, of the A $\rightleftharpoons$ D transition of α- lactalbumin is only 30% of the cooperativity of the transition from the N to the D state.[12,14] The noncooperative thermal transition and the cooperative unfolding by denaturants of the molten globule suggest that the compact globularity of the molecule and the enhanced stability of the secondary structure segments in this state may be brought about by nonspecific hydrophobic interactions. The strength of the hydrophobic interaction increases with increasing temperature[127] while secondary structure segments tend to melt at a high temperature. According to surface–area calculations of known X-ray structures of globular proteins, the formation of secondary structure stabilized by hydrogen bonding buries large amounts of polar surface, mostly from peptide groups, but the assembly of the secondary structure into native tertiary structure buries almost exclusively the hydrophobic surface.[29] These facts, together with the hierarchical organization of the protein structure described previously, suggest that the molten globule may be characterized primarily as a nonspecific assembly of the secondary structure segments. A late stage of the hierarchical organization from the molten globule may thereby be associated with the long-range specific interactions including specific electrostatic interactions, tertiary hydrogen bonding, and specific hydrophobic interactions, all of which come into play in a concerted manner to bring about the specific arrangement of the structured segments and the extensive burial of the hydrophobic surface. This picture of protein folding will be refined by the experimental studies of kinetic refolding in the following.

PROTEIN FOLDING

The Molten Globule as a Transient Folding Intermediate

In the preceding section, the molten globule state was assumed to be equivalent to an early intermediate of protein folding, rather naively, because the state is an equilibrium unfolding intermediate in some proteins and is expected to be related to the hierarchical nature of the protein self-organization. Support for this proposal was, however, previously obtained for α-lactalbumin from the thermodynamics and kinetics of the three-state unfolding and was based on the observations[13] that (1) the A state is more stable than the D state in the native conditions (Fig. 3) and (2) the D to A process is much faster than the A to N process in the unfolding transition zone, so that if this relation holds, the A state accumulates first in refolding in the native conditions. More conclusive evidence for the accumulation of the A state as a transient folding intermediate is provided by direct measurements of the time-dependent changes in the peptide and aromatic CD spectra during the kinetic refolding from the D state induced by a concentration jump of GdnHCl.[61] The results for α-lactalbumin are shown in Figure 4a. Similar refolding studies were also made for lysozyme and the results are shown in Figure 4b and c. For both proteins, almost full changes in ellipticity, expected from the equilibrium differences in the spectra between the N and D states, are seen kinetically in the aromatic region. In the peptide region, however, most of the ellipticity changes of the two proteins occurs within the dead time of the measurement. The results clearly demonstrate the accumulation of an early transient intermediate that is still unfolded when measured by aromatic CD bands but has folded secondary structure as measured by peptide CD bands. The CD spectra of the transient intermediates of the two proteins were obtained by measuring the refolding kinetics at various wavelengths and the stabilities of the intermediates against GdnHCl were obtained by measuring the kinetics at various concentrations of GdnHCl.[15,61] The spectrum and the stability of the intermediate of α-lactalbumin have been shown to be identical to those of the A state observed at equilibrium, and, more importantly, essentially the same intermediate is also accumulated in lysozyme at the first stage of refolding. The stability curve of the lysozyme intermediate, shown by a broken line in Figure 3b, substantiates the previous conclusion about its equilibrium unfolding behavior, i.e., the intermediate (A) state also exists in this protein but is hidden by a higher stability of the N state.

Accordingly, the folding reactions of these proteins can be divided into at least two stages:

$$\mathrm{D} \rightarrow \mathrm{A} \rightarrow \mathrm{N} \qquad (3)$$

The first stage corresponds to rapid formation of the molten globule (D→A), and the second stage is the organization of the specific tertiary structure (A→N). The first stage is known to be complete within a millisecond time range for α-lactalbumin and probably also for lysozyme,[15,57] and the second stage involves the rate-determining step of the folding reaction.

Such a model of protein folding (the *framework model*[9]) is not peculiar to α-lactalbumin or lysozyme. Other proteins, including parvalbumin, ferricytochrome *c*, β-lactoglobulin, ribonuclease A, carbonic anhydrase, staphylococcal β-lactamase, tryptophan synthase α-subunit, and bovine growth hormone, are now known to exhibit similar transient intermediates before organization of the specific tertiary structure.[18,19,87,93,95,103,128–136] Because these proteins are not related to each other,

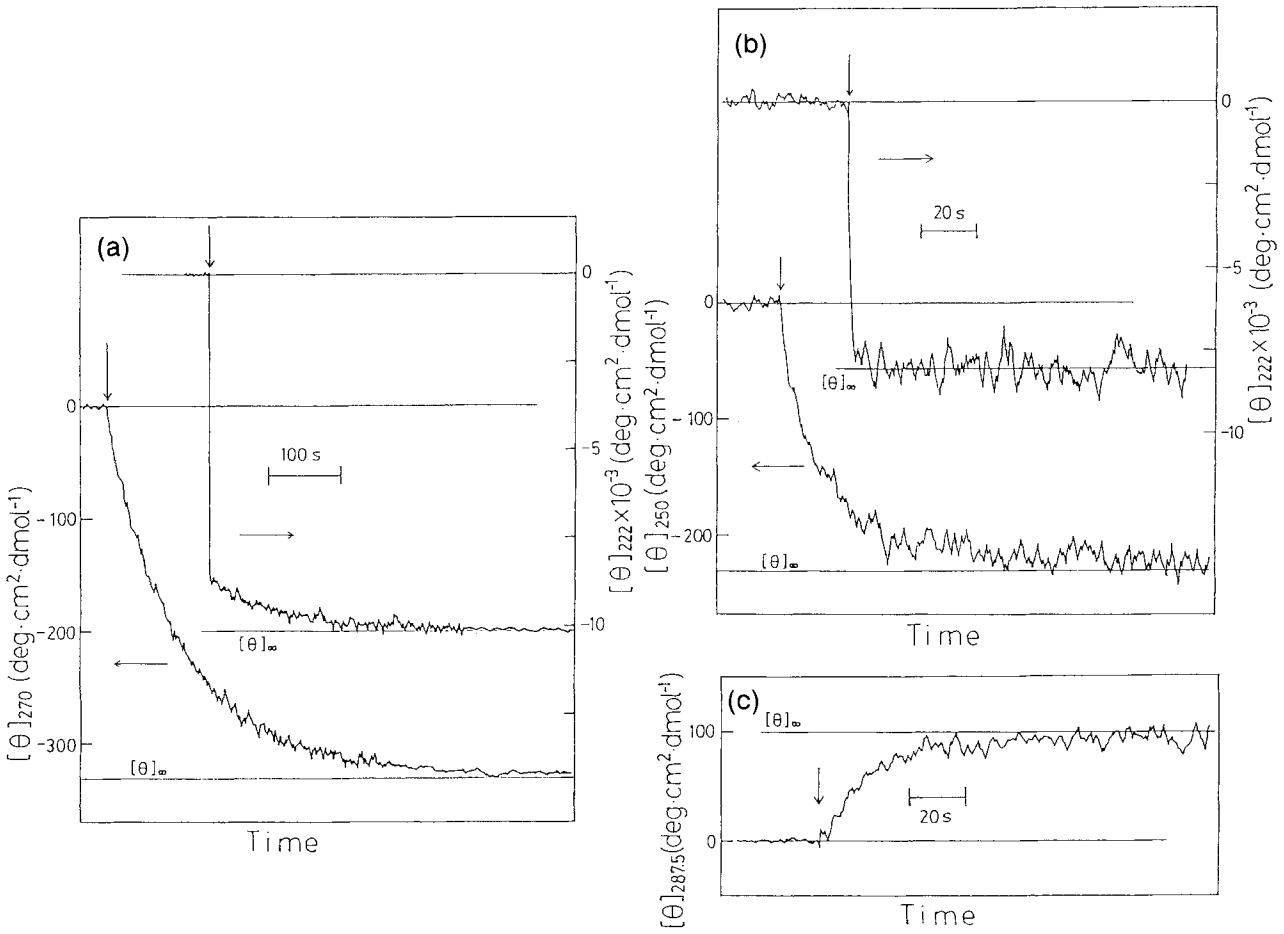


Fig. 4. Kinetic progress curves of refolding measured by CD spectra at different wavelengths at 4.5°C. The refolding was initiated by a concentration jump of GdnHCl from 6.0 to 0.3 M. The figures were taken from ref. 61. (**a**) Apo-α-lactalbumin at 270 and 222 nm (pH 7.0). (**b**) Lysozyme at 250 and 222 nm (pH 1.58). (**c**) Lysozyme at 287.5 nm (pH 1.58). Vertical arrows indicate the zero time at which the refolding was initiated. $[\theta]_\infty$ denotes the ellipticity at equilibrium.

the presence of transient intermediates similar to the molten globule may be common in globular protein folding. The kinetic CD measurements such as stopped-flow CD[84,128,130] and the hydrogen-exchange measurements of peptide NH protons[134] have been used for characterizing the folded backbone structures in the transient intermediates. The urea-gradient gel electrophoresis is also effective and has been used to demonstrate the compact nature of the transient intermediate in a number of globular proteins.[135] It is also known, however, that how much the secondary structure is restored in the first stage of folding varies with the protein species. An empirical measure (α) of the restoration of the secondary structure is given by

$$\alpha = \frac{[\theta]-[\theta]_D}{[\theta]_N-[\theta]_D} \qquad (4)$$

where $[\theta]_D$ and $[\theta]_N$ are the ellipticities of the fully unfolded and the native states, respectively, in the peptide region, and $[\theta]$ is the ellipticity restored in the first stage of folding, so that $[\theta]$ may correspond to $[\theta]_A$ when the intermediate is sufficiently stable in refolding conditions [Eq. (3)]. The α value for parvalbumin is only ~60% as determined by stopped-flow CD measurements between 213 and 240 nm,[84] suggesting that some secondary structure is organized in the late stage of folding or otherwise that the transient intermediate of this protein is not fully stable in refolding conditions, while α is ~200% for β-lactoglobulin,[128] apparently indicating that some structure formed in the transient intermediate must be disorganized in the late stage of folding.

A major question as to the role of the transient intermediate in protein folding is concerned with the correlation between the secondary structure formed in the intermediate and the structure stabilized in the native molecule, i.e., do any parts of the secondary structure segments in the intermediate persist during the late stage of folding? Recently, ^{1}H-NMR measurements with a hydrogen-exchange

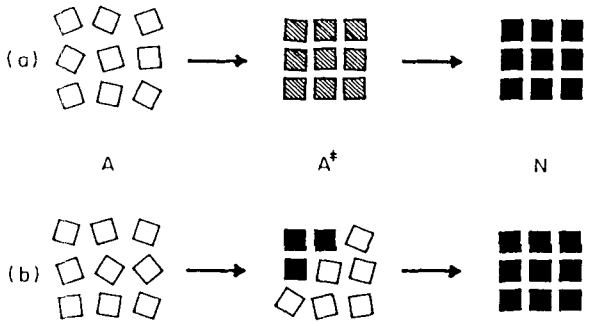


Fig. 5. Two alternative models for describing the activation process of protein folding: (**a**) critical-distortion model; (**b**) critical-substructure model. The mobile secondary structure segments in the molten globule state are shown by open squares. The fixed structural segments in the native state are shown by filled squares. The hatched squares in (**a**) show the distorted structure in the activated state. The assembly of the filled squares in (**b**) indicates the critical substructure. The figure was taken from ref. 16.

labeling technique have been utilized effectively for investigating this problem in folding reactions of ribonuclease A by Udgaonker and Baldwin[137] and cytochrome *c* by Roder and his co-workers.[138] The protons stabilized by the secondary structure in the transient intermediate were labeled specifically by hydrogen-exchange pulse labeling at an early stage of refolding from the fully unfolded state, and then the NMR spectra of the labeled protons were observed in the native state after completion of refolding. At least several peptide NH protons that are located in the β-sheet of native ribonuclease A are extensively stabilized, with more than 10^4-fold retardation of the exchange rates at an early stage of folding (500 msec) but before organization of the tertiary structure (pH 9 and 5°C). The N- and C-terminal α-helices of ferricytochrome *c* are remarkably stabilized, with more than 50-fold protection against hydrogen exchange (pH 9.3 and 10°C), at an early stage (10 msec) of refolding with the other helices and the tertiary hydrogen bonds being still labile. The N- and C-terminal helices are in close contact with each other in the native cytochrome *c* structure, so that a similar tertiary arrangement of the helices probably exists and contributes to stabilization of the helices in the folding intermediate. These results for ribonuclease A and cytochrome *c* provide clear indications that some secondary structure segments in transient intermediates provide the framework of the folding reaction and persist during the late stage of folding.

Critical Activated State in the Late Stage of Folding

The rate-determining step is located at a late stage of folding after the compact molten globule has been formed in refolding conditions. Thus, the next problem to be solved involves the question of how the preformed secondary structure segments in the transient intermediate are organized in the late stages of folding so as to lead finally to the specific tertiary structure of the native molecule. This question has recently been addressed by Kuwajima and his co-workers who have analyzed the folding and unfolding kinetics of α-lactalbumin as a function of concentrations of GdnHCl and free Ca^{2+}.[16] Two alternative models for describing the critical structure in the activated state of folding presented in their paper are shown in Figure 5; one is the *critical-distortion model,* and the other is the *critical-substructure model.* In both models, the state on the unfolded side is the molten globule intermediate, and the preformed secondary structure segments in this state are shown by open squares. The secondary structure segments in the molten globule are highly mobile and not correctly packed against each other, so that a large solvent-accessible surface area is still available in this state. The equilibrium transition between the molten globule and the native state occurs cooperatively and is accompanied by a large ΔH and a large heat capacity change, ΔC_p,[13,53,56] indicating there is extensive exposure of hydrophobic surface in the molten globule state. The organization shown in Figure 5 thereby corresponds to fixing of the mobile secondary structure segments to lead finally to the fully organized native molecule, the rigid structural segments of which are shown by filled squares.

In the critical-distortion model, the structure in the critical activated state is a high-energy distorted form of the native structure and the activated state in folding and unfolding is very close to the native conformation. Previously, equivalent models were postulated by a number of researchers primarily on the basis of the observation that most of the ΔC_p of folding is ascribable to the activation heat capacity change of folding, $\Delta C_p^{\ddagger}$, from the unfolded to the activated state.[13,139–141] This observation suggests, apparently, that most of the hydrophobic surface area may be buried in the interior of the protein in the activated state. It was shown later, however, that the partial reduction in the accessible surface area in the activated state is clearly indicated by the folding and unfolding kinetics of α-lactalbumin and other globular proteins (see below), and hence a part of $\Delta C_p^{\ddagger}$ must be ascribed to a phenomenon other than hydrophobic interactions. A change in $\Delta C_p^{\ddagger}$ is expected to result from a change in the activated-state structure with temperature, so that $\Delta C_p^{\ddagger}$ cannot be used to evaluate a change in the hydrophobic surface area.[16] On the other hand, the critical-substructure model describes the folding reaction as a growing process in which the correctly organized assembly of the preformed structural segments grows during the folding. The growth of the assembly is accompanied by at least two opposite contributions to the free energy: a free-energy increase caused by a decrease in mobility of the assembled structural

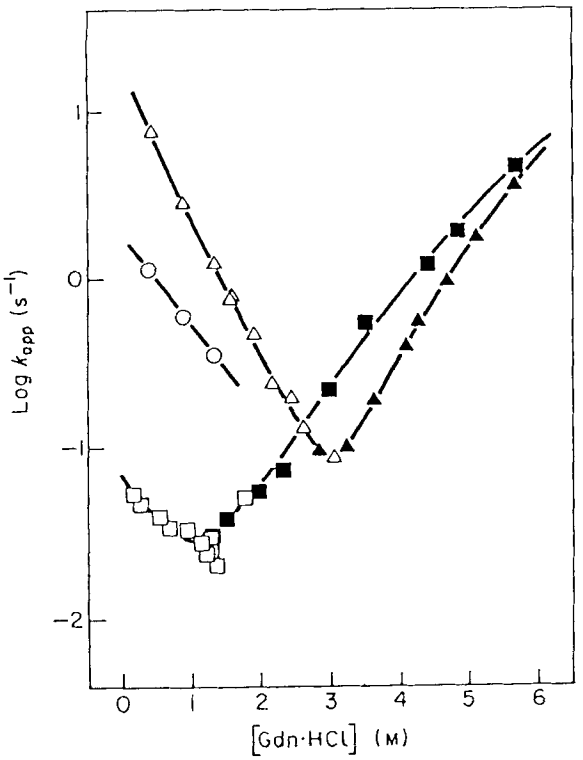
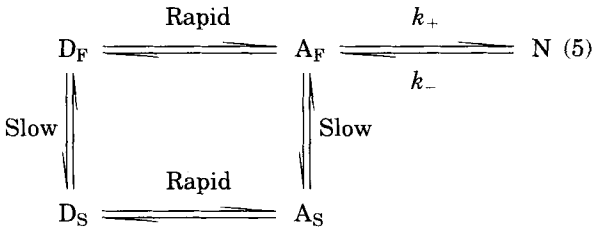


Fig. 6. Dependence of the logarithmic k_{app} of refolding (open symbols) and unfolding (filled symbols) of α-lactalbumin on GdnHCl concentration (pH 7.0 and 25°C): (○, △, ▲) data for the holoprotein at 1 mM $CaCl_2$; (■, □) data for the apoprotein in the absence of Ca^{2+}. The open circles refer to the slow phase in biphasic refolding reactions. The figure was taken from ref. 16.

segments and a free-energy decrease caused by a decrease in the accessible surface area. These, as well as some other contributions to the free energy, depend on the size of the organized structure, and any partially organized forms must be more unstable than the molten globule or the final native state. Thus, the critical size of the structure may exist at the point where the sum of all the contributions to the free energy is maximal. The organized structural region that has the critical size is termed the critical substructure. The folding and unfolding kinetics of α-lactalbumin and other proteins are known to favor strongly the critical-substructure model (see below).

Partial organization in terms of the accessible surface area

The apparent rate constant, k_{app}, of folding and unfolding reactions of α-lactalbumin shows a V-shaped dependence on GdnHCl concentration with a minimum near the midpoint of the unfolding transition (Fig. 6). In the presence of 1 mM $CaCl_2$, where the refolding reaction is accelerated 100-fold by Ca^{2+}, the kinetics are biphasic below 1.5 M GdnHCl owing to a slow isomerization between different kinetic species, presumably arising from the well-known proline isomerization in the unfolded state.[9] Considering this fact, the total reaction scheme of folding and unfolding of the protein has been elaborated[16] and it is expressed by



where the subscripts F and S denote the fast- and slow-folding species, and k_+ and k_- are the rate constants for the folding and unfolding directions, respectively. The slow-folding species are only 10% of the total molecules both in the D and in the A state. From the results of Figure 6 and the equilibrium constants of the three-state unfolding of this protein, k_+ and k_- were obtained as a function of GdnHCl concentration. It has been shown that the V-shaped dependence of the logarithmic k_{app} (Fig. 6) arises from closely linear relationships of the activation free energies, $\Delta G_+^{\ddagger}$ and $\Delta G_-^{\ddagger}$, derived from k_+ and k_-, respectively, to the denaturant concentration.[16] $\Delta G_+^{\ddagger}$ increases and $\Delta G_-^{\ddagger}$ decreases approximately linearly with increasing GdnHCl concentration. Such linear relationships are thus similar to the linear relationship between ΔG of unfolding and the denaturant concentration, found in the equilibrium unfolding reactions of many globular proteins.[31,142] Therefore, by analogy with Schellman's treatment of solvent denaturation of a protein, the differential free energy differences of activation, $\Delta b_+^{\ddagger}$ and $\Delta b_-^{\ddagger}$, are given by

$$\Delta b_+^{\ddagger} = \frac{\partial G_+^{\ddagger}}{\partial[\mathrm{GdnHCl}]} = -RT\left(\frac{\partial \ln k_+}{\partial[\mathrm{GdnHCl}]}\right) \quad (6)$$

and

$$\Delta b_-^{\ddagger} = \frac{\partial \Delta G_-^{\ddagger}}{\partial[\mathrm{GdnHCl}]} = -RT\left(\frac{\partial \ln k_-}{\partial[\mathrm{GdnHCl}]}\right) \quad (7)$$

and relate to thermodynamic binding that includes all modes of interaction of the protein with the denaturant.[31] It has been demonstrated that $\Delta b_+^{\ddagger}$ and $\Delta b_-^{\ddagger}$ are useful measures of changes in the accessible surface area of hydrophobic residues during the activation processes for folding from the A to the activated state and for unfolding from the N to the activated state, respectively; $\Delta b_+^{\ddagger} = 0.52 \sim 0.65$ kcal/mol/M and $\Delta b_-^{\ddagger} = -1.04 \sim -1.32$ kcal/mol/M.[16] The relative degree of the structural organization in the activated state of folding is given by the ratio, $\Delta b_+^{\ddagger}/(\Delta b_+^{\ddagger} - \Delta b_-^{\ddagger})$, and indicates that only a third

of the difference in accessible surface area between the A and N states is buried in the activated state. Although the proportionality of $\Delta b_+^{\ddagger}$ (or $\Delta b_-^{\ddagger}$) to the solvent-accessible surface area is only a rough approximation, the results are sufficient to demonstrate the partial organization of the structure in the critical activated state.

The V-shaped dependence of the logarithmic k_{app} on concentration of a denaturant such as GdnHCl or urea has also been reported in many other globular proteins.[15,84,143–149] Therefore, the partial organization in terms of the accessible surface area in the activated state is a general trend in globular protein folding.

Localized organization of the specific structure

Localized organization of a specific structure in the activated state has been demonstrated by the studies of effect of Ca^{2+} and effect of selective cleavage of a disulfide bond on the folding and unfolding kinetics of α-lactalbumin.[16,69,150] As seen in Figure 6, only the folding rate constant is selectively accelerated by Ca^{2+}. The dependence of k_+ on Ca^{2+} concentration was thus examined. The logarithmic k_+ was shown to exhibit a sigmoidal dependence on log $[Ca^{2+}]$ with a slope of unity in the middle,[16] indicating that one Ca^{2+} is bound by the protein in the activated state, and the binding constant of Ca^{2+} in the activated state was estimated to be 3.1×10^5 M^{-1}. This value is an order of magnitude smaller than the binding constant in the N state ($10^6 \sim 10^7$ M^{-1}) but more than two orders of magnitude larger than the binding constant in the A state (8.8×10^2 M^{-1}).[16] Therefore, the specific structure required for the strong Ca^{2+} binding is already organized in the critical activated state of folding. On the other hand, of the four disulfide bonds in α-lactalbumin, Cys-6–Cys-120 was selectively reduced by dithiothreitol and carboxymethylated by iodoacetate,[69,150] and the effects of the selective cleavage of the disulfide bond on the folding and unfolding kinetics were investigated. The carboxymethylated three-disulfide α-lactalbumin can assume the native structure but its stability against the A state is decreased considerably compared with the disulfide-intact protein. This decrease in stability is found to be mostly ascribable to a 100-fold increase in k_- in the three-disulfide species. Such a selective effect on the unfolding rate constant demonstrates that the Cys-6–Cys-120 disulfide bond is not involved in the organized structural region in the activated state. This disulfide bond and the Ca^{2+}-binding site are 22 Å apart, according to the model structure of α-lactalbumin (Fig. 1).[37,38] These results thus provide clear evidence that the specific structure organized in the activated state of folding must be localized within the molecule. The rate-determining step of folding of α-lactalbumin is well represented by the critical-substructure model.

Accordingly, the critical substructure of folding involves the Ca^{2+}-binding substructure in α-lactalbumin. The crystallographic structure found by X-ray analysis indicates that the Ca^{2+}-binding substructure in this protein consists of two helical segments, corresponding to residues 76 to 82 and 86 to 99, and an "elbow" loop that connects the helices.[38,39] It should thus be noted that at least one of these helical segments is already more stable than the other structured segments and is involved in the kernel of the molecule in the A state shown previously. Therefore, the kernel may serve as the folding initiation site, and the activation process of folding may correspond to the growing process of the organized assembly of the structured segments around the folding initiation site to bring about the critical substructure. The restriction in mobility of the two helices provided by the disulfide linkages (Cys-73–Cys-91 and Cys-61–Cys-77)[16] and the hydrophobic clustering around these helices[75] have been thought to be important for stability of the two helical segments.

At present, the critical-substructure model of folding is substantiated in α-lactalbumin and also in Ca^{2+}-binding parvalbumin.[16,84] As mentioned above, however, the partial organization in terms of the accessible surface area in the critical activated state of folding is expected to be true in many other globular proteins, and these include immunoglobulin C_L fragment, lysozyme, and ribonuclease A. Importantly, in spite of the partial organization in the activated state, the cleavage of the disulfide bond in the C_L fragment[151] and the introduction of extrinsic cross-linking in lysozyme[152] and ribonuclease A[146] are known to bring about a selective effect on the rate constant of folding or unfolding; the refolding rate is selectively affected in the C_L fragment and lysozyme while the unfolding rate is selectively affected in ribonuclease A. These results thus suggest that the folding reactions of these proteins may also conform to the critical-substructure model, and such localized organization of the specific structure in the activated state may be common in the activation process of globular protein folding.

CONCLUSIONS

1. The structural characteristics of the molten globule state have been summarized; primarily these are a compact globule with native-like secondary structure and with slowly fluctuating tertiary structure. The available data for equilibrium unfolding and the kinetics of folding of various proteins clearly demonstrate that the state is a common physical state among different proteins.

2. In the equilibrium transition from the native to the fully unfolded state, the classical two-state

theory may be a phenomenologically simplified version of the three-state unfolding transition with the molten globule as an intermediate. The unfolding of the molten globule is accompanied by only a small or negligible heat absorption, so that the calorimetric measurement of protein unfolding is incapable of detecting the molten globule state.

3. The hierarchical nature of protein structure and the thermodynamic properties of the molten globule suggest that the state may be characterized as a nonspecific assembly of secondary structure segments brought about by nonspecific hydrophobic interactions. Recent data also demonstrate, however, the presence of a localized structural region (kernel) of particular stability within the molecule of the molten globule state.

4. The molten globule state corresponds to the transient intermediate formed at an early stage of kinetic refolding from the fully unfolded state. Some secondary structure segments in the intermediate are known to persist during the late stage of folding.

5. The rate-determining step of kinetic folding is located at a late stage after accumulation of the molten globule state. It is demonstrated that localized organization of the specific tertiary structure with partial reduction of the accessible surface area of the protein molecule leads to the critical activated state of folding. The kernel in the molten globule may act as the folding initiation site at the beginning of this step.

6. The above picture of the protein folding mechanism has, however, been established in a small number of globular proteins and only from an experimental point of view, so that many questions in the folding problem remain to be fully explored.

NOTE ADDED IN PROOF

Subsequent to submission of this manuscript, Brems and Havel reported the evidence for consistency of their observations of folding of bovine growth hormone with the molten globule hypothesis,[154] and Acharya et al. reported the refined X-ray structure of baboon α-lactalbumin at 1.7 Å resolution.[155]

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REFERENCES

1. Anfinsen, C.B., Scheraga, H.A. Experimental and theoretical aspects of protein folding. Adv. Protein Chem. 29: 205–300, 1975.
2. Levinthal, C. Are there pathways for protein folding? J. Chim. Phys. 65:44–45, 1968.
3. Wetlaufer, D.B. Nucleation, rapid folding, and globular intrachain regions in proteins. Proc. Natl. Acad. Sci. U.S.A. 70:697–701, 1973.
4. Karplus, M., Weaver, D.L. Protein-folding dynamics. Nature (London) 260:404–406, 1976.
5. Gō, N. Theoretical studies of protein folding. Annu. Rev. Bophys. Bioeng. 12:183–210, 1983.
6. Harrison, S.C., Durbin, R. Is there a single pathway for the folding of a polypeptide chain? Proc. Natl. Acad. Sci. U.S.A. 82:4028–4030, 1985.
7. Pfeil, W., Privalov, P.L. Thermodynamic investigations of proteins. III. Calorimetric study of lysozyme denaturation by guanidine hydrochloride. Biophys. Chem. 4:33–40, 1976.
8. Privalov, P.L. Stability of proteins. Small globular proteins. Adv. Protein Chem. 33:167–241, 1979.
9. Kim, P.S., Baldwin, R.L. Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. Annu. Rev. Biochem. 51:459–489, 1982.
10. Tanford, C. Protein denaturation. Adv. Protein Chem. 23: 121–282, 1968.
11. Myer, Y.P. Conformation of cytochromes. III. Effect of urea, temperature, extrinsic ligands, and pH variation on the conformation of horse heart ferricytochrome *c*. Biochemistry 7:765–776, 1968.
12. Kuwajima, K., Nitta, K., Yoneyama, M., Sugai, S. Three-state denaturation of α-lactalbumin by guanidine hydrochloride. J. Mol. Biol. 106:359–373, 1976.
13. Kuwajima, K. A folding model of α-lactalbumin deduced from the three-state denaturation mechanism. J. Mol. Biol. 114:241–258, 1977.
14. Ikeguchi, M., Kuwajima, K., Sugai, S. Ca^{2+}-induced alteration in the unfolding behavior of α-lactalbumin. J. Biochem. (Tokyo) 99:1191–1201, 1986.
15. Ikeguchi, M., Kuwajima, K., Mitani, M., Sugai, S. Evidence for identity between the equilibrium unfolding intermediate and a transient folding intermediate: A comparative study of the folding reactions of α-lactalbumin and lysozyme. Biochemistry 25:6965–6972, 1986.
16. Kuwajima, K., Mitani, M., Sugai, S. Characterization of the critical state in protein folding. Effects of guanidine hydrochloride and specific Ca^{2+} binding on the folding kinetics of α-lactalbumin. J. Mol. Biol. 206:547–561, 1989.
17. Dolgikh, D.A., Gilmanshin, R.I., Brazhnikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venyaminov, S. Yu., Ptitsyn, O.B. α-Lactalbumin: Compact state with fluctuating tertiary structure? FEBS Lett. 136:311–315, 1981.
18. Dolgikh, D.A., Kolomiets, A.P., Bolotina, I.A., Ptitsyn, O.B. 'Molten-globule' state accumulates in carbonic anhydrase folding. FEBS Lett. 165:88–92, 1984.
19. Semisotnov, G.V., Rodionova, N.A., Kutyshenko, V.P., Ebert, B., Blanck, J., Ptitsyn, O.B. Sequential mechanism of refolding of carbonic anhydrase B. FEBS Lett. 224:9–13, 1987.
20. Ptitsyn, O.B. Protein folding: Hypotheses and experiments. J. Protein Chem. 6:272–293, 1987.
21. Wong, K.-P., Tanford, C. Denaturation of bovine carbonic anhydrase B by guanidine hydrochloride. A process involving separatable sequential transitions. J. Biol. Chem. 248:8518–8523, 1973.
22. Holladay, L.A., Hammonds, R.G., Jr., Puett, D. Growth hormone conformation and conformational equilibria. Biochemistry 13:1653–1661, 1974.
23. Brems, D.N., Plaisted, S.M., Havel, H.A., Kauffman, E.W., Stodola, J.D., Eaton, L.C., White, R.D. Equilibrium denaturation of pituitary- and recombinant-derived bovine growth hormone. Biochemistry 24:7662–7668, 1985.
24. Ohgushi, M., Wada, A. 'Molten-globule state': A compact form of globular proteins with mobile side-chains. FEBS Lett. 164:21–24, 1983.
25. Lapanje, S. "Physicochemical Aspects of Protein Denaturation." New York: Wiley, 1978.

26. Richardson, J.S. The anatomy and taxonomy of protein structure. Adv. Protein Chem. 34:167–339, 1981.
27. Ghélis, C., Yon, J. "Protein Folding." New York: Academic Press, 1982.
28. Creighton, T.E. "Proteins: Structure and Molecular Principles." New York: Freeman, 1983.
29. Chothia, C. Principles that determine the structure of proteins. Annu. Rev. Biochem. 53:537–572, 1984.
30. Jaenicke, R. Folding and association of proteins. Prog. Biophys. Mol. Biol. 49:117–237, 1987.
31. Schellman, J.A. The thermodynamic stability of proteins. Annu. Rev. Biophys. Biophys. Chem. 16:115–137, 1987.
32. Goldenberg, D.P. Genetic studies of protein stability and mechanisms of folding. Annu. Rev. Biophys. Biophys. Chem. 17:481–507, 1988.
33. Bychkova, V.E., Pain, R.H., Ptitsyn, O.B. The 'molten globule' state is involved in the translocation of proteins across membranes? FEBS Lett. 238:231–234, 1988.
34. Hill, R.L., Brew, K. Lactose synthetase. Adv. Enzymol. Relat. Areas Mol. Biol. 43:411–490, 1975.
35. Phillips, D.C., Acharya, K.R., Handoll, H.H.G., Stuart, D.I. From lysozyme to α-lactalbumin: protein engineering and evolution. Biochem. Soc. Trans. 15:737–744, 1987.
36. Browne, W.J., North, A.C.T., Phillips, D.C., Brew, K., Vanaman, T.C., Hill, R.L. A possible three-dimensional structure of bovine α-lactalbumin based on that of hen's egg-white lysozyme. J. Mol. Biol. 42:65–86, 1969.
37. Warme, P.K., Momany, F.A., Rumball, S.V., Tuttle, R.W., Scheraga, H.A. Computation of structures of homologous proteins. α-Lactalbumin from lysozyme. Biochemistry 13: 768–782, 1974.
38. Stuart, D.I., Acharya, K.R., Walker, N.P.C., Smith, S.G., Lewis, M., Phillips, D.C. α-Lactalbumin possesses a novel calcium binding loop. Nature (London) 324:84–87, 1986.
39. Smith, S.G., Lewis, M., Aschaffenburg, R., Fenna, R.E., Wilson, I.A., Sundaralingam, M., Stuart, D.I., Phillips, D.C. Crystallographic analysis of the three-dimensional structure of baboon α-lactalbumin at low resolution. Biochem. J. 242:353–360, 1987.
40. Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S., Murai, N. α-Lactalbumin: A calcium metalloprotein. Biochem. Biophys. Res. Commun. 95:1098–1104, 1980.
41. Segawa, T., Sugai, S. Interactions of divalent metal ions with bovine, human, and goat α-lactalbumins. J. Biochem. (Tokyo) 93:1321–1328, 1983.
42. Hamano, M., Nitta, K., Kuwajima, K., Sugai, S. Binding strength of calcium ion to bovine α-lactalbumin. J. Biochem. (Tokyo) 100:1617–1622, 1986.
43. Berliner, L.J., Johnson, J.D. α-Lactalbumin and calmodulin. In: "Calcium Binding Proteins, Vol. II, Biological Functions." Thompson, M.P. ed. Boca Raton: CRC Press, 1988: 79–116.
44. Kronman, M.J. Metal-ion binding and the molecular conformational properties of α-lactalbumin. CRC Crit. Rev. Biochem. Mol. Biol., in press.
45. Kuwajima, K., Ogawa, Y., Sugai, S. Role of the interaction between ionizable groups in the folding of bovine α-lactalbumin. J. Biochem. (Tokyo) 89:759–770, 1981.
46. Kronman, M.J., Cerankowski, L., Holmes, L.G. Inter- and intramolecular interactions of α-lactalbumin. III. Spectral changes at acid pH. Biochemistry 4:518–525, 1965.
47. Kronman, M.J., Holmes, L.G., Robbins, F.M. Inter- and intramolecular interactions of α-lactalbumin. VIII. The alkaline conformational change. Biochim. Biophys. Acta 133:46–55, 1967.
48. Kronman, M.J., Blum, R., Holmes, L.G. Inter- and intramolecular interactions of α-lactalbumin. VI. Optical rotation dispersion properties. Biochemistry 5: 1970–1978, 1966.
49. Kronman, M.J., Jeroszko, J., Sage, G.W. Inter- and intramolecular interactions of α-lactalbumin. XII. Changes in the environment of aromatic residues in the goat protein. Biochim. Biophys. Acta 285:145–166, 1972.
50. Sommers, P.B., Kronman, M.J. Comparative fluorescence properties of bovine, goat, human and guinea pig α-lactalbumin. Characterization of the environments of individual tryptophan residues in partially folded conformers. Biophys. Chem. 11:217–232, 1980.
51. Kronman, M.J., Hoffman, W.B., Jeroszko, J., Sage, G.W. Inter- and intramolecular interactions of α-lactalbumin. XI. Comparison of the "exposure" of tyrosyl, tryptophyl and lysyl side chains in the goat and bovine proteins. Biochim. Biophys. Acta 285:124–144, 1972.
52. Mulqueen, P.M., Kronman, M.J. Binding of naphthalene dyes to the N and A conformers of bovine α-lactalbumin. Arch. Biochem. Biophys. 215:28–39, 1982.
53. Dolgikh, D.A., Abaturov, L.V., Bolotina, I.A., Brazhnikov, E.V., Bychkova, V.E., Bushuev, V.N., Gilmanshin, R.I., Levedev, Yu. O., Semisotnov, G.V., Tiktopulo, E.I., Ptitsyn, O.B. Compact state of a protein molecule with pronounced small-scale mobility: Bovine α-lactalbumin. Eur. Biophys. J. 13:109–121, 1985.
54. Damaschun, G., Gernat, Ch., Damaschun, H., Bychkova, V.E., Ptitsyn, O.B. Comparison of intramolecular packing of a protein in native and 'molten globule' states. Int. J. Biol. Macromol. 8:226–230, 1986.
55. Gast, K., Zirwer, D., Welfle, H., Bychkova, V.E., Ptitsyn, O.B. Quasielastic light scattering from human α-lactalbumin: Comparison of molecular dimensions in native and 'molten globule' states. Int. J. Biol. Macromol. 8:231–236, 1986.
56. Pfeil, W., Bychkova, V.E., Ptitsyn, O.B. Physical nature of the phase transition in globular proteins. Calorimetric study of human α-lactalbumin. FEBS Lett. 198:287–291, 1986.
57. Gilmanshin, R.I., Ptitsyn, O.B. An early intermediate of refolding α-lactalbumin forms within 20 ms. FEBS Lett. 223:327–329, 1987.
58. Kuwajima, K., Nitta, K., Sugai, S. Electrophoretic investigations of the acid conformational change of α-lactalbumin. J. Biochem. (Tokyo) 78:205–211, 1975.
59. Nozaka, M., Kuwajima, K., Nitta, K., Sugai, S. Detection and characterization of the intermediate on the folding pathway of human α-lactalbumin. Biochemistry 17: 3753–3758, 1978.
60. Kuwajima, K., Nitta, K., Sugai, S. Intramolecular perturbation of tryptophans induced by the protonation of ionizable groups in goat α-lactalbumin. Biochim. Biophys. Acta 623:389–401, 1980.
61. Kuwajima, K., Hiraoka, Y., Ikeguchi, M., Sugai, S. Comparison of the transient folding intermediates in lysozyme and α-lactalbumin. Biochemistry 24:874–881, 1985.
62. Kuwajima, K., Ogawa, Y., Sugai, S. Application of the pH-jump method to the titration of tyrosine residues in bovine α- lactalbumin. Biochemistry 18:878–882, 1979.
63. Izumi, Y., Miyake, Y., Kuwajima, K., Sugai, S., Inoue, K., Iizumi, M., Katano, S. Folding-unfolding of α-lactalbumin. Physica 120B:444–448, 1983.
64. Hiraoka, Y., Sugai, S. Equilibrium and kinetic study of sodium- and potassium-induced conformational changes of apo-α-lactalbumin. Int. J. Peptide Protein Res. 26:252–261, 1985.
65. Kuwajima, K., Harushima, Y., Sugai, S. Influence of Ca^{2+} binding on the structure and stability of bovine α-lactalbumin studied by circular dichroism and nuclear magnetic resonance spectra. Int. J. Peptide Protein Res. 27:18–27, 1986.
66. Harushima, Y., Kuwajima, K., Sugai, S. Hydrogen exchange of the tryptophan residues in bovine α-lactalbumin studied by UV spectroscopy. Biopolymers 27: 629–644, 1988.
67. Maruyama, S., Kuwajima, K., Nitta, K., Sugai, S. Thermodynamic characterization of partially denatured states in the denaturation process of bovine α-lactalbumin by inorganic denaturants. Biochim. Biophys. Acta 494:343–353, 1977.
68. Ikeguchi, M., Sugai, S. Contribution of disulfide bonds to stability of the folding intermediate of α-lactalbumin. Int. J. Peptide Protein Res. 33:289–297, 1989.
69. Sugai, S., Kuwajima, K., Nitta, K. Characterization of folding intermediates of globular proteins. In: "Protein Design." Hatano, M. ed. Tokyo: Japan Scientific Society Press, to be published.
70. Pfeil, W. Is thermally denatured protein unfolded? The example of α-lactalbumin. Biochim. Biophys. Acta 911: 114–116, 1987.
71. Mets, Ü., Pooga, M., Kask, P., Gast, K. Rotational diffu-

sion studies of native and "molten globule" states of bovine α-lactalbumin. Studia Biophys. 126:87–97, 1988.
72. Hanssens, I., Houthuys, C., Herreman, W., Van Cauwelaert, F.H. Interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles. I. A microcalorimetric and fluorescence study. Biochim. Biophys. Acta 602:539–557, 1980.
73. Kim, J., Kim, H. Fusion of phospholipid vesicles induced by α-lactalbumin at acidic pH. Biochemistry 25: 7867–7874, 1986.
74. Pfeil, W., Sadowski, M.L. A scanning calorimetric study of bovine and human apo-α-lactalbumin. Studia Biophys. 109:163–170, 1985.
75. Baum, J., Dobson, C.M., Evans, P.A., Hanley, C. Characterization of a partly folded protein by NMR methods: Studies on the molten globule state of guinea pig α-lactalbumin. Biochemistry 28:7–13, 1989.
76. Burger, H.G., Edelhoch, H., Condliffe, P.G. The properties of bovine growth hormone. I. Behavior in acid solution. J. Biol. Chem. 241:449–457, 1966.
77. Yutani, K., Ogasawara, K., Sugino, Y. pH dependence of stability of the wild-type tryptophan synthase α-subunit and two mutant proteins (Glu49 →Met or Gln). J. Mol. Biol. 144:455–465, 1980.
78. Adams, B., Burgess, R.J., Carrey, E.A., Mackintosh, I.R., Mitchinson, C., Thomas, R.M., Pain, R.H. The role of folding units in the kinetic folding of globular proteins. In: "Protein Folding." Jaenicke, R. ed. Amsterdam: Elsevier/North-Holland Biomedical Press, 1980: 447–467.
79. Bewley, T.A., Levine, H.L., Wetzel, R. Structural features of human leukocyte interferon A as determined by circular dichroism spectroscopy. Int. J. Peptide Protein Res. 20:93–96, 1982.
80. Bismuto, E., Colonna, G., Irace, G. Unfolding pathway of myoglobin. Evidence for a multistate process. Biochemistry 22:4165–4170, 1983.
81. Dalzoppo, D., Vita, C., Fontana, A. Domain characteristics of the carboxyl-terminal fragment 206–316 of thermolysin: pH and ionic strength dependence of conformation. Biopolymers 24:767–782, 1985.
82. Zhao, J.-M., London, E. Similarity of the conformation of diphtheria toxin at high temperature to that in the membrane-penetrating low-pH state. Proc. Natl. Acad. Sci. U.S.A. 83:2002–2006, 1986.
83. Arakawa, T., Hsu, Y.-R., Yphantis, D.A. Acid unfolding and self-association of recombinant *Escherichia coli* derived human interferon γ. Biochemistry 26:5428–5432, 1987.
84. Kuwajima, K., Sakuraoka, A., Fueki, S., Yoneyama, M., Sugai, S. Folding of carp parvalbumin studied by equilibrium and kinetic circular dichroism spectra. Biochemistry 27:7419–7428, 1988.
85. Wong, K.-P., Hamlin, L.M. Acid denaturation of bovine carbonic anhydrase B. Biochemistry 13:2678–2683, 1974.
86. Brazhnikov, E.V., Chirgadze, Yu., N., Dolgikh, D.A., Ptitsyn, O.B. Noncooperative temperature melting of a globular protein without specific tertiary structure: Acid form of bovine carbonic anhydrase B. Biopolymers 24: 1899–1907, 1985.
87. Jagannadham, M.V., Balasubramanian, D. The molten globular intermediate form in the folding pathway of human carbonic anhydrase B. FEBS Lett. 188:326–330, 1985.
88. Goto, Y., Fink, A.L. Conformational states of β-lactamase: Molten-globule state at acidic and alkaline pH with high salt. Biochemistry 28:945–952, 1989.
89. Robson, B., Pain, R.H. The mechanism of folding of globular proteins. Equilibria and kinetics of conformational transitions of penicillinase from *Staphylococcus aureus* involving a state of intermediate conformation. Biochem. J. 155:331–344, 1976.
90. Balestrieri, C., Colonna, G., Giovane, A., Irace, G., Servillo, L. Equilibrium evidence of non-single step transition during guanidine unfolding of apomyoglobins. FEBS Lett. 66:60–64, 1976.
91. Myer, Y.P., MacDonald, L.H., Verma, B.C., Pande, A. Urea denaturation of horse heart ferricytochrome *c*. *Equilibrium studies and characterization of intermediate forms*. Biochemistry 19:199–207, 1980.
92. Matthews, C.R., Crisanti, M.M. Urea-induced unfolding of the α subunit of tryptophan synthase: Evidence for a multistate process. Biochemistry 20:784–792, 1981.
93. McCoy, L.F., Jr., Rowe, E.S., Wong, K.-P. Multiparameter kinetic study on the unfolding and refolding of bovine carbonic anhydrase B. Biochemistry 19:4738–4743, 1980.
94. Havel, H.A., Kauffman, E.W., Plaisted, S.M., Brems, D.N. Reversible self-association of bovine growth hormone during equilibrium unfolding. Biochemistry 25: 6533–6538, 1986.
95. Creighton, T.E., Pain, R.H. Unfolding and refolding of *Staphylococcus aureus* penicillinase by urea-gradient electrophoresis. J. Mol. Biol. 137:431–436, 1980.
96. Craig, S., Hollecker, M., Creighton, T.E., Pain, R.H. Single amino acid mutations block a late step in the folding of β-lactamase from *Staphylococcus aureus*. J. Mol. Biol. 185:681–687, 1985.
97. Hyde, C.C., Ahmed, S.A., Padlan, E.A., Miles, E.W., Davies, D.R. Three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ multienzyme complex from *Salmonella typhimurium*. J. Biol. Chem. 263:17857–17871, 1988.
98. Shortle, D., Meeker, A.K. Residual structure in large fragments of staphylococcal nuclease: Effects of amino acid substitutions. Biochemistry 28:936–944, 1989.
99. Hughson, F.M., Baldwin, R.L. Use of site-directed mutagenesis to destabilize native apomyoglobin relative to folding intermediates. Biochemistry 28:4415–4422, 1989.
100. Holzman, T.F., Brems, D.N., Dougherty, J.J., Jr. Reoxidation of reduced bovine growth hormone from a stable secondary structure. Biochemistry 25:6907–6917, 1986.
101. Amir, D., Haas, E. Reduced bovine pancreatic trypsin inhibitor has a compact structure. Biochemistry 27: 8889–8893, 1988.
102. Griko, Yu.V., Privalov, P.L., Venyaminov, S.Yu., Kutyshenko, V.P. Thermodynamic study of the apomyoglobin structure. J. Mol. Biol. 202:127–138, 1988.
103. Denton, J.B., Konishi, Y., Scheraga, H.A. Folding of ribonuclease A from a partially disordered conformation. Kinetic study under folding conditions. Biochemistry 21: 5155–5163, 1982.
104. Lynn, R.M., Konishi, Y., Scheraga, H.A. Folding of ribonuclease A from a partially disordered conformation. Kinetic study under transition conditions. Biochemistry 23: 2470–2477, 1984.
105. Crippen, G.M. The tree structural organization of proteins. J. Mol. Biol. 126:315–332, 1978.
106. Lesk, A.M., Rose, G.D. Folding units in globular proteins. Proc. Natl. Acad. Sci. U.S.A. 78:4304–4308, 1981.
107. Ptitsyn, O.B., Rashin, A.A. A model of myoglobin self-organization. Biophys. Chem. 3:1–20, 1975.
108. Tanaka, S., Scheraga, H.A. Hypothesis about the mechanism of protein folding. Macromolecules 10:291–304, 1977.
109. Saitô, N., Shigaki, T., Kobayashi, Y., Yamamoto, M. Mechanism of protein folding: I. General considerations and refolding of myoglobin. PROTEINS: Structure, Function, Genetics 3:199–207, 1988.
110. Kidera, A., Mochizuki, M., Hasegawa, R., Hayashi, T., Sato, H., Nakajima, A., Fredrickson, R.A., Powers, S.P., Lee, S., Scheraga, H.A. Helix-coil transition in multicomponent random copolypeptides in water. 1. Theory, and application to random copolymers of (hydroxypropyl)-L-glutamine, L-alanine, and glycine. Macromolecules 16: 162–172, 1983.
111. Reutimann, H., Luisi, P.L., Holmgren, A. Folding of protein fragments: Conformational and biological studies on thioredoxin and its fragments. Biopolymers 22:107–111, 1983.
112. Dalzoppo, D., Vita, C., Fontana, A. Folding of thermolysin fragments. Identification of the minimum size of a carboxyl-terminal fragment that can fold into a stable native-like structure. J. Mol. Biol. 182:331–340, 1985.
113. Shoemaker, K.R., Kim, P.S., York, E.J., Stewart, J.M., Baldwin, R.L. Tests of the helix dipole model for stabilization of α-helices. Nature (London) 326:563–567, 1987.
114. Marqusee, S., Baldwin, R.L. Helix stabilization by Glu^- . . . Lys^+ salt bridges in short peptides of *de novo* design. Proc. Natl. Acad. Sci. U.S.A. 84:8898–8902, 1987.
115. Jiménez, M.A., Rico, M., Herranz, J., Santoro, J., Nieto,

J.L. ^{1}H-NMR assignment and folding of the isolated ribonuclease 21–42 fragment. Eur. J. Biochem. 175:101–109, 1988.
116. Gooley, P.R., MacKenzie, N.E. Location of an α-helix in fragment 96–133 from bovine somatotropin by ^{1}H NMR spectroscopy. Biochemistry 27:4032–4040, 1988.
117. Wright, P.E., Dyson, H.J., Lerner, R.A. Conformation of peptide fragments of proteins in aqueous solution: Implications for initiation of protein folding. Biochemistry 27: 7167–7175, 1988.
118. Goodman, E.M., Kim, P.S. Folding of a peptide corresponding to the α-helix in bovine pancreatic trypsin inhibitor. Biochemistry 28:4343–4347, 1989.
119. Regan, L., DeGrado, W.F. Characterization of a helical protein designed from first principles. Science 241:976–978, 1988.
120. DeGrado, W.F. Design of peptide and proteins. Adv. Protein Chem. 39:51–124, 1988.
121. Vasquez, M., Scheraga, H.A. Effect of sequence-specific interactions on the stability of helical conformations in polypeptides. Biopolymers 27:41–58, 1988.
122. Pfeil, W. Thermodynamics of α-lactalbumin unfolding. Biophys. Chem. 13:181–186, 1981.
123. Bradbury, J.H., Norton, R.S. Denaturation of proteins. V. N.M.R. study of the arginine residues of lysozyme. Int. J. Peptide Protein Res. 6:295–302, 1974.
124. Matheson, R.R., Jr., Scheraga, H.A. Steps in the pathway of the thermal unfolding of ribonuclease A. A nonspecific photochemical surface-labeling study. Biochemistry 18: 2437–2445, 1979.
125. Sugai, S., Nitta, K., Ohno, N. Hydrophobic domains of maleic acid copolymers. In: "Microdomains in Polymer Solutions." Dubin, P. ed. New York: Plenum, 1985: 13–32.
126. Dill, K.A. Theory for the folding and stability of globular proteins. Biochemistry 24:1501–1509, 1985.
127. Privalov, P.L., Gill, S.J. Stability of protein structure and hydrophobic interaction. Adv. Protein Chem. 39:191–234, 1988.
128. Kuwajima, K., Yamaya, H., Miwa, S., Sugai, S., Nagamura, T. Rapid formation of secondary structure framework in protein folding studied by stopped-flow circular dichroism. FEBS Lett. 221:115–118, 1987.
129. Brems, D.N., Plaisted, S.M., Dougherty, J.J., Jr., Holzman, T.F. The kinetics of bovine growth hormone folding are consistent with a framework model. J. Biol. Chem. 262:2590–2596, 1987.
130. Labhardt, A.M. Folding intermediates studied by circular dichroism. Methods Enzymol. 131:126–135, 1986.
131. Schmid, F.X., Baldwin, R.L. Detection of an early intermediate in the folding of ribonuclease A by protection of amide protons against exchange. J. Mol. Biol. 135:199–215, 1979.
132. Kim, P.S., Baldwin, R.L. Structural intermediates trapped during the folding of ribonuclease A by amide proton exchange. Biochemistry 19:6124–6129, 1980.
133. Beasty, A.M., Matthews, C.R. Characterization of an early intermediate in the folding of the α subunit of tryptophan synthase by hydrogen exchange measurement. Biochemistry 24:3547–3553, 1985.
134. Kim, P.S. Amide proton exchange as a probe of protein folding pathway. Methods Enzymol. 131:136–156, 1986.
135. Creighton, T.E. Detection of folding intermediates using urea-gradient electrophoresis. Methods Enzymol. 131: 156–171, 1986.
136. Bergenhem, N., Carlsson, U., Karlsson, J.-Å. Evidence for an initial fast nucleation process in the folding of human carbonic anhydrase I. Int. J. Peptide Protein Res. 33:140–145, 1989.
137. Udgaonkar, J.B., Baldwin, R.L. NMR evidence for an early framework intermediate on the folding pathway of ribonuclease A. Nature (London) 335:694–699, 1988.
138. Roder, H., Elöve, G., Englander, S.W. Structural characterization of folding intermediates in cytochrome *c* by H-exchange labelling and proton NMR. Nature (London) 335:700–704, 1988.
139. Lumry, R., Biltonen, R. Thermodynamic and kinetic aspects of protein conformations in relation to physiological function. In: "Structure and Stability of Biological Macromolecules." Timasheff, S.N., Fasman, G.D. eds. New York: Dekker, 1969: 65–212.
140. Segawa, S., Sugihara, M. Characterization of the transition state of lysozyme unfolding. I. Effect of protein-solvent interactions on the transition state. Biopolymers 23: 2473–2488, 1984.
141. Goldenberg, D.P., Creighton, T.E. Energetics of protein structure and folding. Biopolymers 24:167–182, 1985.
142. Pace, C.N. Determination and analysis of urea and guanidine hydrochloride denaturation curves. Methods Enzymol. 131:266–280, 1986.
143. Nall, B.T., Landers, T.A. Guanidine hydrochloride induced unfolding of yeast iso-2 cytochrome *c*. Biochemistry 20: 5403–5411, 1981.
144. Goto, Y., Hamaguchi, K. Unfolding and refolding of the constant fragment of the immunoglobulin light chain. J. Mol. Biol. 156:891–910, 1982.
145. Mitchinson, C., Pain, R.H. Effects of sulphate and urea on the stability and reversible unfolding of β-lactamase from *Staphylococcus aureus*. Implication for the folding pathway of β-lactamase. J. Mol. Biol. 184:331–342, 1985.
146. Lin, S.H., Konishi, Y., Nall, B.T., Scheraga, H.A. Influence of an extrinsic cross-link on the folding pathway of ribonuclease A. Kinetics of folding-unfolding. Biochemistry 24:2680–2686, 1985.
147. Kelley, R.F., Wilson, J., Bryant, C., Stellwagen, E. Effects of guanidine hydrochloride on the refolding kinetics of denatured thioredoxin. Biochemistry 25:728–732, 1986.
148. Matthews, C.R. Effect of point mutations on the folding of globular proteins. Methods Enzymol. 154:498–511, 1987.
149. Chen, B., Baase, W.A., Schellman, J.A. Low-temperature unfolding of a mutant of phase T4 lysozyme. 2. Kinetic investigations. Biochemistry 28:691–699, 1989.
150. Ikeguchi, M., Fujino, M., Sugawara, T., Kuwajima, K., Sugai, S. The manuscript in preparation.
151. Goto, Y., Hamaguchi, K. Unfolding and refolding of the reduced constant fragment of the immunoglobulin light chain. Kinetic role of the intrachain disulfide bond. J. Mol. Biol. 156:911–926, 1982.
152. Segawa, S., Sugihara, M. Characterization of the transition state of lysozyme unfolding. II. Effects of the intrachain crosslinking and the inhibitor binding on the transition state. Biopolymers 23:2489–2498, 1984.
153. Koseki, T., Kitabatake, N., Doi, E. Conformational changes in ovalbumin at acid pH. J. Biochem. (Tokyo) 103:425–430, 1988.
154. Brems, D.N., Havel, H.A. Folding of bovine growth hormone is consistent with the molten globule hypothesis. PROTEINS: Structure, Function, Genetics 5:93–94, 1989.
155. Acharya, K.R., Stuart, D.I., Walker, N.P.C, Lewis, M., Phillips, D.C. Refined structure of baboon α-lactalbumin at 1.7 Å resolution. J. Mol. Biol. 208:99–127, 1989.