The Interaction of the Molecular Chaperone, \( \alpha \)-Crystallin, with Molten Globule States of Bovine \( \alpha \)-Lactalbumin\(^*\)

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Small heat shock proteins function in a chaperone-like manner to prevent the precipitation of proteins under conditions of stress (e.g. heat). \( \alpha \)-Crystallin, the major mammalian lens protein, is a small heat shock protein. The mechanism of chaperone action of these proteins is poorly understood. In this paper, the conformational state of a protein when it forms a high molecular weight complex with \( \alpha \)-crystallin is investigated by examining, using NMR spectroscopy and size exclusion high performance liquid chromatography, the interaction of \( \alpha \)-crystallin with \( \alpha \)-lactalbumin and its various intermediate folded (molten globule) states. The complex is formed following reduction of \( \alpha \)-lactalbumin by dithiothreitol in the presence of \( \alpha \)-crystallin, and this interaction has been monitored in real time by \(^1\)H NMR spectroscopy. It is concluded that \( \alpha \)-crystallin interacts with a disordered molten globule state of \( \alpha \)-lactalbumin while it is on an irreversible pathway toward aggregation and precipitation. \( \alpha \)-Crystallin does not interact, however, with molten globule states of \( \alpha \)-lactalbumin that are stable in solution, e.g. the reduced and carboxyamidated species. It is proposed that \( \alpha \)-crystallin distinguishes between the various molten globule states of \( \alpha \)-lactalbumin on the basis of the lifetimes of these states, i.e. the protein must be in a disordered molten globule state for a significant length of time and on the pathway to aggregation and precipitation for interaction to occur.

It has become apparent over the last few years that the folding of proteins in vivo involves the interaction of nascent polypeptides with a diversity of unrelated proteins known as chaperones (reviewed by Buchner (1)). Unfolded polypeptide chains expose to solution hydrophobic surfaces that are potentially available for interaction with each other. As a result, undesirable products, i.e. incorrectly folded and/or aggregated proteins, may result. By sequestering or isolating these juvenile proteins from the surrounding milieu, chaperone proteins encourage the correct folding of proteins. Chaperone proteins are often also known as heat shock proteins (Hsps) because of their increased expression when cells are exposed to elevated temperatures. Thus, proteins such as Hsp90, Hsp60, Hsp70, and Hsp25 are expressed at higher levels under such conditions, and in doing so, they have a stabilizing effect on other proteins in the cell. Hsp70 has a crucial role in protein synthesis whereby it interacts with proteins soon after their synthesis on the ribosome and then passes on the protein to Hsp60 (GroEL) complexes where further folding of the molecule occurs. The role of Hsp90 is more enigmatic, but it seems to be involved in a diversity of tasks such as regulating receptors, interacting with kinases and general protein stabilization (2).

The small heat shock family of chaperones (e.g. Hsp25) is also involved in protein stabilization but only at the crudest level whereby these chaperones stabilize their "substrate" proteins against stress (e.g. heat, heavy metals, oxidation, etc.) via formation of a high molecular weight (HMW) complex with the chaperone protein (3, 4). Thus, these proteins are not involved directly in protein folding. On their own, Hsp25 chaperone proteins do not have a significant ability to release their substrate proteins, although recent evidence suggests that they may be able to do so in vitro, at least partially, via secondary interaction of the HMW complex with Hsp70 (5). Small Hsps have been therefore classified as "junior chaperones" because of their inability, individually, to release proteins once complexed (6). The major eye lens protein, \( \alpha \)-crystallin, is a member of the small heat shock family and shares many properties with Hsp25, e.g. they have extensive amino acid sequence similarity in their C-terminal regions and are both found as large, aggregated complexes of average mass around 800 kDa (7).

The lens is composed almost entirely of proteins known as crystallins. There is very little protein turnover in the lens, e.g. the crystallin proteins in the center of the lens are as old as the individual. Therefore, probably one of the major roles of \( \alpha \)-crystallin in the lens is to act as a stabilizer of the other crystallins to prevent their precipitation from solution and hence their contribution to cataract formation. Furthermore, \( \alpha \)-crystallin is not lens-specific and is found in many other organs (e.g. heart, lung, brain, kidney, etc.), where it has a similar stabilizing role to that of Hsp25 (7). The mechanism of the interaction of \( \alpha \)-crystallin with its substrate protein is not known. In particular, it would be of great interest to ascertain the conformation of the substrate protein when bound to \( \alpha \)-crystallin because, in a general sense, this information may provide insights into how different chaperone proteins interact and differentiate between the various unfolded states of proteins.

In this study, we have used NMR spectroscopy to investigate the conformational state of the substrate protein during its interaction with \( \alpha \)-crystallin. For these studies we have chosen \( \alpha \)-lactalbumin as the substrate protein. \( \alpha \)-Lactalbumin is ideally suited for such an investigation because its various folded and semi-folded states have been extensively studied (reviewed...
Experimental Procedures

Chemicals and Proteins—Bovine apo-\(\alpha\)-lactalbumin was obtained from Sigma. The intermediately folded forms of \(\alpha\)-lactalbumin (R-cam-\(\alpha\)-lactalbumin with its four disulfide bonds reduced and carboxymethylated, and 3SS-\(\alpha\)-lactalbumin with Cys\(^6\)-Cys\(^{120}\) selectively reduced and carboxymethylated) were prepared by literature methods (9, 10). Deuterated dithiothreitol (DTT) was from Cambridge Isotopes Laboratory. Bovine \(\alpha\)-crystallin was prepared by standard methods via size exclusion chromatography from lenses of animals less than one year in age (11). Samples were checked for purity by SDS-polyacrylamide gel electrophoresis and electrospray ionization mass spectrometry.

NMR Spectroscopy—\(^1\)H NMR spectra were acquired at 400 MHz on a Varian Unity-400 spectrometer. All parameters were as described previously (4, 11). The total correlation spectroscopy (TOCSY) spectra used a spin lock period of 30 ms.

Visible Absorption Spectroscopy—The chaperone activity of \(\alpha\)-crystallin upon reduction of \(\alpha\)-lactalbumin with DTT was assessed with a method based on that of Farahbakhsh et al. (12). \(\alpha\)-Lactalbumin (2 mg/ml) in 50 mM sodium phosphate, pH 7.2, 0.1 M NaCl was incubated at 37 °C in the presence of varying amounts of \(\alpha\)-crystallin from 0 to 14 mg/ml. Reduction and precipitation of \(\alpha\)-lactalbumin was induced by the addition of 20 mM DTT. Light scattering at 360 nm and 37 °C was monitored over time using a Spectramax spectrophotometer.

HPLC Analysis—Size exclusion HPLC was performed using a TSK 4000SW column (7.5 mm inner diameter \(\times\) 30 cm) attached to an ICI Kortec K350 pump delivery system, a Knauer variable wavelength monitor, and a Shimadzu C-R6A Chromatopac Integrator. Samples were incubated at 37 °C for 1 h prior to analysis by HPLC. \(\alpha\)-Lactalbumin (0.05 mg) plus the corresponding amount of \(\alpha\)-crystallin from the incubated samples in a volume of up to 40 \(\mu\)l was loaded onto the column and eluted in 50 mM sodium phosphate, pH 7.2, and a range of salt conditions depending on the experiment. The flow rate was 1.0 ml/min, and the absorbance was monitored at 280 nm.

Electrospray Ionization Mass Spectrometry—Spectra were acquired on a VG Quattro mass spectrometer with the methods outlined in Kilby et al. (13).

Results

Monitoring the Reduction of \(\alpha\)-Lactalbumin in the Presence of \(\alpha\)-Crystallin—\(\alpha\)-Lactalbumin has four disulfide bonds that upon reduction (e.g., with DTT) lead to unfolding, aggregation, and precipitation of the protein (10). It is easy to follow this process using visible absorption spectroscopy via the increase in light scattering associated with precipitation of the protein. Similar behavior is observed with other proteins upon reduction of disulfide bonds, e.g., the B chain of insulin precipitates from solution when the disulfide bonds joining it to the A chain are reduced. In the case of the insulin B chain, addition of an approximately stoichiometric amount (on a subunit basis) of \(\alpha\)-crystallin leads to prevention of this precipitation (12). Importantly, DTT does not affect bovine \(\alpha\)-crystallin because it contains no disulfide bonds. Under similar conditions to those for insulin, \(\alpha\)-crystallin also prevents the precipitation of apo-\(\alpha\)-lactalbumin from solution (Fig. 1). Under these conditions (pH 7.2, 0.1 M NaCl, 37 °C), a w/w ratio of \(\alpha\)-crystallin:\(\alpha\)-lactalbumin of around 3:1 provided complete protection against aggregation.

To investigate this interaction at the molecular level, the reaction of apo-\(\alpha\)-lactalbumin with DTT in the absence and the presence of \(\alpha\)-crystallin was followed by \(^1\)H NMR spectroscopy. Being a small and monomeric protein of \(-14\) kDa in mass, bovine \(\alpha\)-lactalbumin has a well defined \(^1\)H NMR spectrum whose aromatic resonances have been mostly assigned (14). Fig. 2A shows the aromatic region of the one-dimensional \(^1\)H NMR spectrum of apo-\(\alpha\)-lactalbumin at pH 7.4 and the same region at various times after addition of deuterated DTT. As is apparent from these spectra, the addition of DTT leads to a rapid alteration in the NMR spectrum. Thus, within the time it took to add the DTT, place the sample in the NMR spectrometer, and commence acquisition (46 s), the spectrum broadened
leads to the formation of an intermediated folded, or molten globule, protein that has the major elements of secondary structure and some elements of tertiary structure present. It is possible to isolate this three-disulfide-bonded species (10), and it has been extensively examined, e.g., in its interactions with GroEL (15). At pH 7.0 and 25 °C in the absence of calcium, further complete reduction with excess DTT of this species over a period of approximately 10 min (the slow phase) leads to greater unfolding, loss of tertiary structure and eventual aggregation and precipitation of α-lactalbumin (10). Dobson and co-workers have proposed that there are disordered and highly ordered molten globules that represent intermediate states early and late, respectively, along the folding pathway (16, 17). It is concluded that the two forms of intermediately folded α-lactalbumin observed in Fig. 2A (represented by spectra at 46 and 183 s) correspond respectively to three-disulfide-bonded α-lactalbumin (the highly ordered molten globule) and a less-structured intermediate containing a mixture of zero, one, and two disulfide bonds (the disordered molten globule).

The same reaction was monitored by 1H NMR in the presence of a slight subunit molar excess of α-crystallin (2:1 w/w α-crystallin:α-lactalbumin), i.e. conditions corresponding to α-lactalbumin being protected approximately 50% from precipitation relative to a control in the absence of α-crystallin (Fig. 1). α-Crystallin has two homologous subunits, A and B, and exhibits a well resolved 1H NMR spectrum due to short, flexible, and unstructured C-terminal extensions arising from the A and B subunits, respectively (11). Fortunately, there are no aromatic amino acids in these regions. The aromatic region of the 1H NMR spectrum of α-crystallin contains only weak and broad resonances from histidine H-2 and phenylalanine aromatic groups that do not complicate the interpretation of effects on the aromatic resonances of α-lactalbumin when it is reduced with DTT in the presence of α-crystallin.

The spectra of α-lactalbumin upon reduction in the presence and absence of α-crystallin were not identical (Fig. 2), i.e. in comparing Fig. 2 (A and B), the highly ordered molten globule intermediate was not observed, with only the disordered molten globule being present. Thus, it would seem that α-crystallin promoted the formation of this species, i.e. it sped up the slow phase step in the reduction of α-lactalbumin. Furthermore, α-crystallin had a significant stabilizing effect on α-lactalbumin when it was reduced, i.e. the broadened resonances from α-lactalbumin remain in solution longer when compared with the spectra of α-lactalbumin reduced in the absence of α-crystallin (Fig. 2). Thus, it took approximately 1000 and 1500 s, respectively, for the upfield resonance at 6.8 ppm from the tyrosine (3, 5) protons of α-lactalbumin to be lost in the absence and the presence of α-crystallin. Very similar stabilizing rates are observed for the other aromatic resonances of α-lactalbumin. The same NMR experiment was undertaken at a ratio of 4:1 or 6:1 w/w α-crystallin:α-lactalbumin. Under both of these conditions, α-crystallin completely stabilizes α-lactalbumin from precipitation (Fig. 1). For these mixtures, a time course of spectra was observed very similar to that in Fig. 2B, i.e. only the disordered molten globule form of α-lactalbumin was observed (not shown).

As is apparent from Fig. 2, resonances of α-lactalbumin after reduction are broad and poorly resolved and do not correspond in chemical shift to those expected for apo-α-lactalbumin in a completely unfolded state in 6 M guanidinium chloride at pH 7.2 (18). Under these conditions, the resonances are sharper and exhibit greater dispersion. In fact, the spectra observed for the disordered molten globule of α-lactalbumin, i.e. 183 s after reduction (Fig. 2A) and α-lactalbumin after reduction in the presence of α-crystallin (Fig. 2B), are identical to the spectrum significantly. There was still some dispersion in the spectrum, however, which was mostly lost (spectra acquired at 78, 114, and 149 s are not shown) because these resonances broadened further and eventually disappeared due to the protein aggregating and precipitating from solution. Indeed, upon removal from the spectrometer after the experiment, a substantial protein precipitate was present in the NMR tube.

Kuwajima et al. (10) have examined this reaction in detail and concluded that the Cys⁶-Cys¹²⁰ disulfide bond of α-lactalbumin is hyper-reactive and its rapid reduction (the fast phase)
observed for apo-α-lactalbumin almost immediately (4.85 s) after initiation of refolding from denaturing agent (18). In this case, the spectrum arises from α-lactalbumin that is early along its folding pathway in which the kinetically formed molten globule state has elements of secondary structure but little tertiary structure (18). Fig. 3 shows the 1H NMR spectrum of α-lactalbumin at pH 1.9 under which conditions the protein adopts a molten globule conformation, the so-called A state (8, 18). Allowing for pH-induced differences in chemical shifts for the downfield histidine H-2 resonances, the A state spectrum in Fig. 3 is equivalent to that for α-lactalbumin early along its folding pathway (18) and late along its unfolding pathway (Fig. 2). Thus, α-lactalbumin adopts the A state conformation prior to its precipitation from solution after addition of DTT (Fig. 2A). Furthermore, from the results presented in Fig. 2B, α-crystallin stabilizes α-lactalbumin in the A state, and because no further changes are observed with time in the NMR spectrum of α-lactalbumin in the presence of α-crystallin (apart from a decrease in intensity of these broadened α-lactalbumin resonances), this disordered molten globule state interacts with α-crystallin.

In previous studies of the heat-induced chaperone interaction of α-crystallin with unfolding proteins, a HMW complex is formed between the two proteins (3, 4). Similarly, a size exclusion HPLC profile of the 2:1 w/w α-crystallin:α-lactalbumin sample following completion of the NMR experiment exhibited a peak associated with a HMW complex and a much smaller peak arising from the monomeric α-lactalbumin (not shown). Thus, the profile was very similar to those of the heat (4) and denaturant-induced (19) HMW complexes between α-crystallin and unfolded proteins. For the HMW complex between α-crystallin and α-lactalbumin, there was no further change in the one-dimensional 1H NMR spectrum 30 min after addition of the DTT, implying that the reaction had gone to completion. Approximately 2 h after addition of the DTT, therefore, a TOCSY spectrum was acquired on the sample. The spectrum was very similar to that observed in previous studies on the HMW complex between α-crystallin and γ-crystallin (19) and the naturally occurring HMW complex obtained from older lenses (20). Thus, it contained cross-peaks from the C-terminal extensions of α-crystallin with no cross-peaks that could be identified from α-lactalbumin, i.e. α-lactalbumin loses its flexibility upon interaction with α-crystallin, which is consistent with its incorporation into a HMW complex.

Comparison of the NMR spectra of α-lactalbumin in the presence and the absence of α-crystallin (Fig. 2) prior to addition of DTT shows that the spectra of α-lactalbumin are noticeably broader in the former, suggesting that there is some association between the two proteins under nonreducing conditions that leads to exchange broadening. Similar effects are observed upon mixing α-crystallin with other proteins, e.g. β- and γ-crystallins (4), reduced and carboxymethylated α-lactalbumin and ovalbumin (19), insulin, and ovotransferrin, which implies that α-crystallin has a weak, nonspecific association with a variety of stable proteins. The interaction between α- and the β- and γ-crystallins is selective, at least in part, for the C-terminal region of these crystallins (4). Furthermore, this weak interaction of α-crystallin with stable proteins may be a prelude to interactions involved in the chaperone action of α-crystallin.

The Interaction of α-Crystallin with Various Intermediately Folded Forms of α-Lactalbumin—From the above results on the reduction of α-lactalbumin with DTT, it seems that during its chaperone action, α-crystallin interacts with the disordered molten globule (A state) of α-lactalbumin to form a HMW complex. The various intermediately folded states of α-lactalbumin have been extensively investigated by a variety of different techniques (summarized by Kuwajima (8)). Importantly, various partially folded states of α-lactalbumin can be prepared as stable entities that can then be used as probes to investigate the interaction of α-crystallin with unfolded proteins.

Initial studies were undertaken on mixtures of α-crystallin, and the well characterized molten globule state of apo-α-lactalbumin that is observed at neutral pH and low salt in the absence of calcium. Fig. 4 shows the aromatic region of the one-dimensional 1H NMR spectra of apo-α-lactalbumin at pH 7.3, 1 mM EDTA (to complex any calcium ions) and 45 °C in the absence and the presence of a 2-fold mass excess α-crystallin. The spectrum of α-lactalbumin on its own under these conditions (Fig. 4A) exhibits little chemical shift distribution and is relatively broad, which is indicative of a molten globule state. In the presence of α-crystallin (Fig. 4B), there is some small alteration in the chemical shifts of resonances, but none are lost. This suggests that there is some weak association between the two proteins but no formation of a HMW complex, i.e. similar to the situation observed with native α-lactalbumin in the presence of α-crystallin (compare the spectrum of α-lactalbumin prior to the addition of DTT in Fig. 2A with the corre-

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sponding one in Fig. 2B). The chaperone ability of α-cryptallin is enhanced at elevated temperatures (21, 22), so the absence of any specific interaction between the two proteins does not arise from a decrease in chaperone action of α-cryptallin at 45 °C.

A comparison of the $^1$H NMR spectrum of apo-α-lactalbumin in the molten globule A state at pH 1.9 and 20 °C (Fig. 3) with that at pH 7.3 and 45 °C (Fig. 4A) reveals that better resolved resonances are observed in the spectrum at neutral pH, i.e. the molten globule states of α-lactalbumin at these two pH values are not identical, with the state at neutral pH being more structured. Indeed, a one-dimensional spectrum of α-lactalbumin at 20 °C and pH 7.3 was much more structured than that at 45 °C (Fig. 4A) with broadened α-CH resonances immediately downfield of the residual water peak and broadened methyl resonances upfield of 0.8 ppm (not shown), i.e. in regions indicative of tertiary structure. These resonances were only very weakly present in the spectrum at 45 °C, implying that higher temperatures at neutral pH favor a more molten globule-like state for α-lactalbumin. It seems, therefore, that there are a variety of molten globule states of α-lactalbumin that are associated with relative degrees of unfolding. In the case of α-lactalbumin, the partially folded states observed at pH 7.3 and 1.9 correspond respectively to the highly ordered and disordered molten globule states. The spectrum in Fig. 4A is similar, therefore, to that observed for α-lactalbumin 46 s after its reduction with DTT (Fig. 2A). From the data presented in Fig. 4, it is apparent that the weak association of α-cryptallin with the highly ordered molten globule state of α-lactalbumin does not lead to the formation of a HMW complex. On the other hand, upon reduction with DTT, the disordered molten globule A state of α-lactalbumin interacts with α-cryptallin to form a HMW complex (Fig. 2B). Finally, it should be noted that α-cryptallin dissociates at pH values below 4 (23), so it was not possible to investigate its interaction with the molten globule A state of α-lactalbumin.

It is possible to isolate intermediately folded, molten globule forms of apo-α-lactalbumin. Thus, α-lactalbumin with its four disulfide bonds reduced (and subsequently blocked via reaction with iodoacetamide to form R-cam-α-lactalbumin) has a molten globule conformation at neutral pH as judged spectroscopically (15). Likewise, α-lactalbumin with its hyper-reactive Cys$^\alpha$ Cys$^\beta$ disulfide bond selectively reduced and carboxymethylated, i.e. 3SS-α-lactalbumin (10), adopts a molten globule conformation. Both derivatives of α-lactalbumin were prepared and were found to have the expected masses as judged by electrospray ionization mass spectrometry (14293 ± 3 Da for 3SS-α-lactalbumin, expected 14292 Da; 14640 ± 2 Da for R-cam-α-lactalbumin, expected 14638 Da). The interactions of these derivatives with α-cryptallin were assessed by NMR spectroscopy and size exclusion HPLC.

The aromatic region of the one-dimensional $^1$H NMR spectra of R-cam-α-lactalbumin at 20 °C in the absence and the presence of a 4-fold mass excess of α-cryptallin is shown in Fig. 5. It is apparent from the spectrum of R-cam-α-lactalbumin in the absence of α-cryptallin that the protein adopts a disordered molten globule conformation, i.e. the spectrum is very similar to that observed for the A state of α-lactalbumin (Fig. 3) and upon reduction of α-lactalbumin with DTT in the presence of α-cryptallin (Fig. 2B). Upon addition of α-cryptallin (in two stages of 2- and 4-fold mass excess) at 20 °C, there was little change in the spectrum in the aromatic region apart from a general broadening (Fig. 5B). Furthermore, there was no loss in signal intensity in this region of the spectrum, implying that little or no HMW complex had formed. In confirmation of this conclusion, a size exclusion HPLC profile of this mixture gave no indication of a HMW species. Furthermore, TOCSY spectra of R-cam-α-lactalbumin in the absence and the presence of α-cryptallin (not shown) indicated little change in the aromatic resonances of α-lactalbumin due to the presence of α-cryptallin.

R-cam-α-lactalbumin binds readily to GroEL, and the interaction is enhanced in the presence of high concentrations of salt (15). Thus, the salt screens electrostatic repulsive forces between R-cam-α-lactalbumin and GroEL, implying that hydrophobic interactions are important in the interaction between the two proteins. Salt (KCl) to a concentration of 0.2 M was therefore added to the mixture of R-cam-α-lactalbumin and α-cryptallin to investigate whether salt had a similar effect on the interaction of these two proteins. No effect, however, was discernable on the one-dimensional $^1$H NMR resonances of either protein upon addition of salt. Furthermore, a size exclusion HPLC profile of this solution exhibited only peaks indicative of the two proteins and a profile that was very similar to a mixture of native α-lactalbumin and α-cryptallin (Fig. 6). In an HPLC profile of the protein in the absence of α-cryptallin, the peak arising from R-cam-α-lactalbumin is broadened compared with that for the native α-lactalbumin, and it has a slightly later elution position (Fig. 6). In the presence of α-cryptallin and 0.2 M KCl, this peak sharpens but still elutes later than native α-lactalbumin. No peak indicative of a HMW complex was observed. These data are consistent with R-cam-α-lactalbumin being of different conformation (possibly of more dynamic character) compared with native α-lactalbumin. It is concluded from the above results, therefore, that α-cryptallin does not interact with the molten globule, R-cam-α-lactalbumin to form a HMW complex.

A similar series of experiments was conducted at 37 °C on 3SS-α-lactalbumin in the absence and the presence of α-cryptallin. The $^1$H NMR spectrum of this α-lactalbumin derivative at 20 °C (not shown) and 37 °C (Fig. 7A) was more structured than that of R-cam-α-lactalbumin, suggesting that it had more of a highly ordered molten globule conformation, i.e. similar to that for apo-α-lactalbumin at neutral pH and high temperature (Fig. 4) and α-lactalbumin 46 s after reduction with DTT (Fig. 2A). As for the R-cam derivative, little alteration was observed in the NMR spectrum upon addition of a 4-fold mass excess of α-cryptallin (Fig. 7B). Thus, there was no complexation between the two proteins to form the HMW complex. The size
exclusion HPLC profile of the NMR mixture was consistent with this conclusion (not shown), i.e. only peaks associated with α crystallin and 3SS-α-lactalbumin were observed. Unlike the HPLC peak for R-cam-α-lactalbumin, however, the 3SS-α-lactalbumin peak had characteristics of a more-structured species, i.e. its shape and elution time were similar to those of native α-lactalbumin.

The overall conclusion, therefore, from these experiments with molten globule forms of α-lactalbumin (Figs. 4–7) is that α crystallin does not interact with the disordered or highly ordered molten globule forms of α-lactalbumin that are stable in solution. From the results on reduced α-lactalbumin (Fig. 2), α crystallin will only interact with the disordered molten globule state of α-lactalbumin when it is on the path to an aggregated, insoluble precipitate.

Recently, the folding of apo-α-lactalbumin following rapid dilution from denaturant has been followed by rapid acquisition one-dimensional 1H NMR spectroscopy (18). It was of interest to examine whether α crystallin interacted with any of the intermediates of α-lactalbumin along its folding pathway. We did not have access to facilities to enable rapid acquisition of spectra as Balbach et al. (18) had done but were able to compare spectra of the folding of α-lactalbumin in the absence and the presence of twice the mass of α crystallin within 40 s of initiation of the reaction via dilution 10-fold from denaturant (6 M guanidinium chloride (GuCl)). In both cases, one-dimensional NMR spectra were acquired over a period of an hour (not shown) and during this period, only resonances from native α-lactalbumin were observed, i.e. no resonances indicative of molten globule states of α-lactalbumin were present. In fact, the NMR spectra of α-lactalbumin refolded under these two conditions (not shown) indicated that there was little difference between them apart from broader resonances being observed following refolding of α-lactalbumin in the presence of α crystallin. Consistent with the results of Balbach et al. (18), the spectra showed that α-lactalbumin folded rapidly and that its folding was almost complete within the first 40 s following dilution from denaturant. Stopped flow circular dichroism and fluorescence measurements indicate a similar time period for refolding of α-lactalbumin (24). It is concluded, therefore, that α crystallin did not alter the rate of folding of α-lactalbumin nor did it interact with intermediates along the folding pathway. As expected from the results detailed above, α crystallin did interact weakly with α-lactalbumin in its native folded form, which led to broadened resonances of α-lactalbumin. TOCSY spectra of both solutions after folding had been completed were identical, apart from broader cross-peaks being observed for α-lactalbumin in the presence of α crystallin (not shown). A size exclusion HPLC profile of the mixture of α-lactalbumin and α crystallin indicated that no HMW complex had formed.

**DISCUSSION**

Ascertaining the nature of the interaction of chaperone proteins with their substrate proteins is fundamental to determining the mechanism of action of this class of proteins. In this paper, we have chosen α-lactalbumin as a model substrate for the chaperone protein, α crystallin. This system has a number of advantages; the various folded states of α-lactalbumin are well understood, these intermediately folded states can be stabilized and isolated, and both α-lactalbumin and α crystallin are easily obtained in the relatively large quantities required for NMR studies of their interaction. We have monitored the interaction between these two proteins under a variety of conditions by NMR spectroscopy and have provided strong evidence that α-lactalbumin interacts with α crystallin to form a
HMW complex when the former is in a disordered molten globule state while on the irreversible pathway to aggregation and precipitation.

It is generally believed that the various intermediates along the folding pathway of a particular protein are similar if not equivalent to those along its unfolding pathway (16). If it is assumed, therefore, that unfolding of a protein occurs via the reverse pathway to its folding pathway, then a hierarchy of protein unfolding would involve the following steps in Scheme 1, where MG indicates molten globule.

\[
\text{folded protein} \rightarrow \text{highly ordered MG} \rightarrow \text{disordered MG} \rightarrow \text{unfolded protein}
\]

\[\downarrow \text{aggregation and precipitation}\]

**SCHEME 1**

The alternative paths in the last step in Scheme 1 lead either to the unfolded protein (that remains in solution) or along an irreversible path to an aggregated and precipitated protein. A very similar scheme to that presented in Scheme 1 has been proposed for the refolding or aggregation of carbonic anhydrase B following dilution from 5 M GuCl (25). Thus, for relatively concentrated solutions of carbonic anhydrase B, a disordered molten globule state forms upon rapid dilution from GuCl, which then aggregates and precipitates. The aggregation route of carbonic anhydrase B is characterized by the formation of dimers and trimers prior to their mutual association into very large (micron-sized) aggregates (25). Similar mechanisms probably occur for other proteins upon aggregation and precipitation.

In this study, α-lactalbumin is present in relatively high concentrations. When α-lactalbumin is reduced with DTT under these conditions, the aggregation and precipitation pathway of Scheme 1 is present. The specificity of α-crystallin for proteins that are on the off-folding pathway makes sense when the role of this chaperone is considered. In the lens, where α-crystallin has a stabilizing role in minimizing protein precipitation and hence cataract formation, α-crystallin would not interact with proteins that still have reasonable elements of structure, i.e., those adopting the highly ordered molten globule state in which most of their secondary structure and some of their tertiary structural features are present. These types of proteins could potentially refold back into their correct conformation. Likewise, proteins that are in the disordered molten globule state but are quite stable in solution would not interact with α-crystallin. Only when a protein is in a disordered molten globule state and on the path to aggregation and precipitation will it be recognized for interaction with α-crystallin and stabilized via formation of a HMW complex. One possible mechanism by which α-crystallin recognizes this aggregated molten globule state of α-lactalbumin is by its large increase in hydrophobicity compared with the molten globule states along the unfolding/folding pathway. Finally, α-crystallin will not recognize unfolded and stable proteins, e.g., reduced and carboxymethylated α-lactalbumin is unfolded and quite stable but has few concentrated regions of hydrophobicity exposed to solution and does not interact with α-crystallin (15, 19). Likewise, α-casein is unfolded and stable and exposes hydrophobic regions to solution but does not interact with α-crystallin (19, 26).

The conformation of proteins bound to α-crystallin has been probed using other techniques (12, 19, 27). To eliminate any complication from intrinsic fluorescence, Das et al. (27) used fluorescence spectroscopy to examine the interaction of rhodanese and γ-crystallin with a mutant of α-crystallin in which the single tryptophan residue had been replaced by a phenylalanine. They concluded that α-crystallin binds proteins that have a low degree of unfolding, i.e., states that are early along the unfolding pathway. Furthermore, they suggested that the conformation of proteins bound to α-crystallin is more structured than that of GroEL-bound proteins. The data presented herein are not in total agreement with these conclusions, because we observe little interaction of α-crystallin with the highly ordered molten globule state of α-lactalbumin. Instead, we conclude that α-crystallin binds proteins in a disordered molten globule state along the off-folding pathway. Das et al. (27) also concluded, however, that proteins bound to α-crystallin have significant elements of secondary structure, which is in agreement with the results of this study. Furthermore, Farahbakhsh et al. (12) used EPR spectroscopy of spin-labeled peptides bound to α-crystallin to show that they bind in a semi-structured arrangement and are separated from each other, i.e., they are not clustered and bind in a nonaggregated state. Finally, our fluorescence studies of the binding of the hydrophobic probe, 8-anilino-1-naphthalene sulfonate, to the HMW complexes of α-crystallin and substrate proteins were consistent with the substrate protein being bound in a molten globule state (19). To summarize these results, therefore, it seems that α-crystallin binds proteins that are not totally unfolded but retain elements of secondary structure.

In previous studies on the interaction of α-crystallin with γ-crystallin unfolded by GuCl (19), γ-crystallin had to be diluted gradually out of the denaturant in the presence of α-crystallin to avoid precipitation of γ-crystallin that occurred upon rapid dilution from denaturant, even in the presence of an excess of α-crystallin. If the dilution was performed incrementally, however, with concomitant partial unfolding of α-crystallin, a HMW complex was formed that was characterized by NMR and HPLC (19). In agreement with this result, Das and Surewicz (26) concluded that α-crystallin did not stabilize rhodanese or βL- or γ-crystallin upon rapid dilution from denaturant, i.e., they all precipitated from solution in such an experiment. α-Lactalbumin, by contrast, does not precipitate from solution upon rapid dilution from denaturant and it refolds rapidly, i.e., α-lactalbumin is intrinsically more stable than these proteins upon such treatment. Furthermore, during this folding process, α-crystallin did not interact with α-lactalbumin to form a HMW complex. It seems, therefore, that α-crystallin in its native state does not interact with proteins along their folding pathway. Das and Surewicz (26) came to the same conclusion.

In the unfolding of α-lactalbumin following reduction with DTT, its interaction with α-crystallin to form a HMW species only occurs when α-lactalbumin is on the way to aggregation and precipitation, i.e., on the off-pathway step in Scheme 1. It is not possible to study the unfolding of α-lactalbumin in the presence of GuCl and α-crystallin in a manner analogous to that used for investigating its folding following dilution from GuCl because α-crystallin will also unfold under these conditions. For α-lactalbumin on its own, however, this reaction was monitored by 1H NMR spectroscopy. In this experiment, the unfolding of α-lactalbumin, following its dilution 10-fold into 6 M GuCl, was found to occur within the 40 s it took to acquire the initial spectrum. Thus, no molten globule state of α-lactalbumin was observed, implying that the unfolding, like the folding, of α-lactalbumin is a very rapid process. By contrast, during the unfolding of α-lactalbumin following reduction with DTT, the disordered molten globule state is stabilized for a long time (up to ~1500 s) prior to precipitation or interaction with α-crystallin to form the HMW complex (Fig. 2). As discussed above, this intermediate is long lived due to the long time required to reduce the remaining three disulfide bonds following rapid reduction of the hyper-reactive Cys8-Cys420 bond (10). Thus, a possible reason why α-crystallin can interact with reduced and aggregated α-lactalbumin in the disordered molten globule
state under these conditions is simply that this state exists for a considerably longer time compared with when fully oxidized α-lactalbumin is either folded or unfolded along the linear pathway as indicated in Scheme 1. In either of these scenarios, the disordered molten globule state is only present transiently. Thus, on this basis, rhodanese and β- and γ-crystallin all precipitate from solution upon rapid dilution out of denaturant in the presence of α-crystallin because their disordered molten globule states are not present long enough to interact with α-crystallin. By contrast, the presence of a relatively long lived disordered and aggregated molten globule state of α-lactalbumin under reducing conditions would give α-crystallin the time to interact with this state. Recall, however, that α-crystallin does not interact with R-cam-α-lactalbumin in the disordered molten globule state (Figs. 5 and 6). Thus, for this state to interact with α-crystallin, in addition to it being present for a long time, it must also be largely aggregated and prone to precipitation from solution.

The implication from the above discussion is that one way α-crystallin differentiates between molten globule states of interacting proteins is on the basis of their lifetimes. This ability makes sense when the function of α-crystallin to act as a stabilizer of destabilized proteins is considered. Thus, in the lens, where there is no protein turnover, the destabilization of the crystallins takes place gradually over a long period of time, which would give α-crystallin the time to interact and prevent the protein precipitating out of solution. α-Crystallin therefore has no ability to stabilize folding, nascent proteins because this process occurs relatively rapidly. Instead, it only interacts with previously functional, mature proteins that are about to aggregate and precipitate from solution.

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