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Structural characterization of the molten globule of α-lactalbumin by solution X-ray scattering

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Abstract

A compact denatured state is often observed under a mild denaturation condition for various proteins. A typical example is the α-lactalbumin molten globule. Although the molecular compactness and shape are the essential properties for defining the molten globule, there have been ambiguities of these properties for the molten globule of α-lactalbumin. Using solution X-ray scattering, we have examined the structural properties of two types of molten globule of α-lactalbumin, the apo-protein at neutral pH and the acid molten globule. The radius of gyration for the native holo-protein was 15.7 Å, but the two different molten globules both had a radius of gyration of 17.2 Å. The maximum dimension of the molecule was also increased from 50 Å for the native state to 60 Å for the molten globule. These values clearly indicate that the molten globule is not as compact as the native state. The increment in the radius of gyration was less than 10% for the α-lactalbumin molten globule, compared with up to 30% for the molten globules of other globular proteins. Intramolecular disulfide bonds restrict the molecular expansion of the molten globule. The distance distribution function of the α-lactalbumin molten globule is composed of a single peak suggesting a globular shape, which is simply swollen from the native state. The scattering profile in the high Q region of the molten globule indicates the presence of a significant amount of tertiary fold. Based on the structural properties obtained by solution X-ray scattering, general and conceptual structural images for the molten globules of various proteins are described and compared with the individual, detailed structural model obtained by nuclear magnetic resonance.

Keywords: α-lactalbumin; compact denatured state; molecular compactness; molten globule; protein folding; solution X-ray scattering

It is now widely accepted that the structural characterization of protein non-native conformations is as important as the determination of the high-resolution 3-dimensional structure of the native state in order to understand protein folding and stability (Dill & Shortle, 1991; Creighton et al., 1996; Shortle, 1996). The high-resolution structure of the native state is obtained by analysis either in crystal by X-ray or in solution by nuclear magnetic resonance (NMR). On the other hand, neither technique can be applied to non-native states such as denatured states and folding intermediates, since these would be ensembles of various conformational states. The solution X-ray scattering technique has been successfully utilized to describe the characteristics of various non-native conformations (Damaschun et al., 1991, 1993; Flanagan et al., 1992, 1993; Kataoka et al., 1993, 1995; Konno et al., 1995; Kataoka & Goto, 1996). Although it is a low-resolution technique, it gives information on molecular size, molecular shape, and, in some cases, tertiary fold, which are essential structural properties for describing the extent of folding. Structural characterization in terms of these properties is also essential for evaluating the various theories or models of folding (Lattman, 1994; Lattman et al., 1994; Kataoka & Goto, 1996).

We can observe two types of non-native conformation experimentally. One is a highly denatured or fully unfolded state and the other is the compact intermediates which have been observed under both transient and equilibrium conditions. The term molten globule (Ohgushi & Wada, 1983) is frequently used to specify compact folding intermediates (Ptitsyn, 1987, 1992, 1995; Kuwajima, 1989). The molten globule is a rigorously defined structural state: (1) substantial secondary structure, (2) absence of native-like tertiary structure, (3) compact and globular, (4) without cooperative thermal denaturation (Ptitsyn, 1987, 1992, 1995; Kuwajima, 1989; Fink, 1995). Since some of the structural properties of the molten globule are quite similar to those of kinetic folding inter-

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Abbreviations: NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; R_g, radius of gyration; R_s, Stokes radius; d_{max}, largest dimension of molecule.

mediates, they are considered to be common folding intermediates of globular proteins (Kuwajima, 1989; Fink, 1995; Ptitsyn, 1995). It has also been argued that the observed transient intermediate is a kinetically trapped state, and not a productive intermediate on the folding pathway (Sosnick et al., 1994, Fersht, 1995). Thus, the exact role of the molten globule in protein folding seems controversial at present. The structural characterization of the compact denatured states is essential and important for the better understanding of the protein folding and stability. Throughout this paper, we will use the term molten globule to describe the compact denatured state of cytochrome *c*, α-lactalbumin, and apomyoglobin, because these have been regarded as typical molten globules, although we understand that the real structure of the so-called molten globule is different from the original definition (Baum et al., 1989; Hughson et al., 1990; Alexandrescu et al., 1993; Chyan et al., 1993; Kataoka et al., 1995; Loh et al.; 1995, Privalov, 1996).

We have characterized the molten globule and compact denatured states of cytochrome *c*, apomyoglobin, and staphylococcal nuclease by small-angle X-ray scattering (SAXS) (Flanagan et al, 1992, 1993; Kataoka et al., 1993, 1994, 1995; Nishii et al., 1994, 1995). We observed that the radius of gyration (R_g) for the molten globule is 20–30% larger than that for the native state (Kataoka et al., 1995). On the other hand, Ptitsyn and co-workers reported that R_g of the molten globule of α-lactalbumin was almost identical to the value for the native state (Dolgikh et al., 1981, 1985). Nevertheless, they have also reported an increase in the Stokes radius (R_s) for the molten globule of α-lactalbumin (Gast et al., 1986, 1994; Ptitsyn, 1992). Thus, the information on the compactness of the molten globule of α-lactalbumin is still obscure, although compactness is an essential property to define the molten globule.

In this paper, we describe the structural characterization of the molten globule of α-lactalbumin revealed by SAXS measurements. We revealed that the molten globule is not as compact as the native state. The structural properties of the α-lactalbumin molten globule were then compared with those of molten globules of other proteins to provide conceptual images of various types of molten globules. We also describe the more concrete structural image of the molten globule of α-lactalbumin through the comparison of SAXS structural view with the structural image obtained by NMR (Baum et al., 1989; Alexandrescu et al., 1993; Chyan et al., 1993; Shulman et al., 1995).

Results

Guinier plot and the radius of gyration

Figure 1 shows the concentration dependence of Guinier plots for various conformational states of α-lactalbumin. Holo-α-lactalbumin (A) is in the native state, while apo-α-lactalbumin (B) is in a molten globule state. Inter-particle interference effects are significant for the acid molten globule (C), as reflected by downward curvature toward zero angle, and the resulting maximum shifts toward the higher angle side at higher protein concentration. This has already been shown by Ptitsyn and coworkers (Dolgikh et al., 1981). On the other hand, apo-α-lactalbumin shows little interparticle interference up to 10 mg/mL, and holo-α-lactalbumin shows essentially none.

The R_g values at infinite dilution were obtained by extrapolation to zero protein concentration on the plot of R_g^2 vs protein concentration (Fig. 2). Figure 2 was obtained by a combination of two independent experiments carried out on different protein preparations. The R_g value for holo-α-lactalbumin is 15.7 Å, which is almost the same as that (15.6 Å) reported by Dolgikh et al. (1981). The R_g values for the two different molten globules converge at the same value, 17.2 Å (Fig. 2). Similar R_g values were obtained from

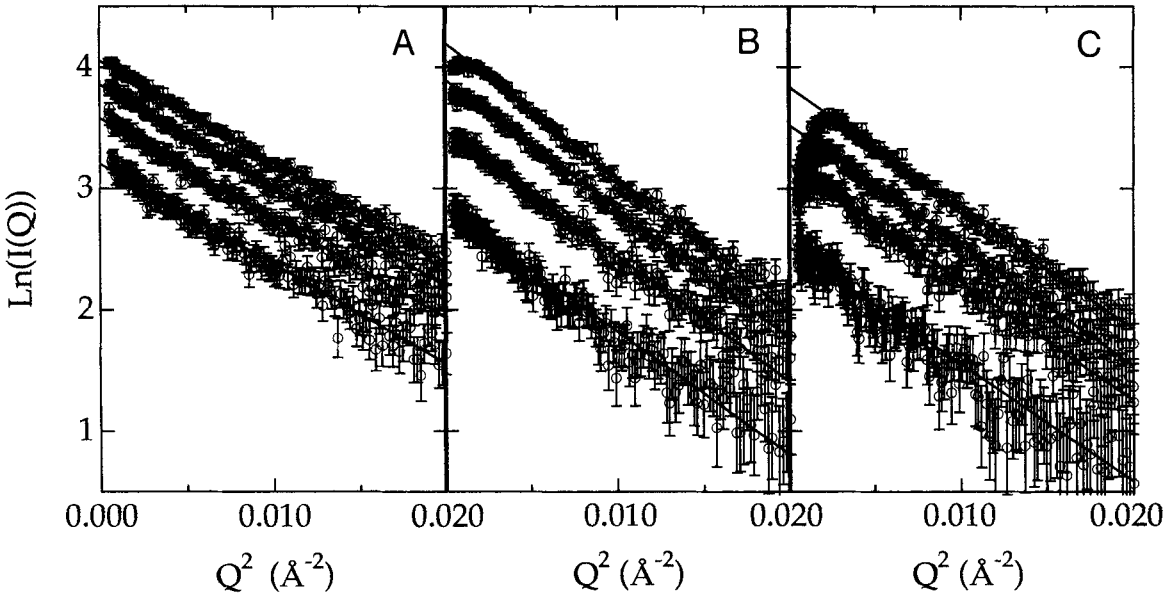

Fig. 1. Concentration series of Guinier plots for various conformational states of α-lactalbumin. **A:** Holo-α-lactalbumin in the native state dissolved in 20 mM Tris-HCl buffer containing 2 mM $CaCl_2$ (pH 8.0). Protein concentration for each plot, from top to bottom, is 10.6 mg/mL, 8.5 mg/mL, 6.4 mg/mL, and 4.3 mg/mL, respectively. **B:** Apo-α-lactalbumin in the molten globule state dissolved in 20 mM Tris-HCl buffer containing 1 mM EGTA (pH 8.0). Protein concentration for each plot, from top to bottom, is 9.7 mg/mL, 7.3 mg/mL, 4.9 mg/mL, and 2.4 mg/mL, respectively. **C:** α-Lactalbumin in the acid molten globule state dissolved in 10 mM HCl (pH 2.0). Protein concentration for each plot, from top to bottom, is 9.5 mg/mL, 7.1 mg/mL, 4.7 mg/mL, and 2.4 mg/mL, respectively.

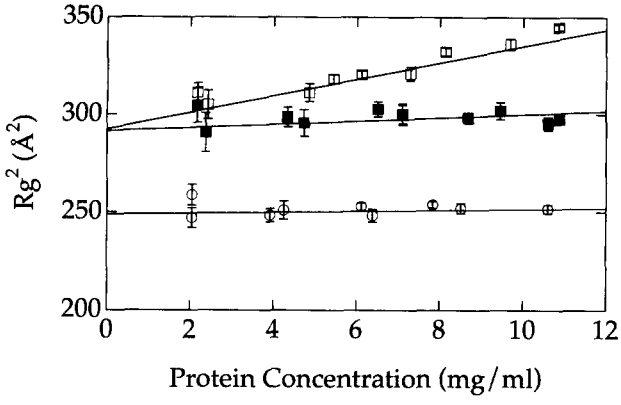

Fig. 2. Concentration dependence of R_g^2 for various conformational states of α-lactalbumin. (○) Holo-α-lactalbumin in the native state, (□) apo-α-lactalbumin, and (■) acid molten globule state.

Guinier plots of scattering curves extrapolated to zero protein concentration. The difference in concentration dependence of R_g for apo protein and the acid molten globule suggests that their surface properties differ, which is consistent with the difference in interparticle interference effect. The R_g value for the urea-unfolded state of intact α-lactalbumin obtained by 8 M urea was 30.0 Å (data not shown).

Association or aggregation would be one possible reason for the larger R_g value. To exclude the possibility, we examined the forward scattering, $I(0)$, which is a measure of molecular weight. The $c/I(0)$ should be proportional to the protein concentration if the system is monodisperse (Zimm, 1948). The plots (Fig. 3) are linear and do not show any significant decrease with increasing c, supporting no aggregation. The plots for holo- and apo-α-lactalbumin converge to almost the same value, as shown in Fig. 3. These results clearly indicate that the aggregation state of the molten globule is identical to that of the native state, i.e., monomeric. Therefore, the increase in R_g for the molten globule state is not due to molecular aggregation.

Shape of molten globule

Expression of the scattering profile in the form of a Kratky ($Q^2I(Q)$ vs. Q) plot (Kratky, 1982) is quite useful for protein folding studies

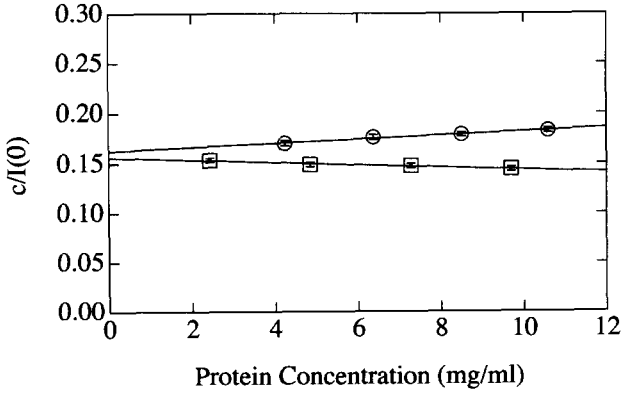

Fig. 3. Concentration dependence of $I(0)$ for holo-α-lactalbumin (○) and apo-α-lactalbumin (□). $kc/I(0) = 1/MW + 2A_2c$, where MW and A_2 are the molecular weight and the second virial constant, respectively, and k is a constant.

and thus widely utilized to describe the structural characteristics of various conformational states (Kataoka et al., 1993, 1994, 1995; Lattman, 1994; Flanagan et al., 1993; Damaschun et al., 1991, 1993; Konno et al., 1995). In folding studies, a peak in a Kratky plot is an expression of a compact globular structure (Kataoka et al. 1993, 1995; Lattman, 1994). The Kratky plot of a real chain in good solvent has a plateau in the moderate angle region and then tends to approach a straight line through the origin (Kratky, 1982).

Figure 4 shows Kratky plots of various conformational states of α-lactalbumin. The scattering curves for the native state and two different molten globules show a distinct peak in each plot. Further, the profiles for the apo-protein and acid molten globule are indistinguishable, indicating that these two different molten globules have essentially identical overall shapes and conformations, despite of the difference in the surface properties. The peak position at higher Q corresponds to the smaller size. Therefore, the peak shift toward the lower Q side for the molten globule from the native state corresponds to the increase of R_g. Thus, we can conclude that the molten globule is compact and globular, but swollen and expanded from the native state. The urea-denatured state does not show a clear peak in the Kratky plot, suggesting an expanded chain-like conformation.

Another important feature of Figure 4 is the fact that the scattering curve for the denatured state intersects the curves for the native state and molten globules at different points. These findings strongly indicate that the molten globule is an independent conformational state, but not an equilibrium mixture of the unfolded and native states.

Information on shape and size can also be obtained from the distance distribution function, $P(r)$. The $P(r)$ functions for the native and two different molten globule states of α-lactalbumin are shown in Figure 5. The two molten globules gave substantially identical $P(r)$ functions. The $P(r)$ functions for both the native and molten globule states show a single peak. The maximum chord of

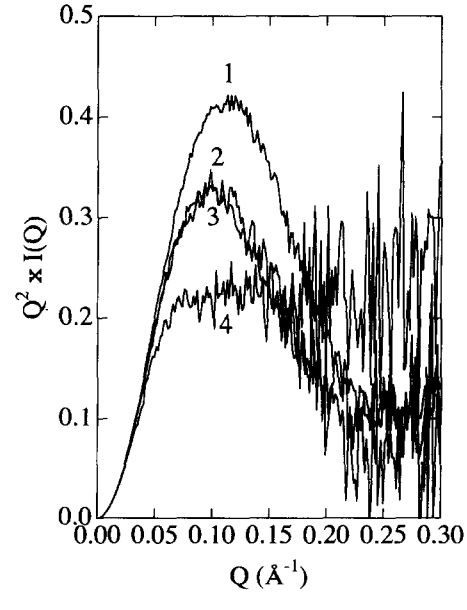

Fig. 4. Kratky plots of scattering profiles from various conformational states of α-lactalbumin. Curve 1, holo-α-lactalbumin; curve 2, apo-α-lactalbumin; curve 3, acid molten globule state; curve 4, urea-denatured state of intact α-lactalbumin. Note that curves 2 and 3 are essentially identical.

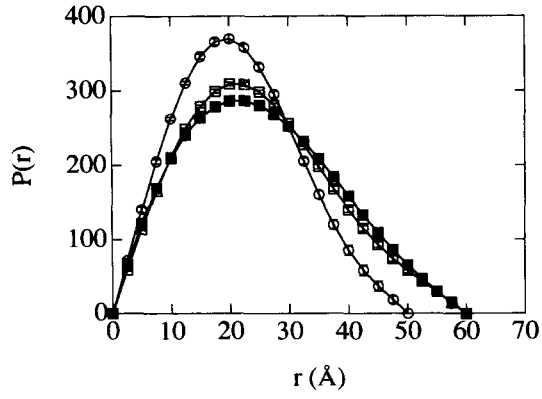

Fig. 5. Comparison of distance distribution function, $P(r)$, for the native and molten globule states of α-lactalbumin. (○) holo-α-lactalbumin in the native state, (□) apo-α-lactalbumin, and (■) acid molten globule state. $P(r)$ function of apo-α-lactalbumin is substantially identical to $P(r)$ of the acid molten globule. Errors are smaller than the symbol size, except where otherwise described.

the molecule, d_{max}, is obtained from the point where the function approaches zero. The d_{max} clearly increased from the native state to the molten globule state (Table 1). Therefore, it is again concluded that molten globule is expanded from the native state. The calculated d_{max} based on the crystal structure (Acharya et al., 1989) is 53 Å, which is similar to the observed value of 50 Å, and confirms that the scatterer is monomeric.

Tertiary fold

α-Lactalbumin is composed of two subdomains (Acharya et al., 1989): an α-helical domain and a β-sheet domain. According to the definition of the molten globule, it has native-like secondary structures. Using ^{1}H-NMR combined with the hydrogen exchange technique, Dobson and coworkers revealed that the α-helical domain possesses some native-like helices, whereas the β-sheet domain is significantly unfolded (Baum et al., 1989; Alexandrescu et al., 1993; Chyan et al., 1993). It is important to address whether there is definite spatial packing among the secondary structural elements of the molten globule or whether the elements are randomly condensed in the molecule. The formation of disulfide bonds suggests that the tertiary fold in the α-domain would be native-like (Peng & Kim, 1994).

The X-ray scattering profile in the moderate to high Q region ($0.2 < Q < 1.0$) reflects the fluctuation of electron density distribution within a protein molecule, i.e., the inner structure, rather than global shape (Kataoka et al., 1995). The protein inner structure appearing in these regions is determined largely by interaction between secondary structural elements, especially α-helical segments. In fact, the mode of packing of the α-helices was first recognized from the low-resolution crystal structure of myoglobin solved with diffraction spots up to $Q = 1.0$ (Kendrew et al., 1960).

The scattering profiles in the high Q region for the holo- and apo-proteins are shown in Figure 6. Two distinct broad maxima are observed for the holo-protein, while one distinct broad maximum is clearly evident for the apo-protein. The acid molten globule of human α-lactalbumin also shows a similar scattering profile in this region (Ptitsyn, 1987, 1992) to that of apo-protein. Since X-ray scattering gives ensemble- and time-averaged structural information, we cannot expect to observe a distinct maximum as seen in Figure 6 if the packing pattern of the secondary structural elements differs from molecule to molecule, as suggested by Ewbank and Creighton (1991). Therefore, the result in Figure 6 indicates the existence of the definite tertiary fold in the molten globule. The scattering function calculated based on the α-domain cannot explain the observed scattering profile, suggesting that the tertiary fold formed in the molten globule is not necessarily identical to that in the native state.

A similar broad but less prominent maximum was also observed for the molten globule of apomyoglobin (Kataoka et al., 1995) (see Fig. 8). This suggests that the packing of the secondary structural elements in the molten globule would be more evident for α-lactalbumin than for apomyoglobin. Further, the scattering profile at high Q for the α-lactalbumin molten globule is substantially the same as that for the apomyoglobin molten globule (Figs. 6, 8), suggesting that the tertiary fold in the molten globule is composed of a nonspecific α-helical bundle, and that the tertiary fold would be more or less disordered (Kataoka et al., 1995). Structural parameters and properties obtained in this study are summarized in Table 1.

Comparison of the structural properties with molten globules of other globular proteins

The $P(r)$ functions and high Q scattering profiles of the native and molten globules for cytochrome *c* and apomyoglobin are compared in Figures 7 and 8. The salt-induced molten globule of cytochrome *c* shows a $P(r)$ with a single peak (Fig. 7) similar to the $P(r)$ of the molten globule of α-lactalbumin (Fig. 5). Such a unimodal $P(r)$ function is a characteristic for a globular shape. On the other hand, the $P(r)$ for the molten globule of apomyoglobin has a peak and a shoulder (Kataoka et al., 1995; Gast et al., 1994). Two other examples of such a bimodal $P(r)$ function for a compact denatured

Table 1. *Structural parameters and properties for various conformational states of α-lactalbumin*

Conformational State	R_g (Å)	d_{max} (Å)	$I(0)$[a]	Shape	Tertiary fold
Holo-protein (native)	15.7 ± 0.2	50 ± 3	1.0	Globular	++
Apo-protein (molten globule)	17.2 ± 0.2	60 ± 3	1.05 ± 0.10	Globular	+
Acid molten globule	17.2 ± 0.3	60 ± 3	0.93 ± 0.13	Globular	+[b]
Urea denatured (intact)	30.0 ± 0.7	n.d.[c]	0.97 ± 0.21	Chain	n.d.[c]

[a]Values are relative to the value of holo-protein.
[b]Data taken from Ptitsyn (1992).
[c]Not determined.

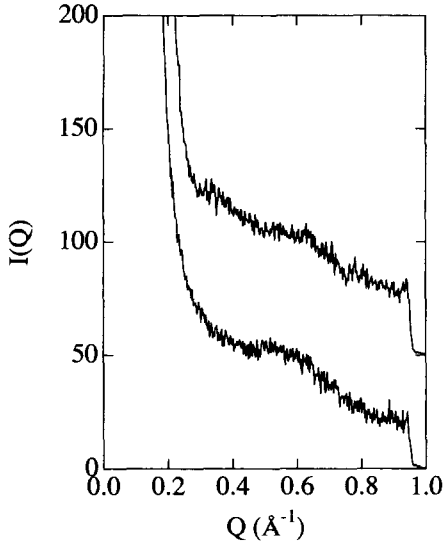

Fig. 6. Comparison of solution X-ray scattering profile in the high Q region for holo- (top curve) and apo-α-lactalbumin (bottom curve). Top curve has been shifted along the $I(Q)$ axis for clarity.

state are staphylococcal nuclease fragment (Flanagan et al., 1992, 1993) and thermally denatured RNase A (Sosnick & Trewhella, 1992). Thus, the solution structural property of the molten globule can be classified into two categories, one giving a unimodal $P(r)$ function and the other a bimodal $P(r)$ function.

The scattering profile in the high Q region for the molten globule of α-lactalbumin (Fig. 6) indicates a significant amount of tertiary fold. The molten globule of cytochrome c gives a quite similar high Q scattering profile to the native state (Fig. 8), indicating that substantial native-like tertiary folds have already formed in the molten globule. On the other hand, as mentioned above, the tertiary fold of the apomyoglobin molten globule would not be significant in comparison with those of α-lactalbumin. Staphylococcal nuclease fragment does not show any distinct fine structures in its high Q scattering profile (Flanagan et al., 1993). Thus, we can find a close correlation between the shape of the $P(r)$ function and the tertiary fold, in that the molten globule with a unimodal $P(r)$ contains a significant amount of tertiary fold, whereas the molten globule giving a bimodal $P(r)$ has rather a small amount of tertiary fold. We interpret that the structure of a molten globule with a bimodal $P(r)$ is composed of a core with flaring tail(s) (Kataoka et al., 1995).

Since the molten globule with a unimodal $P(r)$ function possesses more tertiary fold than that with a bimodal $P(r)$ function, the former resides at the later stage of folding than the latter. Based on the lattice model, Lattman et al. (1994) suggested that a structure which gives a bimodal $P(r)$ function is a common property of an early folding intermediate or a compact denatured state, although their model is not necessarily consistent with our interpretation. Some of their models are apparently dumbbell-shaped and others have a core with a tail (Lattman et al., 1994).

Discussion

Radius of gyration of the molten globule

We demonstrated an increase in R_g for the molten globule of α-lactalbumin. The increase was confirmed by three independent

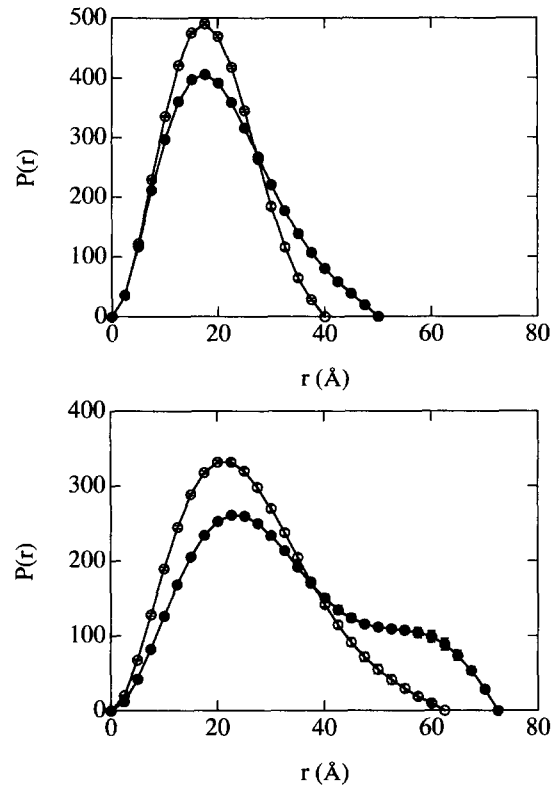

Fig. 7. Comparison of $P(r)$ functions for the native and molten globule states between cytochrome c (top panel) and apomyoglobin (bottom panel). For both panels, open symbols describe the native state and solid symbols, the salt-induced molten globule state. The molten globule state of cytochrome c is stabilized in 10 mM HCl, 100 mM NaCl (pH 2.0). The data for apomyoglobin are taken from Kataoka et al. (1995).

measurements with two different molten globules. In contrast, other investigators have reported that the R_g value for the acid molten globule of this protein is identical to that of the native state (Dolgikh et al., 1981, 1985; Izumi et al., 1983). However, all groups obtained identical R_g value for the native state.

We measured three different states (native state, apo-protein, and acid molten globule) within 3 hours to avoid deterioration or deformation of the scattering curves due to subtle changes in optics and the other instrumental conditions as well as possible changes in the protein solution. The association state for the molten globule is identical to that for the native state in our case (Fig. 3). We also observed the increase in d_{max} (Fig. 5). The peak appearing in the Kratky plot shifted toward the lower angle side from the peak of the native state (Fig. 4), corresponding to a size increase for the molten globule. All of our observations are self-consistent and confirm the increase in R_g for the molten globule.

Gast et al. (1994) reported that the R_s values for the native state and acid molten globule of α-lactalbumin were 17.7 Å and 19.9 Å, respectively, while the R_g values were identical. R_s and R_g are different measures of compactness displaying the hydrodynamic dimensions and the second moment of the electron density distribution within the molecule. We could not give any reasonable explanation for the increase in R_s with constant R_g. The increment in R_s is 12.4%, which is comparable to the increment in R_g reported here, 9.6%. The increments in R_s were also comparable to the increments in R_g for the molten globules of cytochrome c and apomyoglobin (Gast

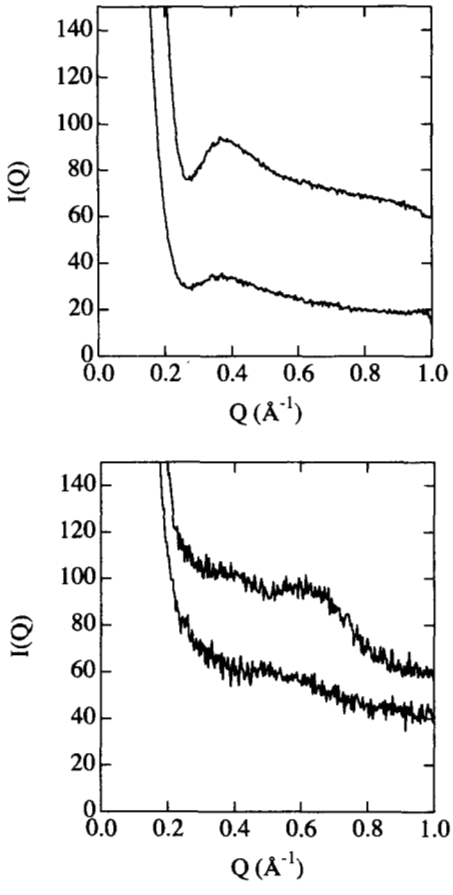

Fig. 8. Comparison of X-ray solution scattering profiles in the high Q region from the native and molten globule states of cytochrome *c* (top panel) and apomyoglobin (bottom panel). For both panels, the top curve is the scattering profile from the native state and the bottom curve is that for the salt-induced molten globule. Top curve in each panel has been shifted along the $I(Q)$ axis for clarity. The data for apomyoglobin are taken from Kataoka et al. (1995).

et al., 1994). Thus, we can conclude that the R_g for the molten globule is larger than that for the native state for α-lactalbumin.

Table 2 summarizes the sizes for the native and molten globule states for various proteins we have measured so far. Apparently, the R_g values for molten globule states are larger than those for native states. Based on their SAXS results for α-lactalbumin, Ptitsyn and coworkers claimed that the increase in R_g for the molten globule should be less than 10% (Dolgikh et al., 1981; Ptitsyn, 1987). Our measurements, however, indicate that the increment ratio differs from protein to protein and ranges up to 30%. The increment for α-lactalbumin is limited to 10%, but this is rather exceptional. Among the four proteins examined, only α-lactalbumin possessed intra-molecular disulfide bonds. The smaller increment in R_g for the molten globule of α-lactalbumin compared with the other three proteins would thus be attributable to the intramolecular disulfide bonds. We carried out SAXS measurements for the reduced apo-α-lactalbumin. Although we could not determine the exact R_g value because of aggregation, the estimated R_g value for the reduced apo-α-lactalbumin was between 20 and 24 Å. This supports the assumption that the intramolecular disulfide bonds restrict the molecular expansion of the molten globule. Thus, we conclude that the molten globule is not as compact as the native state.

Table 2. *Comparison of the radius of gyration, R_g, between the native and molten globule states, and its increment in the molten globule for various proteins*

Protein	R_g for N state (Å)	R_g for MG state (Å)	$\Delta R_g/R_g$(N) (%)
Cytochrome *c*[a]	13.5	17.4[d]	28.8
		17.0[e]	25.9
Holomyoglobin[b]	17.5		12.6[j]
Apomyoglobin[b]	19.7	23.1[f]	17.3[k]
			32.0[l]
Staphylococcal nuclease[c]	16.2	21.2[g]	30.9
α-Lactalbumin	15.7	17.2[h]	9.6
		17.2[i]	9.6

[a]Data taken from Kataoka et al. (1993).
[b]Data taken from Kataoka et al. (1995).
[c]Data taken from Flanagan et al. (1992).
[d]The molten globule is stabilized at pH 2.0 with 100 mM NaCl.
[e]The molten globule is stabilized at pH 2.0 with the acetylation of all lysine residues.
[f]The molten globule is stabilized at pH 2.0 with 50 mM sodium trichloroacetate.
[g]The R_g value for the fragment which lacks 13 amino acid residues from the C-terminus.
[h]The R_g value for apo-α-lactalbumin at pH 8.0.
[i]The molten globule is stabilized at pH 2.0.
[j]Increment from holomyoglobin native state to apomyoglobin native state.
[k]Increment from apomyoglobin native state to molten globule state.
[l]Increment from holomyoglobin native state to molten globule state.

Formation of the molten globule

Thermodynamic property is another important factor for defining the molten globule. The thermal unfolding of the molten globule state of cytochrome *c* and apomyoglobin involves a cooperative transition with an enthalpy change and heat capacity change of unfolding (Potekhin & Pfeil, 1989; Kuroda et al., 1992; Hagihara et al., 1994; Hamada et al., 1994; Nishii et al., 1994, 1995). Cooperative thermal unfolding with distinct heat absorption has also been reported for the staphylococcal nuclease fragment (Gittis et al., 1993; Griko et al., 1994a). These observations imply that hydrophobic interaction is major force for stabilizing the molten globules of cytochrome *c* and apomyoglobin, and the fragment of staphylococcal nuclease.

Because of the significant amount of tertiary fold, we can assume a large heat capacity change upon thermal unfolding of the α-lactalbumin molten globule, as shown by some thermodynamic studies (Xie et al., 1991, 1993; Griko et al., 1994b). The other study demonstrated that the heat capacity of the molten globule state is similar to that of the unfolded state in the absence of salt (Yutani et al., 1992). The discrepancy between these observations has been discussed in detail (Kuwajima, 1996; see also Griko et al., 1994b). These studies suggest that factors other than hydrophobic interaction would also contribute to the formation of the α-lactalbumin molten globule. We assume that intramolecular disulfide bonds play a role in stabilizing the structure, since they are a major factor for maintaining the molecular compactness of α-lactalbumin in the molten globule state. Taking into account

these considerations and SAXS results, models of the structures and formations of molten globules are summarized in Figure 9.

There have now been numerous studies on the molten globule structure of various proteins based on NMR data. It is useful to examine the relationship between the conceptual models in Figure 9 and the individual real models reported so far. In the case of α-lactalbumin, an accepted common view for the molten globule is a bipartite structure with an α-helical domain containing substantial secondary structure and native tertiary fold, and with a disordered β-sheet domain (Baum et al., 1989; Alexandrescu et al., 1993; Chyan et al., 1993; Peng & Kim, 1994; Shulman et al., 1995). Its compactness suggests that the disordered β-sheet domain would be collapsed. The conceptual model shown in Figure 9 combined with the bipartite structural model suggest a more detailed concept in which (1) the tertiary fold formed in the α-helical domain is stabilized by two disulfide bonds as well as by hydrophobic interaction, (2) the disordered β domain is collapsed by one disulfide bond, (3) the two domains are also connected by another disulfide bond to maintain molecular compactness, and (4) the disordered part cannot fluctuate and extend so randomly.

In the molten globule of cytochrome *c*, the N-terminal helix and C-terminal helix are already formed and packed into a native-like conformation (Roder et al., 1988), which is consistent with the native-like scattering profile in the high Q region (Fig. 8). It is also argued that our observed scattering curve for the cytochrome *c* molten globule (Kataoka et al., 1993) may be explained as a partially folded and partially unfolded structure (Doniach et al., 1995). Thus, in the case of cytochrome *c*, helices and the unstructured parts, if any, would be collapsed to form a compact structure by hydrophobic interactions between polypeptides, and between heme and polypeptide (Hamada et al., 1993, 1996).

In the molten globule of apomyoglobin, which we examined by X-ray solution scattering, helices A, B, G, and H are formed and the remaining parts are unstructured (Loh et al., 1995). A bimodal $P(r)$ function suggests a structure with a core and flaring tail(s)

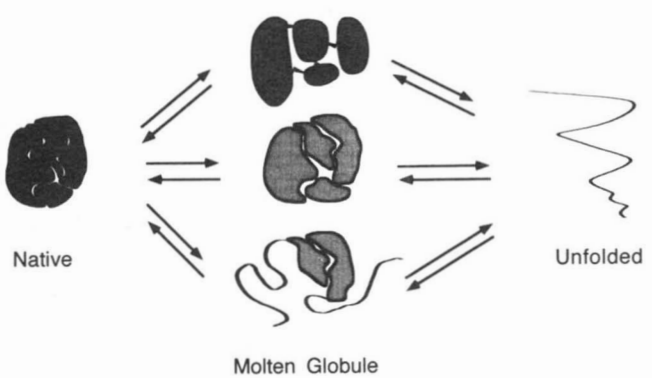

Fig. 9. Schematic description of various types of molten globule derived from the results of solution X-ray scattering. (top) The molecular compactness and tertiary fold are retained by intra-molecular disulfide bonds as well as by hydrophobic interactions. The molten globule of α-lactalbumin is categorized into this type. (middle) The molecular compactness and tertiary fold are retained mainly by hydrophobic interactions. The molten globule of cytochrome *c* is categorized into this type. (bottom) The structure is composed of a hydrophobic core and flaring tail(s). The molten globule of apomyoglobin is categorized into this type. The upper two types give a unimodal $P(r)$, while the bottom type gives a bimodal $P(r)$. In each model, the shaded region corresponds to a secondary structure or structural domain. For the upper two, the connecting loop between secondary structural elements should be disordered but not so extended.

(Kataoka et al., 1995; Lattman et al., 1994). Thus, the hydrophobic core is composed of helices A, B, G and H, and the rest loop is corresponding to a flaring tail. In the case of staphylococcal nuclease fragment, the N-terminal β-sheet is largely retained, while the rest is disordered (Shortle & Abeygunawardana, 1993; Ermácora et al., 1996). Our interpretation of the bimodal $P(r)$ function is consistent with these observations. Thus, the model in Figure 9 describes a general concept for the fragment of staphylococcal nuclease, if one tail is omitted. A similar view has also been proposed for the folding intermediate of staphylococcal nuclease based on thermodynamic measurements (Carra et al., 1994).

Concluding remarks

Is the term molten globule appropriate to describe a compact denatured state? Although the models in Figure 9 are quite rough, they describe the essence of the structures of the so-called molten globules. It is clear that the term molten globule is used to describe a wide range of non-native conformations, none of them completely consistent with the original definition. This structural diversity is partly responsible for the conflicting data obtained on the thermodynamic properties of the molten globule. The subdomain model might be appropriate for describing some of the molten globule structures. However, our data as well as others indicate the substantial increase in flexibility even in the structured regions, still similar to the original molten globule picture. In conclusion, we should keep in mind the fact that we still do not fully understand the conformation and stability of compact non-native states when we use the term molten globule.

Materials and methods

Materials

Bovine α-lactalbumin was prepared from fresh milk as described previously and contained one bound Ca^{2+} per molecule (Kuwajima et al., 1976). It was dissolved in 20 mM Tris-HCl (pH 8.0) buffer containing 2 mM $CaCl_2$. Apo-α-lactalbumin was prepared as described (Okazaki et al., 1994), and dissolved in 20 mM Tris-HCl (pH 8.0) buffer containing 1 mM EGTA. The acid molten globule was obtained by dissolving holo α-lactalbumin in 10 mM HCl (pH 2.0). To obtain the urea-denatured state, holo α-lactalbumin was dissolved in 8 M urea, 20 mM HCl (pH 2.0) buffer. These solutions were dialyzed against the same buffers. The dialysates were concentrated with a Centricon-10 (Amicon). Protein concentration was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 28,500 (Kuwajima et al., 1985).

Methods

Solution X-ray scattering experiments were carried out at the solution scattering station (SAXES) installed at BL-10C, the Photon Factory, Tsukuba, Japan, using synchrotron radiation (Ueki, 1991; Kataoka et al., 1991). The sample-to-detector distance was either 90 or 200 cm for measurements of SAXS or about 50 cm for measurements of the scattering profile in the high Q region, which were calibrated by meridional diffraction from dried chicken collagen. The sample cell had a volume of 50 μL with 15-μm thick quartz windows, and a 1-mm path length. The sample cell was kept at 20 °C by circulating temperature-controlled water through the sample cell holder. For SAXS measurements, protein concentrations were varied within the range 1.0–12.0 mg/mL, and the

measurement time was 10 min. For higher-angle scattering measurements, the protein concentrations were 20 mg/mL and the measurement time was 15 min. In both cases, the final dialysis fluids were measured as background and subtracted from the data.

Data processing was carried out with both an ACOS 2000 at the computer center, Osaka University, and a PC9801 personal computer (NEC). The R_g was estimated by the Guinier approximation, $I(Q) = I(0)\exp(-R_g^2Q^2/3)$, where Q and $I(0)$ are the momentum transfer and intensity at zero scattering angle, respectively (Guinier & Fournet, 1955; Glatter & Kratky, 1982). Q is defined as $q = 4\pi \sin\theta/\lambda$ where 2θ and λ are the X-ray scattering angle and wavelength (1.488 Å), respectively. We applied a criterion of $QR_g < 1.3$ (Olah et al., 1993) for the Guinier fit. We have shown that this criterion is appropriate for estimating the R_g value even for a denatured state (Kataoka et al., 1995). The distance distribution function, $P(r)$, was calculated using an indirect Fourier transform method reported by Moore (1980). $P(r)$ function was also evaluated using the GNOM program (Svergun et al., 1988), which was kindly supplied by Dr. D. Svergun. Both methods gave essentially the same $P(r)$ function.

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