Compactness of the kinetic molten globule of bovine α -lactalbumin: A dynamic light scattering study

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Abstract

During folding of globular proteins, the molten globule state was observed as an equilibrium intermediate under mildly denaturing conditions as well as a transient intermediate in kinetic refolding experiments. While the high compactness of the equilibrium intermediate of α -lactalbumin has been verified, direct measurements of the compactness of the kinetic intermediate have not been reported until now. Our dynamic light scattering measurements provide a complete set of the hydrodynamic dimensions of bovine α -lactalbumin in different conformational states, particularly in the kinetic molten globule state. The Stokes radii for the native, kinetic molten globule, equilibrium molten globule, and unfolded states are 1.91, 1.99, 2.08, and 2.46 nm, respectively. Therefore, the kinetic intermediate appears to be even more compact than its equilibrium counterpart. Remarkable differences in the concentration dependence of the Stokes radius exist revealing strong attractive but repulsive intermolecular interactions in the kinetic and equilibrium molten globule states, respectively. This underlines the importance of extrapolation to zero protein concentration in measurements of the molecular compactness.

Keywords: α -lactalbumin; circular dichroism; dynamic light scattering; hydrophobic collapse; molecular compactness; molten globule; protein folding

α -Lactalbumin (α -LA) has played a key role in developing the concept of the molten globule (Kuwajima, 1989; Ptitsyn, 1992, 1995; Kuwajima, 1996), which has considerably influenced theoretical and experimental work in studying the process of protein folding. In the case of α -lactalbumin, the molten globule was observed as both an equilibrium intermediate under mildly denaturing conditions, and a transient intermediate in kinetic refolding experiments. The equilibrium intermediates detectable at low pH or at neutral pH, low ionic strength and in the absence of Ca^{2+} have properties consistent with the original definition of the molten globule state (Ptitsyn, 1992): they are nearly as compact as the native state, have native-like secondary structure, and differ from the native state mainly by the lack of a rigid tertiary structure.

Equilibrium molten globules of α -LA have been studied for several species including human, bovine, baboon, guinea pig, and goat. The best studied molten globule is the low pH-form of α -LA

(A-state). Hydrogen-exchange measurements and NMR (Baum et al., 1989; Alexandrescu et al., 1993) as well as protein dissection studies (Peng & Kim, 1994) revealed a nonuniform loss of native structure in the molten globule. While the α -helical domain is essentially structured, the β -sheet domain is significantly more unfolded. Further unfolding of the molten globule is a highly non-cooperative process (Shimizu et al., 1993; Schulman et al., 1997).

A transient folding intermediate having properties similar to the equilibrium molten globule according to near-UV and far-UV circular dichroism (CD) as well as tryptophan absorption data has been detected (Kuwajima et al., 1985; Ikeguchi et al., 1986a). Later it was shown that the NMR spectrum of the transient intermediate resembles closely that of the stable A-state (Balbach et al., 1995). Interestingly, the acquisition of the native main-chain fold during the transition from the kinetic molten globule to the native state proceeds in a very cooperative manner in both subdomains (Balbach et al., 1995, 1996). Investigations with different methods (Arai & Kuwajima, 1996) supplied further evidence that the burst-phase intermediate in α -lactalbumin folding has features of the equilibrium molten globule.

One of the most important physical properties of the molten globule state is that it is almost as compact as the native protein (Ptitsyn, 1992). The high compactness of the equilibrium molten globule of α -LA has been demonstrated by several methods (Dolgikh

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Abbreviations: α -LA, α -lactalbumin; CD, circular dichroism; DLS, dynamic light scattering; A-state, molten globule state at acid pH; N-state, native-like state of apo- α -lactalbumin; GuHCl, guanidine hydrochloride; R_S , Stokes radius, R_G , radius of gyration.

et al., 1985; Gast et al., 1986; Kataoka et al., 1997). Among the molten globule states found for different proteins the α -LA molten globule is most compact.

Up to now, direct measurements of the compactness of the kinetic molten globule of α -LA have not been reported. Therefore, we have performed dynamic light scattering (DLS) investigations in order to characterize the compactness of the kinetic molten globule of bovine α -LA in terms of the hydrodynamic Stokes radius R_S . Refolding was studied in the absence of Ca^{2+} , because the transition from the molten globule to the native state is a single-exponential process in this case and the rate is remarkably slower than the refolding rate of the holoprotein (Kuwajima et al., 1989). There are some differences in the dimensions of α -lactalbumin molecules from different species in the native as well as in the equilibrium molten globule states. Accordingly, we cannot compare the Stokes radius of the kinetic molten globule of bovine α -LA with the Stokes radii of human α -LA in particular equilibrium states. Because of the lack of corresponding data for bovine α -LA, we also had to perform equilibrium DLS experiments. The kinetic molten globule has a Stokes radius 5% greater than that of the native state and is, therefore, at least as compact as the protein in the A-state. The remarkable concentration dependence of the apparent Stokes radius is an indication for strong attractive intermolecular interactions of the kinetic molten globule, which do not exist in the case of the A-state.

Results

Stokes radii of bovine α -lactalbumin in different equilibrium states

The following equilibrium states were investigated: the holoprotein (in the presence of excess Ca^{2+}), the Ca^{2+} -free native-like folded apoprotein (in the presence of 0.1 M Na^+), the A-state at pH 2, the neutral molten globule state, and the unfolded protein in the presence of 5 M GuHCl. If not otherwise stated, we used as standard buffer 50 mM sodium cacodylate, pH 7 containing additionally 50 mM NaCl ensuring identical or comparable solvent conditions with respect to earlier equilibrium (Ikeguchi et al., 1986b) and kinetic (Kuwajima et al., 1989; Arai & Kuwajima, 1996) unfolding-refolding investigations. In the case of the apoprotein, the buffer contained additionally EGTA. It must be pointed out that the term apo- α -LA is used here for the Ca^{2+} -free protein at any concentration of monovalent cations, Na^+ in the present case. At sufficiently high concentrations of monovalent ions (e.g., 0.1 M Na^+) the apoprotein has a conformation hardly distinguishable from that of the native holoprotein according to the near- and far-UV CD (Ikeguchi et al., 1986b). This becomes evident comparing the corresponding near-UV CD spectra in Figure 1. Consequently, this state is regarded as the native state (N-state) of apo- α -LA and will be taken as a reference state for the kinetic experiments. The near-UV CD is very sensitive to even minor changes in the rigid tertiary structure. Therefore, we have measured additionally near-UV CD spectra in order to check whether the protein in a distinct state has a native-like, rigid conformation or is in the molten globule state. Despite of minor structural differences, the holoprotein and the Na^+ -loaded apoprotein of bovine α -LA differ strongly in thermal unfolding (Pfeil, 1998) and in their refolding kinetics (Kuwajima et al., 1989). Removal of Ca^{2+} leads to a considerable decrease in thermal stability lowering the melting temperature from 66 °C to 42 °C (Pfeil, 1998). To check whether the protein has essentially released Ca^{2+} , we have studied the

Fig. 1. Near-UV CD spectra of bovine α -LA in standard buffer 50 mM sodium cacodylate, pH 7, 50 mM NaCl, 2 mM EGTA (1–4), and 50 mM KCl/HCl, pH 2 (5) at 20 °C. Additional solvent components are given in parentheses. (1) Holo- α -LA (+12 mM CaCl_2), $c = 0.72$ mg/mL; (2) N-state of apo- α -LA, $c = 0.73$ mg/mL; (3) apo- α -LA (+0.5 M GuHCl), $c = 0.68$ mg/mL, the protein was transferred from 0 to 0.5 M GuHCl without unfolding by high GuHCl concentrations; (4) apo- α -LA (+0.5 M GuHCl), $c = 0.8$ mg/mL, after refolding from 5 M GuHCl; (5) A-state, $c = 0.5$ mg/mL.

thermal unfolding of the apoprotein in the presence of 0.1 M Na^+ monitoring the CD signal at 269 nm. The measured transition temperature $T_{trs} = 41.6 \pm 0.5$ °C and transition enthalpy $\Delta H = 217 \pm 10$ kJ/mol agree very well with the results of scanning calorimetry, $T_{trs} = 42.0 \pm 0.4$ °C and $\Delta H = 227 \pm 5$ kJ/mol (Pfeil, 1998).

DLS measurements were performed at 20 °C and different protein concentrations. The translational diffusion coefficients D obtained from the measured autocorrelation functions for holo- α -LA, the N-state of apo- α -LA, the A-state, and the unfolded protein are shown in Figure 2. In each case, linear extrapolation to zero protein concentration was performed using the relation $D = D_0(1 + k_D c)$, where k_D is the diffusive virial coefficient and c is the protein concentration in mg/mL. From the diffusion coefficient extrapolated to zero concentration D_0 , the Stokes radius R_S was calculated using the Stokes–Einstein equation. The data are summarized in Table 1. The diffusive virial coefficient k_D originates from excluded volume effects and intermolecular interactions. A detailed quantitative analysis is rather complicated. Therefore, the sign and the magnitude of k_D will be used only for a crude characterization of intermolecular interactions between protein molecules. Negative values of k_D indicate prevalent attractive interactions. Predominant repulsive intermolecular interactions were observed for the A-state and the molten globule state at pH 7 and low ionic strength. The k_D values obtained from the slopes in Figure 2 are also shown in Table 1.

Apparent molar masses were calculated from the relative total scattered light intensity and extrapolated to zero protein concentration. This yielded molar masses (Table 1), which are close to the molar mass calculated from the amino acid composition (14,186 g/mol) confirming that quantities of the monomeric protein could be obtained under our experimental conditions.

Fig. 2. Concentration dependence of the translational diffusion coefficient D of bovine α -LA in different equilibrium states at 20 °C. The solvents are described in the legend of Figure 1. $\circ$ Holo- α -LA, $\blacksquare$ N-state of apo- α -LA, $\blacklozenge$ α -LA unfolded by 5 M GuHCl, $\bullet$ A-state.

Refolding kinetics measured by DLS and near-UV CD

As the starting conformation for refolding investigations, we have used the protein unfolded in 5 M GuHCl with the disulfide bonds intact. All refolding experiments were performed at 20 °C. In the case of kinetic DLS measurements, refolding was initiated by 10-fold dilution of denaturant in the stopped-flow device. The dead time of mixing was about 20 ms, and data acquisition was started 100 ms after mixing using an acquisition time of 2 s. In order to cover the time range until folding is complete, 85 data acquisition intervals had to be recorded. Because the signal-to-noise ratio of a correlation function acquired within 2 s is too low to calculate a reliable distribution function of diffusion coefficients using the program CONTIN (Provencher, 1982), the stopped-flow experiment was repeated about 100 times. This yielded a sufficiently

high signal-to-noise ratio allowing to determine the diffusion coefficient of the protein with an experimental error of $\pm 3\%$ at any time point in our kinetic experiments. The diffusion coefficient of the protein was obtained from the major peak of the distribution function of diffusion coefficients, which also showed a minor peak representing much larger particles with an average Stokes radius $R_S \sim 65$ nm. The minor peak was extremely small or absent in the equilibrium DLS experiments. In the stopped-flow DLS experiments, the major peak contributed to the total light scattering intensity by 65–69%. Because the radii belonging to these peaks differ by a factor of 30, very stable numeric solutions were obtained using the program CONTIN. Furthermore, as the scattering power of the large particles, which probably consist of highly aggregated protein, is greater by a factor of $\sim 30^x$ ($1 < x < 3$), their weight concentration did not exceed 1% of the total amount of protein. Accordingly, the formation of these aggregates is insignificant for the refolding process. But, these aggregates have to be clearly distinguished from oligomeric structures, which could be formed at concentrations higher than that used in our experiments. Kinetic DLS experiments were performed at final protein concentrations of 0.80, 1.35, and 1.80 mg/mL. The kinetic changes of the apparent Stokes radius measured at 1.35 mg/mL are shown in Figure 3. Fitting this curve with a single exponential yielded a time constant of 43 ± 6 s and an apparent initial radius of 2.15 nm. According to this apparent initial radius, the predominant change in the radius proceeds within the dead time of the experiment. However, the exact magnitude of the dead time change depends on the knowledge of the Stokes radius of the unfolded protein under refolding conditions and the initial radius extrapolated to zero protein concentration. Measurements within the post-transition region at 4, 5, and 6 M GuHCl revealed a moderate dependence of R_S on GuHCl concentration. Extrapolation to refolding conditions (0.5 M GuHCl, data not shown) yielded $R_S = 2.42$ nm, which is close to the value 2.46 nm measured at 5 M GuHCl (see Table 1).

Refolding time constants of 43 ± 6 s and 45 ± 6 s were obtained from fitting the kinetic traces at protein concentrations of 0.8 and 1.8 mg/mL, respectively. The integrated light scattering intensity was essentially constant during all three refolding experiments pointing to the absence of transiently formed oligomers during refolding. Indeed, the molar masses of α -LA in the kinetic molten

Table 1. Stokes radii R_S , translational diffusion coefficients D , diffusive virial coefficients k_D , and molar masses M of bovine α -LA in different states at 20 °C

State	Condition	R_S (nm)	$D \times 10^7$ (cm ² /s)	k_D (mL/g)	$M \times 10^{-3}$ (g/mol)
Holo- α -LA	Std. b., ^a 12 mM CaCl ₂	1.88 ± 0.02	11.4 ± 0.1	-7 ± 1	14.5 ± 1.5
Apo- α -LA	Std. b., 2 mM EGTA	1.91 ± 0.02	11.2 ± 0.1	-4 ± 1	14.1 ± 1.5
Unfolded	Std. b., 2 mM EGTA, 5 M GuHCl	2.46 ± 0.05	8.7 ± 0.2	-9 ± 1	n.d. ^b
A-state	50 mM KCl/HCl, pH2	2.08 ± 0.03	10.3 ± 0.1	15 ± 2	14.9 ± 1.5
Neutral MG ^c	5 mM Na-Cacod., pH7, 1 mM EGTA	2.04 ± 0.03	10.5 ± 0.1	62 ± 6	15.4 ± 1.5
Kinetic MG	Std. b., 2 mM EGTA, 0.5 M GuHCl	1.99 ± 0.04	10.8 ± 0.02	-60 ± 6	14.2 ± 1.5
Refolded	Std. b., 2 mM EGTA, 0.5 M GuHCl	1.87 ± 0.04	11.4 ± 0.2	-36 ± 4	13.7 ± 1.5
Control ^d	Std. b., 2 mM EGTA, 0.5 M GuHCl	1.91 ± 0.02	11.2 ± 0.1	-3 ± 1	14.0 ± 1.5

^aStandard buffer: 50 mM sodium cacodylate, pH7, 50 mM NaCl22.

^bNot determined because of the low scattering contrast in solutions of high GuHCl concentrations.

^cMG: molten globule state.

^dControl: protein under refolding conditions without prior unfolding in 5 M GuHCl.

Fig. 3. Refolding kinetics of bovine apo- α -LA monitored by DLS at 20°C in 50 mM sodium cacodylate, pH 7, 50 mM NaCl, 2 mM EGTA, 0.5 M GuHCl. Refolding was initiated by a concentration jump of GuHCl from 5 M to 0.5 M performed in a SFM-3 stopped-flow device. The final protein concentration was 1.35 mg/mL. The experimental curve was fitted by a single exponential yielding a time constant of 43 ± 6 s.

Fig. 4. Concentration dependence of the initial (●) and final (■) diffusion coefficients of apo- α -LA obtained from fitting the kinetic refolding curves measured by DLS after a concentration jump of GuHCl from 5 to 0.5 M. The concentration dependence for apo- α -LA transferred from 0 to 0.5 M GuHCl without unfolding by high GuHCl concentrations is also shown (□). The solvent was 50 mM sodium cacodylate, pH 7, 50 mM NaCl, 2 mM EGTA, 0.5 M GuHCl.

globule and the refolded state differ only by 4% according to the observed changes in the relative integrated scattering intensity (Table 1). The molar mass of the refolded protein was determined in a subsequent equilibrium DLS experiment. While the refolding time constants were practically identical at all three concentrations, the initial and final diffusion coefficients and the corresponding apparent Stokes radii exhibited a considerable concentration dependence (Fig. 4). Though the concentration dependence is weaker in the final state according to the values of k_D (Table 1), it is still much stronger as compared to that found for the N-state of apo- α -LA. It becomes evident from Figure 4 that the apparent radius obtained during a single experiment at finite concentration does not provide valuable information about the dimensions of the kinetic molten globule due to the strong concentration dependence of D or $R_{s,app}$. From extrapolation to zero protein concentration we obtained initial and final Stokes radii of 1.99 and 1.87 nm, respectively. The initial radius is the Stokes radius of the kinetic molten globule, and the strong concentration dependence is reasonable because of possible attractive interactions of the protein molecules in this state. However, for the final state a weaker concentration dependence was expected similar to that observed for holo- α -LA and the N-state of apo- α -LA (Fig. 2), because the protein should be still in a native-like folded state at 0.5 M GuHCl according to equilibrium unfolding experiments (Ikeguchi et al., 1986b). The strong concentration dependence of the apparent final radius was confirmed by additional manual mixing experiments and subsequent equilibrium DLS measurements. Interestingly, the expected weak concentration dependence was observed (Fig. 4), when the apoprotein was transferred from 0 to 0.5 M GuHCl without prior unfolding.

In order to check whether the initial state in the kinetic DLS experiments is indeed the molten globule state and the final state obtained during the refolding experiments has a native-like rigid

tertiary structure, we have performed kinetic CD experiments in the near-UV region. Refolding was initiated by manual mixing with a typical dead time of 7 s. The time course of the recovery of the CD signal at 269 nm is shown in Figure 5. A time constant of 30 ± 4 s was obtained, which is somewhat smaller than that

Fig. 5. Refolding kinetics of bovine apo- α -LA monitored by CD at 269 nm in 50 mM sodium cacodylate, pH 7, 50 mM NaCl, 2 mM EGTA, 0.5 M GuHCl and at $T = 20^\circ\text{C}$. Refolding was initiated by a concentration jump of GuHCl from 5 M to 0.5 M (manual mixing). The final protein concentration was 0.8 mg/mL. The experimental curve was fitted by a single exponential yielding a time constant of 30.4 ± 4.0 s.

measured in the kinetic DLS experiments. The entire near-UV CD spectrum measured after folding was complete is shown in Figure 1. The CD amplitude is only 10% smaller than that of the N-state of apo- α -LA, showing that the rigid tertiary structure is essentially regained. On the other hand, the small difference may have the same causes, like the differences between the final state and the N-state of apo- α -LA observed in the kinetic DLS experiments. Furthermore, we have measured the CD spectrum of the apoprotein that was transferred from 0 to 0.5 M GuHCl without prior unfolding. This spectrum was not fully identical but much closer to the spectrum of the N-state of apo- α -LA. In order to exclude the possibility that the unfolded protein is not capable of refolding completely due to partial degradation, we have subjected the protein refolded in 0.5 M GuHCl to gel chromatography in buffer lacking GuHCl. The near-UV spectrum and the Stokes radius of the protein transferred to zero GuHCl concentration in this way were identical with those of the N-state of apo- α -LA.

Discussion

The results obtained in this study provide a complete set of the hydrodynamic dimensions of bovine α -LA in different conformational states with special emphasis to the kinetic molten globule. Additional new information concerning the solution behavior and the intermolecular interactions comes from the concentration dependence of the measured diffusion coefficients.

Before discussing the properties of the kinetic molten globule in more detail, it is worthwhile to relate our data concerning the native folded state and the equilibrium intermediates to existing data for human and bovine α -LA. The Stokes radii of holo- α -LA and the N-state of apo- α -LA obtained in this work differ only by 1.5%, showing that these states are practically identical also according to their molecular dimensions. The concentration dependence of D is essentially the same in both states. Accordingly, noticeable differences in the net charge or the exposure of hydrophobic surface areas do not exist. It is reasonable, therefore, to take the N-state of apo- α -LA as the reference state to compare the Stokes radii in different equilibrium denatured states and in the kinetic intermediate state with the native folded state.

It turned out that the Stokes radius of native bovine α -LA is 7% larger than $R_S = 1.77$ nm of human holo- α -LA (Gast et al., 1986). It was important in this regard to perform parallel measurements of the molecular mass of the N-state of bovine α -LA, which have clearly demonstrated the monomeric state of the protein. Otherwise, the larger Stokes radius of the bovine protein could be caused by the presence of multimers. We cannot give a definite explanation for the observed difference in R_S . Crystal structures of α -lactalbumins from different species have been obtained (Pike et al., 1996) and revealed similar overall topologies. However, a remarkable structural feature is the so-called flexible loop in the C-terminal tail. Conformational differences between human and bovine α -LA within this region could be the cause of the deviation in the hydrodynamic dimensions.

Next, we compare the hydrodynamic radius R_S with the radius of gyration R_G , which has been obtained recently for the native and equilibrium molten globule states of bovine α -LA (Kataoka et al., 1997). Both quantities are different measures of the compactness, and the connection between these quantities must be considered carefully. Knowing R_G and R_S one can calculate the ρ -factor = R_G/R_S , which is a conformational sensitive parameter (Damaschun et al., 1991). $\rho = 0.8$ has been calculated as an average value over a large

number of compactly folded proteins contained in the Protein Data Bank (Damaschun et al., 1993). Taking $R_G = 1.57$ nm obtained by Kataoka et al. (1997) for the native holoprotein, one gets a ρ -factor of 0.82. This value is typical for a globular protein.

R_G , R_S , and ρ obtained for the native state can now be used as a firm basis to consider the expansion of the equilibrium molten globule relative to the N-state. For the A-state of bovine α -LA, the Stokes radius of 2.08 nm displays a linear expansion by a factor of 1.09. From $R_G = 1.72$ nm (Kataoka et al., 1997) a linear expansion by a factor of 1.10 is obtained. Thus, the hydrodynamic and geometric dimensions change at the same extent on the transition from the native to the molten globule state leading to a nearly unchanged ρ -factor of 0.83. The essentially constant ρ -factor and the unimodal type of the distance distribution function obtained from small-angle X-ray scattering (Kataoka et al., 1997) indicate that the general globular structure is maintained in the molten globule of bovine α -LA. It is known from NMR data (Baum et al., 1989; Alexandrescu et al., 1993) that the structural changes accompanying the transition from the native to the molten globule state are more pronounced within the β -domain. Nevertheless, the picture emerging from the dynamic light scattering and X-ray scattering data is that in the case of bovine α -LA the expansion of the molten globule is weak (about 10%) and not completely but rather uniform. A stronger and much more nonuniform expansion has been observed for apomyoglobin: R_S increased by a factor of 1.21, ρ increased from 0.84 to 0.93 (Gast et al., 1994), and the distance distribution function changed from a unimodal to a bimodal type (Gast et al., 1994; Kataoka et al., 1997).

It has been shown (Gast et al., 1986) that for human α -LA the Stokes radius in the A-state is larger by a factor of 1.12 than in the native state. This means that the expansion during unfolding to the molten globule state is nearly the same, despite of the somewhat smaller Stokes radius in the native state (1.77 nm).

The transition from the N-state to the A-state or to the neutral molten globule state is accompanied by a remarkable change in the diffusive virial coefficient clearly indicating the increase in repulsive intermolecular interactions. Since the theoretically calculated isoelectric point of bovine α -LA is 4.8, the protein molecules must carry a net charge both in the A-state at pH 2 and in the molten globule state at pH 7. Below pH 4 an increase in the exposure of hydrophobic surface area accompanying the transition from the native to the A-state has been shown (Lala & Kaul, 1992). But, in the A-state the electrostatic effects dominate in the intermolecular interactions resulting in $k_D > 0$. It is reasonable to assume that the molten globule states at low pH or pH 7 and low ionic strength are brought about by intramolecular electrostatic repulsion.

The Stokes radius of α -LA unfolded by 5 M GuHCl, frequently termed the U-state, can be considered as a further reference value, because it defines the starting point for refolding in the kinetic experiments. R_S of the highly unfolded protein is 29% larger than in the native state. Measurements of the radius of gyration in the random chain-like conformation (constrained by four disulfide bonds) in the presence of 8 M urea yielded $R_G = 3.0$ nm (Kataoka et al., 1997). Despite of possible differences in the chain dimensions in the presence of either urea or GuHCl, one obtains a ρ -factor of 1.22 being considerably larger than in the folded conformation. While for proteins lacking disulfide bonds a ρ -factor for the random chain conformation of 1.55 is expected theoretically and has been verified experimentally (Damaschun et al., 1991), its value for proteins covalently linked by disulfide bridges has not been verified so far. It is probably smaller than 1.55, but depends cer-

tainly on the number and the specific location of the disulfide bridges (Damaschun et al., 1997).

The properties of the kinetic molten globule will be discussed now. According to the data in Table 1, the Stokes radius is at most 5% larger than in the N-state of apo- α -LA. Hence, the kinetic molten globule is even slightly more compact than its equilibrium counterpart, the A-state. The initial CD amplitude at 269 nm is about $-50 \text{ deg} \cdot \text{cm}^2/\text{dmol}$ (Fig. 5) being identical with the corresponding CD amplitude in the A-state (Fig. 1). This ensures that the initial Stokes radius is indeed that of the kinetic molten globule. A remarkable feature of the molten globule is the strong concentration dependence of D and/or R_s with $k_D < 0$. This is a clear indication for attractive intermolecular forces, obviously caused by the exposure of hydrophobic surface areas. This behavior could be expected from the known ability of the kinetic molten globule to bind hydrophobic dyes (Engelhard & Evans, 1995). Within the investigated concentration range (0.8–1.8 mg/mL), a remarkable irreversible aggregation process could not be observed. We have to point out that from measurements within a rather narrow concentration range it is not simply possible to distinguish between unspecific attractive intermolecular forces and the existence of a concentration dependent monomer-dimer (or higher oligomer) equilibrium, which demands specific intermolecular interactions. However, it is most important in the present case that our data allow an extrapolation to properties of the monomeric protein at zero protein concentration, irrespective of whether the intermolecular interaction are specific or nonspecific. At least an influence of protein concentration on the folding rate could not be observed. By contrast, Engelhard and Evans (1995) found that ANS bound to α -LA affects the folding kinetics.

According to earlier refolding investigations (Ikeguchi et al., 1986a; Kuwajima et al., 1989; Arai & Kuwajima, 1996) and the observed kinetic traces, the final GuHCl concentration of 0.5 M is sufficiently low for a proper characterization of the kinetic molten globule. However, it is surprising that the final conformation after refolding in 0.5 M GuHCl must be slightly different from that in the N-state at 0 M GuHCl: the near-UV CD amplitude is about 10% smaller, k_D is still noticeable larger, but R_s is rather smaller than in the N-state. We have shown that these differences are not due to partial unfolding at 0.5 M GuHCl or due to the inability of the protein to refold correctly. The reason for this behavior is not clear. GuHCl tightly bound to the protein could be responsible for the observed effect, which has obviously a remarkable influence on the intermolecular interactions. Further investigations are needed to achieve a more definite explanation.

It is interesting to compare our results with kinetic NMR data (Balbach et al., 1995). These investigations revealed two interesting features of the transition from the kinetic molten globule state to the N-state. The native state is formed cooperatively, and the formation of the closely packed tertiary structure appears simultaneously in the α - and the β -domain.

Key issues connected with the concept of the molten globule are questions concerning the forces that stabilize the molten globule and how the preformed native-like fold of the molten globule is transferred into the unique native structure (Kuwajima et al., 1989). The question concerning the forces stabilizing the molten globule is of general interest for understanding the process of protein folding and is also of particular interest to explain how the molten globule can be brought about in different ways. Such molten globules have essential common features but also show minor differences in distinct properties. For example, the kinetic molten globule appears to be

more compact than the A-state, and both molten globules differ considerably in the intermolecular and probably also in the intramolecular interactions. But, that might be just the repulsive electrostatic interactions existing in the A-state leading to a slightly more expanded conformation. Presently, nonspecific hydrophobic interactions, particularly solvent-separated hydrophobic interactions (Arai & Kuwajima, 1996), are discussed as the main stabilizing forces of the molten globule. This is supported by the finding that the stability of the molten globule is increased by amino acid substitutions that raise the hydrophobicity (Uchiyama et al., 1995) and recent experiments (Wu & Kim, 1997) demonstrating that the replacement of specific hydrophobic amino acid residues by a unique one (leucine) does not influence the overall fold essentially.

Conclusions

Our data underline that during refolding of α -lactalbumin the hydrophobic collapse into the molten globule, which is nearly as compact as the native state, plays an important role. Such a strong hydrophobic collapse is typical of α -LA and certainly also for related proteins. The molten globule further folds to the N-state in a rate-limiting step. Under conditions used in this work, the molten globule is much less prone to aggregation as it widely believed and is, therefore, no dead end product of folding. However, the observed strong attractive intermolecular interactions could demand folding assistance at concentration as high as found within the cell. Furthermore, alternative folding pathways of α -LA escaping the molten globule state have not been observed showing that the molten globule of α -LA is an on-pathway intermediate. However, evidence is accumulating that proteins can fold fast without detectable intermediates to the native state (Schindler et al., 1995) pointing to the existence of alternative folding pathways avoiding the molten globule state. Our observations indicate that in the case of α -LA a compact state is already formed within the burst-phase of folding according to the decrease in R_s from 2.46 to 1.99 nm. However, this is not the case for all proteins. During folding of RNase A, we did not observe such a compaction at early stages of folding (Nöppert et al., 1998).

Our experiments have clearly shown that intermolecular interactions in intermediate states may differ considerably from that in the folded and in the highly unfolded states. From the methodological point of view, it is, therefore, very important to extrapolate the results concerning the compactness of intermediate states to zero protein concentration. Figure 2 and particularly Figure 4 clearly show that the ratio between the diffusion coefficients and the corresponding Stokes radii in various states at zero concentration is totally different from that at concentrations $c > 3 \text{ mg/mL}$. A similar behavior has been shown for the reciprocal radii of gyration R_G^{-1} of unfolded and partially folded proteins (Damaschun et al., 1998).

Materials and methods

Materials

Calcium depleted bovine α -lactalbumin (Sigma, type III) was used in all experiments, which were performed under the following solvent conditions: (1) 50 mM sodium cacodylate, pH 7, 50 mM NaCl, 12 mM CaCl_2 (holo α -LA); (2) 50 mM sodium cacodylate, pH 7, 50 mM NaCl, 2 mM EGTA in the absence and presence of GuHCl (apo α -LA and unfolded protein); (3) 50 mM KCl/HCl,

pH 2 (A-form); (4) 5 mM sodium cacodylate, pH 7, 1 mM EGTA (neutral molten globule). Ultra pure GuHCl was purchased from ICN Biomedical Inc.. All other materials were analytical grade. GuHCl concentrations were controlled refractometrically (Nozaki, 1972). The lyophilized protein was dissolved in the different solvents and was then purified by gel filtration using an FPLC column (Superose 12 HR 10/30, Pharmacia, Uppsala, Sweden). Protein concentrations were determined spectrophotometrically using a molar extinction coefficient at 280 nm of $28,500 \text{ M}^{-1} \text{ cm}^{-1}$ (Kronman & Andreotti, 1964). For refolding experiments starting with samples containing 5 M GuHCl and protein concentrations up to 20 mg/mL peak fractions were collected and concentrated by centrifugation in concentrators (Centricon-10, Amicon Inc., Massachusetts).

DLS measurements

The DLS apparatus and the adaptation to the SFM-3 stopped-flow system (Bio-Logic, France) has been described in detail (Gast et al., 1997). The laboratory-built DLS set-up mainly consists of an argon laser Lexel 3500 (Lexel Laser Inc., California) operating at 514.5 nm wavelength and about 1 W output power, a photon detection system and a 90 channel multibit multiple- τ correlator, which calculates the homodyne time-autocorrelation functions of the scattered light intensity. The detection system was linked either to the standard sample head for equilibrium investigations or to the observation head of the SFM-3 device. All measurements were performed at a scattering angle of 90° and at 20°C .

For equilibrium investigations the solvent and protein solutions were filtered through 100-nm pore-size Anotop filters (Whatman, UK) directly into 100- μL flow-through cells (Hellma, Germany). The total scattered light intensity was also measured in order to estimate molecular masses using Benzene as a reference sample. Simultaneous measurements of the hydrodynamic radius and the molecular mass are very important in order to check whether the observed changes in the radius are influenced by molecular association (Gast et al., 1997).

In kinetic DLS experiments refolding was initiated by 10-fold dilution of the solution containing the protein unfolded by 5 M GuHCl with buffer (50 mM sodium cacodylate, pH 7, 50 mM NaCl, 2 mM EGTA). The final GuHCl concentration (0.5 M) was checked by measuring the index of refraction of the waste. Prior to the experiments the entire stopped-flow system was flushed with water and the corresponding solvents filtered through 100-nm pore-size filters. Typically, 100 kinetic experiments were superimposed to achieve a sufficiently high signal-to-noise ratio of the measured autocorrelation functions.

Distribution functions of the translational diffusion coefficient D were derived from the autocorrelation functions using the program CONTIN (Provencher, 1982). The diffusion coefficients D were converted into Stokes radii R_S via the Stokes-Einstein equation, $R_S = k_B T / (6\pi\eta_0 D)$, where k_B is Boltzmann's constant, T is the temperature in K, and η_0 is the solvent viscosity. Solvent viscosities and densities were determined by means of a viscometer Viscoboy (Lauda, Germany) and a digital densitometer DMA 58 (Anton Paar KG, Austria), respectively. All data at finite protein concentrations are to be considered as apparent quantities.

CD measurements

Equilibrium and kinetic CD measurements were carried out in a J-720 (Jasco, Japan) CD spectrometer, which was calibrated with

(+)-camphorsulfonic acid at 290.5 and 192.5 nm (Johnson, 1990). For measurements in the near-UV region, 5- and 10-mm path-length cells were used. Kinetic refolding experiments were performed by manual mixing with a typical dead time of 7 s. Thermal unfolding transitions were recorded using an RT-100 (Neslab, New Hampshire) temperature control unit and the temperature-scan software supplied by Jasco. A mean residue weight of 115.3 was used to calculate mean residue molar ellipticities $[\theta]$.

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