The molten globule state of α-lactalbumin

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ABSTRACT **The molten globule state of α-lactalbumin is the best-characterized folding intermediate of globular proteins and has been studied intensively by various spectroscopic and physicochemical techniques, including stopped-flow CD and fluorescence spectroscopies, a hydrogen-exchange technique, ^{1}H-NMR spectroscopy, disulfide-exchange chemistry, site-directed mutagenesis, and calorimetric techniques. This review summarizes recent studies. Major findings about the structure of the molten globule state are: *1*) It is highly heterogeneous, having a highly structured α-helical domain with the β-sheet domain being significantly unfolded; and *2*) it is not a nonspecific, collapsed polypeptide but already has a native-like tertiary fold. These structural characteristics are essential to fully understand the thermodynamic properties of the molten globule state, which are described in connection with a recently proposed computational approach to predict the structure of the molten globule state of a protein. Mutant proteins in which the stability of the molten globule state was changed were constructed. Studies of the equilibrium unfolding and kinetic refolding of the mutant proteins will provide further insight into the molten globule state as a folding intermediate. In spite of an initial expectation that the structure recognized by an *Escherichia coli* chaperone, GroEL, is the molten globule, the interaction of GroEL with α-lactalbumin in the molten globule state is much weaker than the interaction with more unfolded states of α-lactalbumin, a disulfide-reduced form, and disulfide rearranged species.—Kuwajima, K. The molten globule state of α-lactalbumin.—*FASEB J.* 10, 102–109 (1996)**

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THE MOST ESSENTIAL QUESTION IN protein folding studies is how an unstructured polypeptide of a protein can fold into its unique native conformation in a reasonable period of time even though there are an astronomically large number of possible conformational states (1). This so-called Levinthal's paradox has directed researchers in this field to try to detect and characterize possible intermediates between the native and fully unfolded states (2–11). The presence of a unique pathway of folding, on which a limited number of specific intermediates are well populated, seems to provide an answer to the paradox. Our understanding of the folding intermediates has made great advances during the last 20 years. Intermediates that were characterized in only a few proteins 20 years ago (12) were regarded as rather exceptional (13, 14). Now we find many reports on the structure of the folding intermediates even at a level of atomic resolution. The availability of new experimental techniques was responsible for this advance in protein folding studies. Studies by stopped-flow circular dichroism (CD)[2] techniques have unequivocally shown the presence of kinetic refolding intermediates, which have appreciable amounts of secondary structure and accumulate within 10 ms after the refolding starts in a globular protein (7, 15, 16). Hydrogen-exchange labeling combined with 2D NMR spectroscopy has provided a powerful technique by which we can characterize the structure of refolding intermediates at the level of amino acid residue resolution (6, 17).

Many excellent reviews have appeared on folding intermediates of globular proteins characterized by the advanced techniques mentioned above(6, 7, 15–21). This review therefore does not attempt to be so comprehensive as to include all aspects of kinetic folding intermediates. It confines the subject to the molten globule state of α-lactalbumin because this intermediate has been studied intensively in the last few years. α-Lactalbumin displays the best-characterized molten globule state and is the best model protein in protein folding studies.

α-LACTALBUMIN

α-Lactalbumin is a small Ca^{2+}-binding protein (14,200 Da) present in mammalian milk and functions as a specificity modifier of an enzyme, galactosyltransferase (22–24). It is genetically and structurally homologous to c-type lysozyme (22, 24–26), and its high-resolution X-ray crystallographic structure has already been reported (27–30) (**Fig. 1**). The structure of α-lactalbumin consists of two subdomains: an α-helical domain and a β-sheet domain. There are four α-helices—A [residues 5-11], B [23–34], C [86-99], and D [105-109]—and three 3_{10}-helices [12-16, 101-104, and 115-119] in the α-helical

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[2]Abbreviations: CD, circular dichroism; 2D NMR, 2-dimensional NMR; GdnHCl, guanidine hydrochloride; 2D NOESY, 2-dimensional nuclear Overhauser effect spectroscopy.

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domain; and an anti-parallel β-sheet [40-50] and a 3_{10}-helix [76-82] in the β-sheet domain. The structural and functional properties of α-lactalbumin and its interrelationships with lysozyme are excellently reviewed by McKenzie and White (24) and by Kronman (31). A remarkable property of α-lactalbumin as a model of protein folding studies is the high stability of its molten globule state (4), which is observed in the following conditions (4): *1)* an equilibrium unfolding intermediate at a moderate concentration of a strong denaturant [guanidine hydrochloride (GdnHCl) or urea], *2)* the acid-denatured state, and *3)* a partially unfolded state produced by removal of the bound Ca^{2+} at neutral pH and low salt concentration. The known structural characteristics of the molten globule state are: *1)* native-like secondary structure, *2)* compact structure with a radius only 10–20% larger than that of the native molecule, and *3)* the absence of the specific tertiary packing interactions of amino acid side chains (4). Our group studied the equilibrium and kinetics of the unfolding-refolding reactions among the three states—the native, the molten globule, and the fully unfolded states (12, 32, 33)—and proposed a folding model based on the three-state unfolding of α-lactalbumin (34). This model was later verified, using a kinetic CD technique (35, 36), by direct observation of a transient refolding intermediate that is identical with the molten globule state. These studies have been reviewed by the author (4, 15, 16, 37) and by Sugai and Ikeguchi (38). In this review, only the most recent progress in the studies of the molten globule state of α-lactalbumin will be described.

HOW NATIVE-LIKE IS THE MOLTEN GLOBULE?

Although the molten globule state has native-like secondary structure as measured by the far UV CD spectra, it is important to ask whether the locations of the secondary structure segments along the polypeptide chain are the same as in the native state. Dobson and co-workers (39–41) used ^{1}H-NMR spectroscopy and a hydrogen exchange technique for investigating the structure of the molten globule state of α-lactalbumin, finding that the B and C helices are formed and have protected amide protons. The structure of the molten globule is highly heterogeneous, having the highly structured α-helical domain formed by loose hydrophobic interactions whereas the β-sheet domain is significantly more unfolded. This picture of the molten globule state, organization of the native secondary structure segments in a part of a protein molecule by loose tertiary contacts, is also revealed in other globular proteins by the hydrogen-exchange technique combined with ^{1}H-NMR spectroscopy (6, 17, 19, 42–46). It is, however, important to note that the hydrogen exchange technique can detect only native secondary structure. The amide protons protected in the nonnative secondary structure, if present, will be exchanged out during the NMR measurement done in the native state. Formation of nonnative α-helices at an early stage of refolding has been suggested in β-lactoglobulin by equilibrium and kinetic CD studies (16, 47).

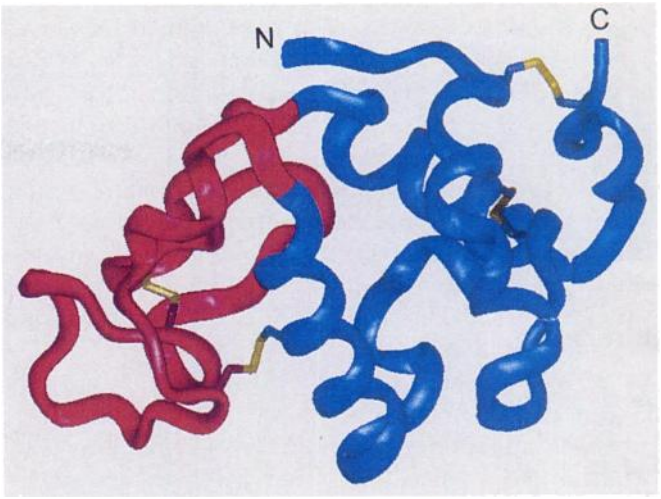

Figure 1. Schematic representation of the structure of baboon α-lactalbumin generated from the structure proposed by Acharya et al. (27), using coordinates 1ALC of the Brookhaven Protein Data Bank. The α-helical domain (residues 1-37 and 85-123) is shown in blue and the δ-sheet domain (residues 38-84) in red. Four yellow sticks indicate the four disulfide bonds of the protein.

Alexandrescu et al. have reported the 2-dimensional nuclear Overhauser effect spectroscopy (2D NOESY) spectra of α-lactalbumin in the molten globule state (40). They have shown that the side chains of Tyr^{103}, Trp^{104}, and His^{107} form a hydrophobic cluster in the molten globule state, but that the structure in this region may differ from the corresponding region in the fully native protein. Hydrogen exchange of tryptophan indole protons, photochemically induced dynamic nuclear polarization, and paramagnetic perturbation of the NMR spectra have also shown that Trp^{26} and Trp^{104}, buried in the native state, are buried in the molten globule (41, 48) so that some form of a hydrophobic core in the α-helical domain of the native structure may persist in the molten globule state. Hamada et al. (49) have reported that the cleavage of the peptide bond between Asp^{66} and Pro^{67} located at the edge of the β-sheet domain makes the α-lactalbumin molecule characteristic of the molten globule with respect to CD and fluorescence spectra and 1-anilino-naphthalene-8-sulfonate (ANS) binding. Nölting et al. (50) have shown the presence of significant conformational relaxation at a time scale faster than 500 ns in the acidic molten globule state by ultrasonic velocimetry.

Another important issue as to the structure of the molten globule state is: Is it a nonspecific collapsed polypeptide or does it already have a native-like tertiary fold? Ikeguchi et al. (51) found that selective reduction of the 6-120 disulfide bond of bovine α-lactalbumin reduces the stability of the molten globule state by 0.8~1.2 kcal/mol, and have suggested that some ordered structure is present within the loop moiety formed by this disulfide.

Creighton and Ewbank (52–54) have studied the three-disulfide species of human α-lactalbumin and found that the three-disulfide form with free thiols on Cys^{6} and Cys^{120} can adopt the molten globule conformation and then spontaneously rearrange its three disulfide bonds to many isomers that maintain similar conformations with respect to spectroscopic and hydrodynamic properties. They concluded that the molten globule state of α-lactalbumin does not maintain the native-like topology of the polypeptide backbone but is more like a collapsed form of an unfolded protein.

A series of recent papers by Kim and co-workers (55–58) also illuminates this issue, although their conclusion is apparently contradictory to that drawn by Creighton and Ewbank (54). They constructed a single-chain, recombinant model of the α-helical domain of human α-lactalbumin, the α-domain, which consists of residues 1-39 and 81-123 of α-lactalbumin connected by a short linker of three glycines (55). The α-domain that has two native disulfide bonds (6-120 and 28-111) shows characteristics of the molten globule state with respect to the CD and ^{1}H-NMR spectra and the diffuse thermal transition, showing that this mini-"protein" cannot form a rigid tertiary structure under the same condition that the intact protein forms the native structure. The disulfide-exchange reaction of α-domain in a redox buffer solution, however, produced predominantly the native disulfide bonds, although the other two nonnative disulfides have much higher probabilities if they are formed in a random-pairing model of a fully unfolded polypeptide. The results clearly indicate that α-domain has a native-like tertiary fold in spite of its molten globule characteristics. They have also studied two kinds of the full-length species [α-LA(α) and α-LA(β)] (56). α-LA(α) contains the same two disulfide bonds as in the α-domain, with the cysteines for the other two disulfides replaced by alanines, whereas α-LA(β) contains the 61-77 disulfide in the β-sheet domain and the interdomain 73-91 disulfide bond, with the cysteines in the α-helical domain replaced by alanines. α-LA(α) shows the same equilibrium preferences of formation of the native disulfides as observed in the α-domain, but α-LA(β) does not show a strong preference for the native disulfide formation. Thus, the molten globule state of α-lactalbumin has a heterogeneous structure in which the α-helical domain resembles an expanded native-like protein and the β-sheet domain is largely unstructured. These results are consistent with the hydrogen exchange results of Dobson and co-workers (41) and also with the study of the three-disulfide species by Ikeguchi et al. (51). The apparent contradiction with the results of Creighton and Ewbank (54) may be interpreted in terms of differences in the number of possible disulfide pairings between the proteins used by the two groups. Intact α-lactalbumin, used by Creighton's group, contains all eight cysteines, so that the preference for the native disulfides in the α-helical domain may be obscured by much more random distribution of disulfide pairings possible in the intact protein.

Peng et al. (57, 58) have recently reported the structural specificity of local regions surrounding specific disulfide bonds in the molten globule state. They determined the effective concentrations for disulfide bond formation for the two native disulfide bonds (6-120 and 28-111) in the α-helical domain and four nonnative disulfides (6-28, 6-111, 28-120, and 111-120) by using the single-disulfide mutants of full-length α-lactalbumin. The effective concentration for formation of the native 28-111 disulfide bond was more than 10-fold higher than the concentration for formation of any other native or nonnative disulfide bond and more than 1000-fold higher than the concentration expected in the random pairing model. Thus, the local region surrounding the 28-111 disulfide bond has a high preference for adopting a native-like structure in the molten globule state.

THERMODYNAMIC PROPERTIES

A remarkable property of the molten globule state of α-lactalbumin is its diffuse thermal transition. Apparently, there is no cooperative heat absorption peak in scanning calorimetric measurements when the heating is started from the molten globule state at acid pH (35, 59, 60). This observation suggests that the molten globule and the thermally unfolded states are thermodynamically indistinguishable, so that there is no thermal unfolding when we start from the molten globule state. In fact, the thermally unfolded state of α-lactalbumin shares common characteristics with the molten globule state—i.e., the native-like secondary structure and the compactness of the molecule—especially when the thermal unfolding is realized at a relatively low temperature by destabilizing the native state (35, 60, 61).

Physical interpretation of the absence of the cooperative thermal transition of the molten globule state has, however, been controversial. By using multidimensional analysis of the heat capacity surface obtained from scanning calorimetric measurements of α-lactalbumin at different concentrations of GdnHCl, Xie et al. (62) have suggested that the intrinsic enthalpy change from the molten globule to the thermally unfolded state must be much higher (24 kcal/mol at 25°C) than that observed experimentally in the presence of GdnHCl or at an acid pH; it is even higher than the enthalpy change from the native to the molten globule state (only 8 kcal/mol by their estimate at 25°C). This conclusion has, however, been disputed by Yutani et al. (63), who have examined the heat capacity function of apo-α-lactalbumin in the molten globule state at neutral pH without GdnHCl by differential scanning microcalorimetry and have shown the absence of the thermal transition. Although Xie et al. (64), opposing the conclusion of Yutani et al. (63), later attributed the absence of the thermal transition to ionic strength dependence of the thermal transition temperature, their argument is misleading because the thermal transition observed at a high ionic strength is not the

transition from the molten globule state but rather the transition from the native to the molten globule state (65).

An extensive study of the energetics of the α-lactalbumin states has recently been reported by Griko et al. (66). At pH values close to neutral, the thermal unfolding from the native state occurs at high temperature and yields a fully unfolded polypeptide with no measurable population of partly folded intermediates. At lower pH values, however, the unfolding occurs at lower temperatures, and a progressively higher population of the molten globule-like intermediates was observed. Because apo-α-lactalbumin unfolds at a low temperature even at a neutral pH, their observation of the population of the molten globule after the thermal unfolding is consistent with the results of Yutani et al. (63). Griko et al. (66) have suggested that the molten globule state has a higher enthalpy than the unfolded state below 45°C, so that when the thermal unfolding occurs below 45°C the thermally unfolded state corresponds to the molten globule state, and once the molten globule is formed, the structure of the protein gradually unfolds upon heating and shows a gradual increase in heat capacity.

The unfolding of the molten globule state induced by a denaturant, urea or GdnHCl, is, however, known to show a cooperative transition, and Ptitsyn and Uversky (67) recently demonstrated that the transition from the molten globule to the fully unfolded state is a two-state transition. The thermal unfolding of α-lactalbumin has also been studied by fluorescence spectroscopy and binding of a hydrophobic fluorophor (68–72). Shimizu et al. (73) have studied the urea-induced unfolding of the molten globule state of α-lactalbumin by titration of aromatic resonances in the ^{1}H-NMR spectra and demonstrated that the unfolding of the molten globule is not a cooperative two-state process.

Xie and Freire (74) have recently investigated partly folded structures of α-lactalbumin, which are consistent with the experimental thermodynamic data, by an interesting computational approach. They generated many partly folded structures from the known X-ray crystallographic structure of α-lactalbumin, using a combinatorial unfolding algorithm. The thermodynamic properties of the partly folded states have been predicted by calculating the accessible surface area and by using an empirically derived structural parameterization. Partly folded states were chosen that satisfy the thermodynamic properties of the molten globule state. The criteria used to choose these states are a lower heat capacity and a higher enthalpy at low temperatures than those in the fully unfolded state (66, 74). Several partly folded states were chosen. All of them have the A, B, and C helices folded, whereas the β-sheet and the D helix were unfolded. Their results are thus consistent with the NMR and hydrogen exchange studies by Dobson's group (41), who have shown the B and C helices to be formed in the molten globule state. Their results are, however, not fully consistent with the results of Peng et al. (58), who have shown that the region surrounding the 28-111 disulfide bond, which may include the D helix, is formed in the molten globule state. In all their calculations, Xie and Freire (74) have assumed that the folded regions in the partly folded states preserve the conformation that exists in the native structure. This is clearly an oversimplification. Quantitative agreement in the thermodynamic parameters between the theory and experiment, they have reported, is thus rather surprising. They have recently applied their computational approach to investigating the molten globule states of other globular proteins (75–77).

Studies of the α-lactalbumin mutants in which the stability of the molten globule state is changed by the mutations will be very useful for elucidating the molecular mechanism of stabilization of the molten globule state. Recently, Uchiyama et al. (78) have reported studies of such mutants of goat α-lactalbumin. They introduced a Thr to Ile mutation at residues 29 and 33, and a Ala to Ile or Ala to Thr mutation at residue 30. The mutation sites are located in the B helix, and the mutations change the hydrophobicity of the core of the α-helical domain formed by the B helix. The choice of these mutation sites is based on the fact that guinea pig α-lactalbumin shows the most stable molten globule state among the three α-lactalbumin species from goat, bovine, and guinea pig. The stabilization free energies of the molten globule state have been estimated to be 3.1, 1.5, and 0.7 kcal/mol for the guinea pig, bovine, and goat proteins, respectively (78). The amino acid residues at these sites are all threonines in the bovine protein; residue 30 is replaced by an alanine in the goat protein, with the other two residues being the same as in the bovine protein, and the three residues are all replaced by hydrophobic isoleucines in the guinea pig protein. The stability of the molten globule state of the five mutants (T29I, A30T, A30I, T33I, and A30I+T33I) of goat α-lactalbumin has been investigated by urea-induced unfolding transition at pH 2.0, where the molten globule state is stable without urea. The molten globule state is stabilized by the amino acid substitutions that raise the hydrophobicity of the residues, suggesting that the hydrophobic core in a globular protein plays an important role in stabilization of the molten globule state. The increase in stability of the molten globule state is much smaller than the difference in stability between the guinea pig and goat proteins and less than 10% of the change expected from the transfer free energy of amino acids from water to hydrophobic environment, e.g., the A30I+T33I double mutation increases the stability of the molten globule state only by 0.5 kcal/mol (78). Thus, the amino acid substitutions other than those at the three sites just referred to are also responsible for the remarkable stability of the molten globule state of the guinea pig protein. It has also been concluded that the hydrophobic core in the molten globule state is highly hydrated and much looser than the core of the native molecule (78).

Pardon et al. (79) have recently constructed an interesting chimera of human lysozyme and bovine α-lactalbumin. In this chimera, amino acid residues 76-102 of

lysozyme were replaced by α-lactalbumin 72-97, which represents the Ca^{2+}-binding loop and the central helix C. The chimera protein has acquired strong Ca^{2+}-binding capacity and shows the ability to form a stable molten globule state under relatively mild conditions. Pardon et al. (79) have suggested that the peculiar aspartate-rich sequence of the Ca^{2+}-binding site is one contribution to the stability of the molten globule state and that the C helix of the α-lactalbumin molecule itself carries important features that determine the molten globule propensity.

THE MOLTEN GLOBULE AS A MODEL TARGET OF MOLECULAR CHAPERONES

Discovery of molecular chaperones has led to the understanding that various cell-biological phenomena are closely related to the problem of protein folding (80, 81). Among various chaperone proteins, the best-characterized chaperone from a physicochemical point of view is the *Escherichia coli* protein GroEL (14-mer of 60,000 Da subunits), which is a member of the chaperonin 60 family. From their observation of in vitro folding of dihydrofolate reductase and rhodanese on the surface of GroEL, Hartl and co-workers (82–84) have suggested that the conformation of GroEL-bound polypeptides corresponds to the molten globule state and have proposed a model in which *E. coli* chaperones—DnaK, DnaJ, and GroEL—act successively along the pathway of chaperone-mediated protein folding in vivo. Because α-lactalbumin exhibits the best-characterized molten globule state, this protein should provide a pertinent model to observed directly the interaction between GroEL and the molten globule state.

Okazaki et al. (85) have thus investigated the interactions between GroEL and two kinds of nonnative α-lactalbumin—the molten globule state and the disulfide reduced form—by molecular sieve chromatography and hydrogen exchange measurements. The molten globule state was produced by removal of the bound Ca^{2+} at neutral pH where GroEL is in the fully native state. Disulfide-reduced α-lactalbumin assumes a more relaxed and expanded structure than the molten globule state of the disulfide-intact protein (85). It is rather surprising that the results of Okazaki et al. (85) report that the interaction between GroEL and apo-α-lactalbumin in the molten globule state cannot be observed by molecular sieve chromatography, which indicates that the interaction, if any, must be weak. Their results also show that disulfide-reduced α-lactalbumin is strongly bound to GroEL when 50 mM KCl is present. Therefore, the protein state recognized by GroEL may be more unfolded and expanded than the typical molten globule state of α-lactalbumin.

Hayer-Hartl et al. (86) reported essentially the same experimental results of the interactions between GroEL and nonnative α-lactalbumin as reported by Okazaki et al. (85), but their conclusion is apparently contradictory. Hayer-Hartl et al. (85) have concluded that GroEL interacts with the hydrophobic surfaces exposed by proteins in a flexible compact intermediate or molten globule state. The discrepancy between the conclusions is ascribable to different meanings of the term "molten globule" used by the two groups. Hayer-Hartl et al. (87) are using this term in a wider context, to include the conformational states of disulfide-reduced or disulfide-rearranged species of α-lactalbumin. Such extension of the term is, however, very confusing and misleading (88). As we have seen before, an important characteristic of the molten globule state is its native-like tertiary fold, which is difficult to realize in the disulfide-reduced species and never realized in the disulfide-rearranged species.

Robinson et al. (89) have recently investigated the conformation of a three-disulfide derivative of α-lactalbumin bound to GroEL by directly monitoring its hydrogen exchange kinetics using electrospray ionization mass spectrometry. The bound protein weakly protected amide protons from exchange to an extent closely similar to that of an uncomplexed molten globule state of the three-disulfide protein with native disulfide bonds, apparently suggesting that the protein bound to GroEL resembles a molten globule state. The three-disulfide derivative of α-lactalbumin they used, however, again was the disulfide-rearranged species. Moreover, the three-disulfide protein with native disulfides in the molten globule state is not strongly bound by GroEL (86), so that once the molecule acquires a native-like tertiary fold with native disulfide bonds, it may not be recognized strongly by GroEL. Recently, we have studied hydrogen exchange kinetics of tritium-labeled α-lactalbumin with the four disulfide bonds being fully reduced, according to tritium-exchange measurements (Okazaki et al. unpublished data). The exchange kinetics measured in the absence and presence of GroEL have been found coincident with each other, although there is some secondary structure exhibiting protected amide protons in this partly folded state of α-lactalbumin. Thus, in spite of strong binding of disulfide reduced α-lactalbumin to GroEL, the secondary structure segments are not recognized and stabilized by GroEL. Therefore, what is recognized by GroEL is not the secondary structure nor a native-like tertiary fold, although both are typical characteristics of the molten globule state.

CONCLUDING REMARKS

This review summarizes recent progress in the studies of the molten globule state of α-lactalbumin. The structural characteristics of the molten globule state of this protein are now much more clearly defined. The most important characteristic emerging from recent studies is the native-like tertiary fold, the formation of which, at least in a part of the molecule, has also been suggested in the molten globule states of other globular proteins (6, 17, 19, 42–46) and is probably a common characteristic of the molten globule state for globular proteins similar in size

α-lactalbumin. It is also well documented that the number of tertiary folds that exist in natural globular proteins is not very large and most of the proteins are classified into only around 1000 protein families (90, 91). As it is likely that the number of tertiary folds is limited by physical constraints of protein structure (92), an important next step in protein folding studies is to elucidate the physical constraints or interactions that are necessary and sufficient to select specifically one of these tertiary folds for a particular protein. In this respect, studies of the molecular mechanism of stabilization of the molten globule state must be considered important.

It is also remarkable that the formation of the molten globule state from the fully unfolded state occurs very rapidly, within 10 ms after the refolding of α-lactalbumin begins. Recently, we have reexamined the refolding reactions of α-lactalbumin from the GdnHCl-induced, fully unfolded state by stopped-flow CD spectroscopy (Arai and K. Kuwajima, unpublished data). An early folding intermediate formed within the dead time (~10 ms) of the stopped-flow measurements has the same spectral properties and the same stability against GdnHCl-induced unfolding as the equilibrium molten globule state. Very rapid refolding within an interval of a few millisecond has recently been observed for several small globular proteins with approximately 60 amino acid residues (93–98), and it has been proposed that the direct folding to the native state is a much more rapid process than previously was expected (98, 99). Because the α-helical domain of α-lactalbumin is similar in size to these small globular proteins, the rapidity of formation of the molten globule state might be analogous to the rapidity of folding of these proteins. If this is the case, the slow folding of α-lactalbumin may be rate-limited by rearrangement of the already partly organized α-helical domain and the more unfolded β-sheet domain.

The experimental study by Griko et al. (66) and the theoretical calculation by Xie and Freire (74) seem to provide a reasonable interpretation of the thermodynamic characteristics of the molten globule state. Studies of the mutant proteins that stabilize or destabilize the molten globule state will provide a more concrete insight into the molecular interpretation of the thermodynamic properties and their relationship to the structure in the molten globule state. Kinetic refolding studies of these mutant proteins must be important for answering the question whether the molten globule is an obligatory intermediate in kinetic folding or is merely a species produced by a kinetic trap. Despite great advances in our understanding of the molten globule state, this important question remains unsolved.

The molten globule state and the disulfide-reduced form of α-lactalbumin have been shown to be good model targets for the chaperonin GroEL. The interaction between GroEL and the molten globule of disulfide-intact α-lactalbumin has recently been investigated more quantitatively by comparing the refolding kinetics of the protein in the absence and presence of GroEL (100; K. Katsumata et al., unpublished data). The binding constant estimated as around 10^5 M^{-1} (pH 7 and 25°C at 50 mM sodium cacodylate) is shown to be two orders of magnitude smaller than the binding constant between GroEL and disulfide-reduced α-lactalbumin and too weak to detect by molecular sieve chromatography. Although such chaperone studies are not directly connected with elucidation of the molecular mechanism of stabilization of the molten globule state, the studies are important for understanding the relationships between the in vitro and in vivo folding reactions. Many other chaperone proteins are required in the correct folding of a protein in vivo (80, 81), and physicochemical studies of the interactions between the chaperone proteins and a model target will become progressively important. The well-characterized molten globule state of α-lactalbumin will also be useful in such studies, and will be the favorite among many protein folders. FJ

Note added in proof: After completion of this article, a few interesting papers (101–103) concerning the molten globule state of α-lactalbumin have been published. The results described in these papers are consistent with the picture of the molten globule described here.

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