The molecular chaperone α-crystallin is in kinetic competition with aggregation to stabilize a monomeric molten-globule form of α-lactalbumin

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In vivo, α-crystallin and other small heat-shock proteins (sHsps) act as molecular chaperones to prevent the precipitation of 'substrate' proteins under stress conditions through the formation of a soluble sHsp–substrate complex. Using a range of different salt conditions, the rate and extent of precipitation of reduced α-lactalbumin have been altered. The interaction of α-crystallin with reduced α-lactalbumin under these various salt conditions was then studied using a range of spectroscopic techniques. Under conditions of low salt, α-lactalbumin aggregates but does not precipitate. α-Crystallin is able to prevent this aggregation, initially by stabilization of a monomeric molten-globule species of α-lactalbumin. It is proposed that this stabilization occurs through weak transient interactions between α-crystallin and α-lactalbumin. Eventually a stable, soluble high-molecular-mass complex is formed between the two proteins. Thus it appears that a tendency for α-lactalbumin to aggregate (but not necessarily precipitate) is the essential requirement for α-crystallin–α-lactalbumin interaction. In other words, α-crystallin interacts with a non-aggregated form of the substrate to prevent aggregation. The rate of precipitation of α-lactalbumin is increased significantly in the presence of Na_2SO_4 compared with NaCl. However, in the former case, α-crystallin is unable to prevent this aggregation and precipitation except in the presence of a large excess of α-crystallin, i.e. at mass ratios more than 10 times greater than in the presence of NaCl. It is concluded that a kinetic competition exists between aggregation and interaction of unfolding proteins with α-crystallin.

Key words: Hofmeister salts, small heat-shock protein, spectroscopy.

INTRODUCTION

Stress conditions such as heat, oxidation and exposure to heavy metals result in destabilization of proteins, which can lead to aggregation and precipitation. α-Crystallin is the principal lens protein and is also found in a number of other tissues. Under stress conditions, α-crystallin and other members of the family of small heat-shock proteins (sHsps) prevent precipitation of unfolding proteins through the formation of a stable, soluble high-molecular-mass complex with the compromised 'substrate' protein. The interaction occurs primarily via hydrophobic interactions [1,2], similarly to other chaperone–substrate protein interactions [3]. α-Lactalbumin is used frequently as a substrate in chaperone activity studies of α-crystallin and other sHsps [4–6], as its precipitation can be induced at physiological temperatures [by reduction of its four disulphide bonds with dithiothreitol (DTT)] without the complication of alteration in the structure of α-crystallin, which does not contain any disulphide bonds. In addition, the folding and unfolding of α-lactalbumin, as well as its partially folded, molten-globule intermediates have been well characterized (reviewed in [7]).

Using α-lactalbumin as a model substrate, we have shown previously that α-crystallin binds preferentially to a molten-globule-like state of the reduced 'substrate' protein that is on an irreversible path to aggregation and precipitation [4,5]. Under these conditions, α-lactalbumin is classified as being unstable. α-Crystallin does not interact with stable (i.e. monomeric and non-precipitation-prone) molten-globule intermediates along the reversible protein folding/unfolding pathway. These intermediates are recognized by other chaperones (e.g. Hsp70 and Hsp60) which have the capacity to facilitate correct protein folding. Nor does α-crystallin complex with chemically modified forms of substrate proteins that adopt molten-globule conformations [4,8]. These states are neither prone to aggregation nor in reversible equilibrium with the native state. Thus it appears that α-crystallin and the sHsps specifically interact with substrates that are on an off-folding pathway destined for aggregation and precipitation. However, many questions still remain regarding the nature of this interaction. For example, how do sHsps distinguish between intermediately folded forms of proteins that are stable in solution and those that are prone to aggregation and precipitation? Does the substrate protein bind in a monomeric or aggregated form? Why is interaction with a sHsp preferable to aggregation and precipitation, a pathway that leads to an energy minimum? In this paper, we have attempted to address these questions. To do this, we have altered the conditions under which aggregation and precipitation of α-lactalbumin occur by varying the type and concentration of salt present, according to the Hofmeister series [9]. We have then examined its interaction with α-crystallin.

In general, protein aggregation and precipitation can be modified or regulated by the addition of salts from the Hofmeister series, which 'ranks' salts according to their effectiveness in disrupting the structure of water, i.e. $SO_4^{2-} = HPO_4^{2-} > F^- > Cl^- > Br^- > I^-$ $(= ClO_4^-) > SCN^-$ [9]. The first ions in the series (e.g. SO_4^{2-}) increase the surface tension of water and the structure of water molecules around hydrophobic groups, thereby decreasing the solubility of non-polar molecules. Consequently, hydrophobic interactions are favoured that stabilize proteins. In contrast, the last ions in the series decrease the surface tension of water, thereby increasing the solubility of non-polar molecules and thus destabilizing proteins. For this study we have used a neutral, constant cation (Na^+) and SO_4^{2-} and Cl^- anions to alter the rate and extent of aggregation and pre-

Abbreviations used: ANS, 8-anilino-1-naphthalenesulphonic acid; DTT, dithiothreitol; ESI-MS, electrospray ionization MS; sHsp, small heat-shock protein; 1D, one-dimensional.

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cipitation of α-lactalbumin under reducing conditions. Specifically, we have examined the interaction between α-lactalbumin and α-crystallin in the presence of Hofmeister series anions in an attempt to understand better the interaction between sHsps and unfolding proteins.

EXPERIMENTAL

Materials

Bovine α-lactalbumin (Ca^{2+}-depleted; > 85 % pure), 8-anilino-1-naphthalenesulphonic acid (ANS) and DTT were obtained from Sigma. ^2H_2O (99.9 % pure) and 1,4-D,L-DTT-d_{10} (98 % deuterated) were purchased from Cambridge Isotope Laboratories. Pre-cast polyacrylamide mini gels (12 % acrylamide) for Tris/glycine SDS/PAGE were purchased from Gradipore (Sydney, Australia). Sephacryl S-300 was obtained from Pharmacia (Uppsala, Sweden). Bovine lenses were obtained from a local abattoir. Unless otherwise stated, all experiments were performed in 50 mM sodium phosphate, pH 7.2/0.02 % NaN_3 alone, or with the addition of 0.1 M NaCl or Na_2SO_4.

Purification of α-crystallin

Lenses were extracted from calf eyes obtained from the local abattoirs and stored at −20 °C. Crystallins (α and β) were purified from bovine lenses based on the method of Slingsby and Bateman [10] with the following modifications. Lenses were homogenized in 50 mM Tris buffer, pH 7.2, containing 5 mM EDTA, 1 mM DTT, 0.04 % NaN_3 and 0.1 mM PMSF (3 ml per lens). Following centrifugation at 12000 rev./min (12000 ***g***) at 4 °C in an Eppendorf microfuge, the supernatant (usually 5 ml at a time) was loaded on to a gel-filtration column containing Sephacryl S-300 (column size 3.0 cm × 90 cm). Protein was eluted using 50 mM Tris buffer, pH 7.5/5 mM EDTA/0.04 % NaN_3 at a rate of approx. 20 ml/h, and monitored at 280 nm. Those fractions shown to contain either α- or β-crystallin were concentrated to approx. 3 ml and then rechromatographed on the same column. SDS/PAGE showed that crystallin preparations were > 95 % pure.

Visible absorption spectroscopy

The ability of α-crystallin to prevent DTT-induced aggregation and precipitation was monitored using the method of Horwitz et al. [6]. α-Lactalbumin (1.0 or 2.0 mg/ml) in 50 mM sodium phosphate, pH 7.2/0.02 % NaN_3, with or without the addition of 0.1 M salt was incubated at 37 °C in the presence of increasing amounts of α-crystallin. Reduction and precipitation of α-lactalbumin was induced by the addition of DTT to a final concentration of 20 mM and the subsequent increase in light scattering monitored at 360 nm using a Molecular Devices Spectramax 250 spectrophotometer.

Gel filtration

The interaction between α-lactalbumin and α-crystallin resulting in formation of a sHsp–substrate high-molecular-mass complex was assessed using gel filtration. Samples (usually 200 μl containing 2.9 mg of α-lactalbumin and 5.8 mg of α-crystallin, or 350 μl containing 2.5 mg of α-lactalbumin and 5.0 mg of α-crystallin) were loaded on to a column (1.5 cm × 70 cm) containing Sephacryl S-300 and eluted with the appropriate buffer, i.e. 50 mM sodium phosphate, pH 7.2/0.02 % NaN_3 with or without addition of 0.1 M NaCl or Na_2SO_4. Protein was eluted at a rate of 20 ml/h and monitored at 280 nm.

ANS fluorescence binding experiments

α-Lactalbumin or α-crystallin were incubated with DTT (29 mM) in 50 mM sodium phosphate, pH 7.2/0.02 % NaN_3 with and without the addition of 0.1 M salt for 1 h at 37 °C in a final volume of 140 μl. Following incubation, samples were diluted to 2.0 ml using the same buffer (final concentration of α-lactalbumin and α-crystallin was 10 and 20 μM, respectively). Control solutions containing 20 μM α-crystallin or 10 μM α-lactalbumin, either with or without DTT, were also prepared. The fluorescence of the hydrophobic probe ANS was measured as described previously [5].

CD spectroscopy

CD spectra were acquired on a Jobin-Yvon CD-6 spectrophotometer with a Haake recirculating water bath at 37 °C. Spectra of α-crystallin and α-lactalbumin at 4 mg/ml were acquired using a 0.1 cm-pathlength cell in the far-UV region (200–240 nm) and a 1.0 cm-pathlength cell in the near-UV region (240–320 nm). All experiments were performed in 50 mM sodium phosphate, pH 7.2/0.02 % NaN_3 with or without addition of 0.1 M salt and 20 mM DTT. Five scans were acquired and averaged for each sample.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed using an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with absorbance optics. Experiments were performed at 25000 rev./min (45000 ***g***) at 25 °C using α-lactalbumin (5 mg/ml) in 20 mM sodium phosphate, pH 7.2/0.02 % NaN_3/20 mM DTT. Radial scans were acquired at 280 nm every 20 min, with a total of six scans for each experiment.

Electrospray ionization MS (ESI-MS)

ESI-MS was carried out on a VG Quattro mass spectrometer (VG BIOTECH, Altrincham, Cheshire, U.K.) as described previously [4].

^{1}H-NMR spectroscopy

One-dimensional (1D) ^{1}H-NMR spectra were acquired at 400 MHz on a Varian Unity-400 spectrometer. Samples containing α-lactalbumin (10 mg) with or without α-crystallin (20 mg) were prepared in a final volume of 0.7 ml. Data were acquired over 16000 data points and a spectral width of 4500 Hz. Spectra represent a total of 16 scans, with a line broadening of 1 Hz being applied prior to Fourier transformation.

RESULTS

The reduction of α-lactalbumin in the presence of various Hofmeister salts

Figure 1 shows aggregation and precipitation of α-lactalbumin in the presence of various sodium salts and α-crystallin after the addition of DTT. As expected [4], α-lactalbumin (at 2 mg/ml) precipitates from solution in the presence of 0.1 M NaCl and this precipitation can be prevented by the addition of α-crystallin at a 2:1 (w/w) ratio of α-crystallin/α-lactalbumin (Figure 1a). α-Lactalbumin also precipitates to a similar extent in the presence of 0.1 M Na_2SO_4 but this precipitation occurs much more rapidly than in the presence of NaCl (Figure 1b), i.e. precipitation commences around 18 min after addition of DTT, compared with 35 min in the presence of NaCl. Interestingly, in the presence

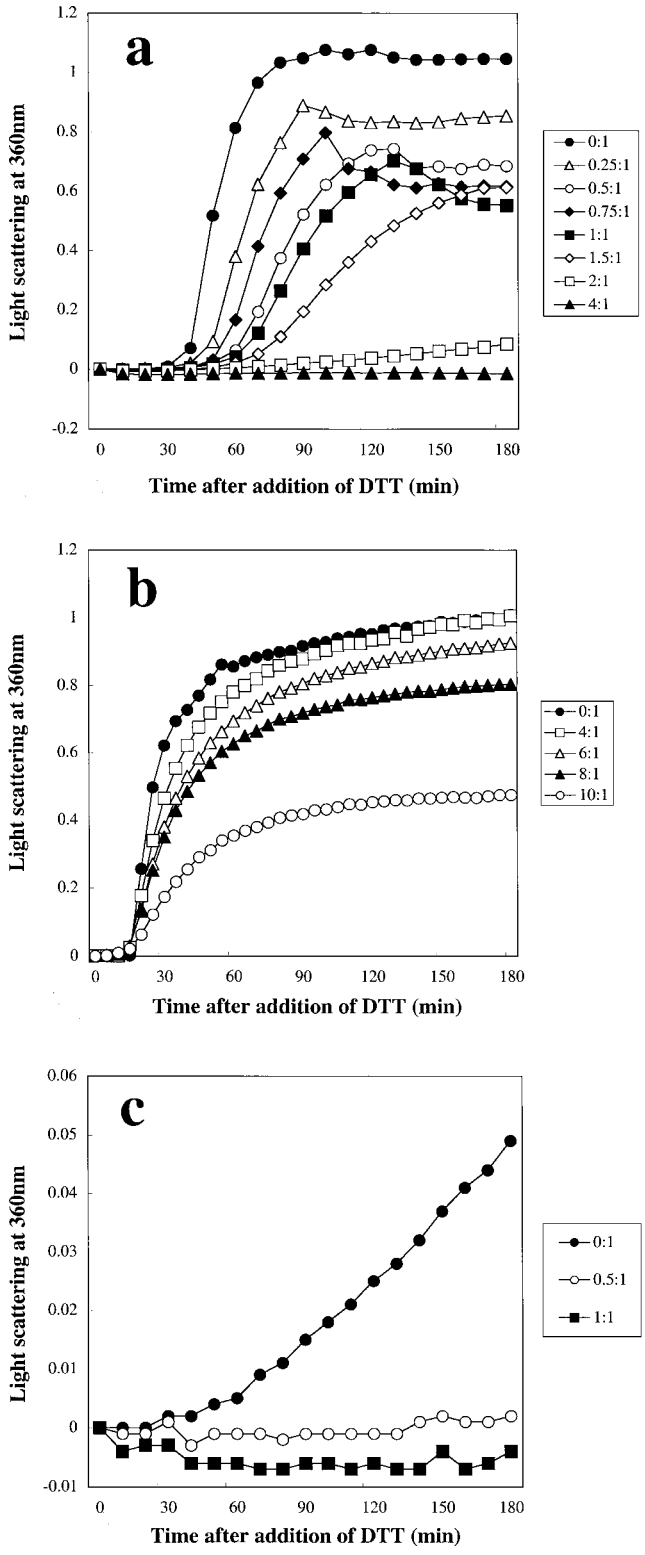


Figure 1 Protein aggregation and precipitation (monitored by light scattering at 360 nm) with time for mixtures of α-lactalbumin (2 mg/ml) and increasing concentrations of α-crystallin following the addition of 20 mM DTT

Experiments were performed in 50 mM sodium phosphate, pH 7.2/0.02% NaN_3 and (**a**) 0.1 M NaCl, (**b**) 0.1 M Na_2SO_4 or (**c**) no added salt. The ratios indicated are of α-crystallin/α-lactalbumin (w/w). Data were acquired at 37 °C.

of Na_2SO_4, α-crystallin was not able to prevent the precipitation of α-lactalbumin at w/w ratios that gave complete suppression in the presence of NaCl. Even at a ratio of 10:1 (w/w) α-crystallin/α-lactalbumin, only approx. 50% protection was observed.

Similar experiments were undertaken in the absence of any added salt (Figure 1c). In this case, no precipitation of α-lactalbumin was observed, but a slight increase in absorbance occurred in the absence of α-crystallin that corresponds to aggregation of α-lactalbumin. A very low w/w ratio of α-crystallin/α-lactalbumin prevented this small increase in absorbance. Sedimentation velocity experiments confirmed that, at pH 7.2 in the absence of added salt, α-lactalbumin aggregates upon addition of 20 mM DTT with a change in sedimentation coefficient from 1.93 S for the native state [11] to 26.3 S (results not shown), i.e. reduced α-lactalbumin in the absence of salt is highly aggregated. Thus in the absence of added salt, precipitation of DTT-reduced α-lactalbumin is prevented. However, aggregation still occurs.

Size-exclusion chromatography was performed on mixtures of α-lactalbumin and α-crystallin in the presence and absence of DTT under the buffer conditions used for visible absorption spectroscopy in order to determine whether any interaction was occurring between the two proteins, as determined by formation of a high-molecular-mass complex. Figure 2 shows the elution profiles obtained under these various conditions. The experiments were performed at a 2:1 (w/w) ratio of α-crystallin/α-lactalbumin unless otherwise stated. A higher concentration of protein was used relative to visible absorption studies in Figure 1 in order to improve the signal-to-noise ratio of the data. In the presence of NaCl, addition of DTT results in the formation of a high-molecular-mass complex between α-crystallin and α-lactalbumin which elutes at approx. 150 min (Figure 2a). These data are in agreement with previous results [4]. SDS/PAGE confirmed the presence of both α-lactalbumin and α-crystallin in this high-molecular-mass peak (results not shown). In the presence of Na_2SO_4, a ratio of greater than 10:1 (w/w) α-crystallin/α-lactalbumin was necessary to obtain significant protection from precipitation of α-lactalbumin (Figure 1b). Figure 2(b) shows the elution profile of a 15:1 (w/w) solution of α-crystallin/α-lactalbumin following incubation for 2 h in the presence of 20 mM DTT. Under these conditions, some precipitation still occurs (shown by SDS/PAGE to be α-lactalbumin only). Only the supernatant was analysed further by size-exclusion chromatography. Formation of a high-molecular-mass species with an elution time of 150 min was observed by size-exclusion chromatography (Figure 2b). Both SDS/PAGE and ESI-MS showed the presence of α-crystallin and α-lactalbumin in this peak, indicating high-molecular-mass sHsp–substrate complexation.

α-Crystallin can prevent aggregation of reduced α-lactalbumin without formation of a high-molecular-mass complex

In the absence of added salt, where no precipitation of α-lactalbumin occurs in the absence of α-crystallin, no formation of an α-crystallin–substrate complex was observed by size-exclusion chromatography following incubation for 2 h (Figure 2c). Similar results were also observed by Rajaraman et al. [12] at pH 7.2 in the absence of salt. ESI-MS analysis of peaks from Figure 2(c) following 2 h of incubation in the presence of DTT did not detect any α-lactalbumin eluting with the α-crystallin peak at approx. 170 min (results not shown), i.e. only mass spectral peaks corresponding to the αA- and αB-crystallin subunits were observed. Furthermore, under these conditions, another interesting feature was observed from size-exclusion

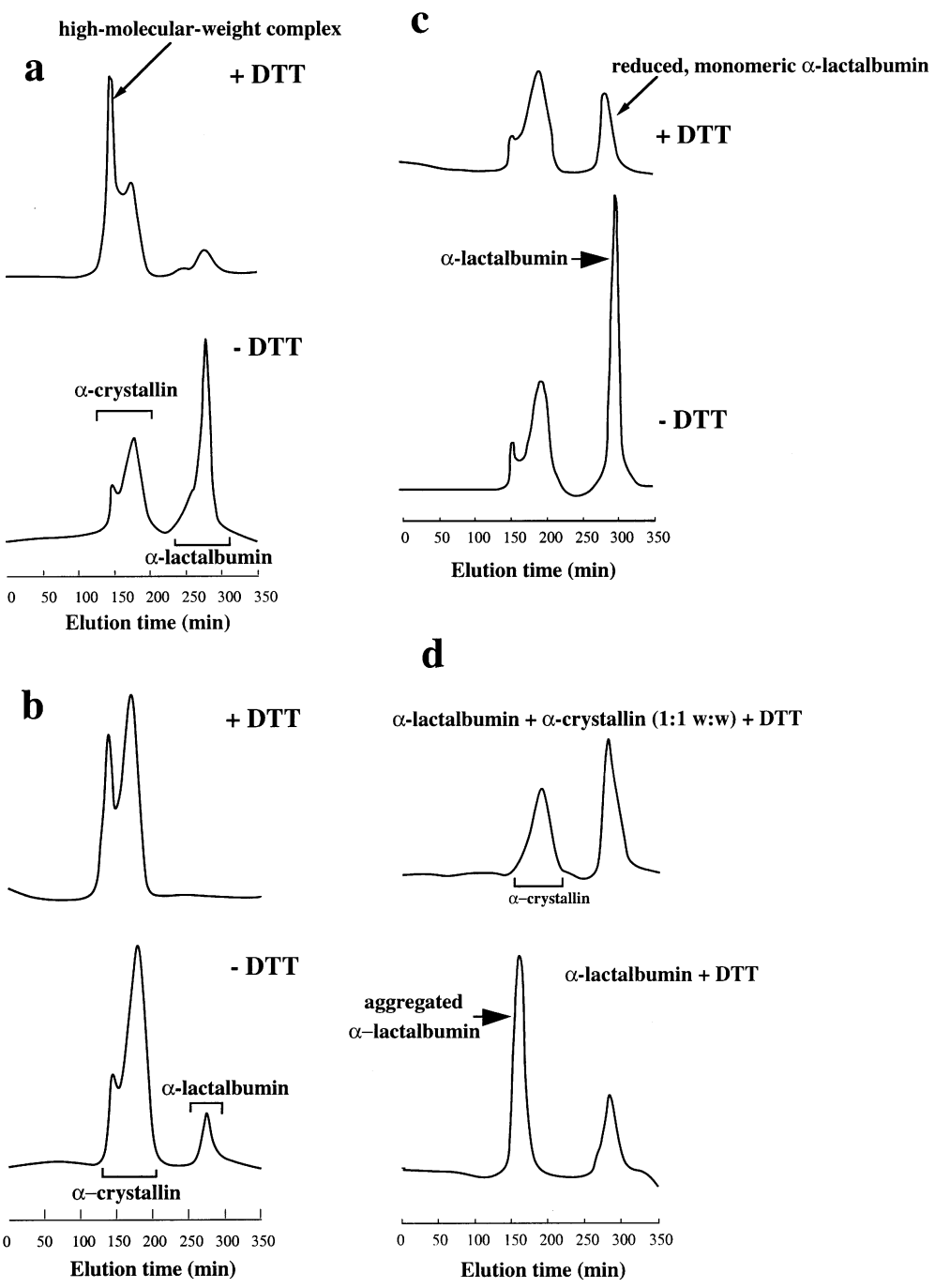


Figure 2 Size-exclusion chromatography on Sephacryl S-300 of α-lactalbumin (2.5 mg) with and without the addition of α-crystallin and DTT (20 mM) in a final volume of 350 μl of 50 mM sodium phosphate, pH 7.2/0.02% NaN_3 and (a) 0.1 M NaCl, 2:1 α-crystallin/α-lactalbumin, (b) 0.1 M Na_2SO_4, 15:1 α-crystallin/α-lactalbumin, 200 μl loaded, (c) no added salt, 2:1 α-crystallin/α-lactalbumin and (d) no added salt, 1:1 α-crystallin/α-lactalbumin (top panel) and reduced α-lactalbumin (bottom panel)

All samples were incubated for 2 h at 37 °C, then centrifuged to remove any precipitate, prior to loading the supernatant on to the column. Note that in (**a**–**c**) a small fraction of highly aggregated α-crystallin was present, giving rise to a small shoulder in the profile of native α-crystallin in the absence of DTT.

chromatography. Reduced α-lactalbumin, in the absence of salt and α-crystallin, formed a high-molecular-mass aggregated species that eluted at approx. 150 min as well as a broad peak at an elution time (270 min) close to that of the native species (Figure 2d). The presence of a large amount of aggregated, reduced α-lactalbumin is consistent with the sedimentation velocity results mentioned above. In the presence of α-crystallin, however, the aggregation of reduced α-lactalbumin did not occur (Figures 2c and 2d), i.e. separate peaks arising from α-crystallin and reduced, monomeric α-lactalbumin only were observed in the size-exclusion chromatography profiles following 2 h of incubation. In addition, an increase in the area of the peak

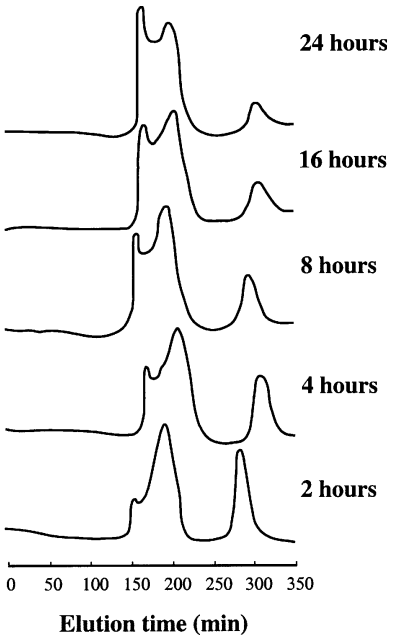


Figure 3 Size-exclusion chromatography on Sephacryl S-300 of α-lactalbumin (2.5 mg) and α-crystallin (5 mg) and DTT (20 mM) in a final volume of 350 μl of 50 mM sodium phosphate, pH 7.2/0.02% NaN_3

Samples were identical to those prepared for Figure 2(c) except that they were incubated for a range of times indicated up to 24 h following addition of DTT. Samples were incubated at 37 °C prior to centrifugation and chromatography of the supernatant. No precipitation was observed under these conditions.

(2–3-fold) arising from monomeric α-lactalbumin occurred in the presence of α-crystallin (Figure 2d), i.e. the monomeric form of reduced α-lactalbumin is stabilized by the presence of α-crystallin. This may be the result of weak, transient interactions between α-crystallin and α-lactalbumin. Consequently, it is concluded that α-crystallin protects the monomeric form of reduced α-lactalbumin from aggregation.

Stabilization of a monomeric substrate occurs prior to complexation with α-crystallin

Figure 3 shows the size-exclusion chromatography profiles for 2:1 (w/w) α-crystallin/α-lactalbumin in 'no salt' conditions at various incubation times from 2 to 24 h after the addition of DTT. At longer times of incubation, an α-crystallin–substrate complex is formed in contrast to after 2 h of incubation, where no interaction was observed (compare with Figure 2c). Thus after long incubation (e.g. 24 h), the reduced, monomeric state of α-lactalbumin has been almost completely incorporated into a complex with α-crystallin, suggesting that stabilization by α-crystallin of a non-aggregated species of α-lactalbumin occurs prior to its incorporation into a complex with α-crystallin.

β-Crystallin has no effect on the aggregation of reduced α-lactalbumin

In order to show that this aggregation-preventative property of α-crystallin was not a general property of all proteins, we repeated the above experiments in the absence of added salt, using lens βH-crystallin instead of α-crystallin. βH-Crystallin is a mixture of β-crystallin subunits of molecular masses from 20 to 30 kDa whose size and chromatographic properties are unaffected by reduction. βH-Crystallin has no chaperone capabilities but shares similar gross secondary-structural features with α-crystallin. Addition of βH-crystallin did not prevent aggregation of α-lactalbumin (as determined by size-exclusion chromatography with a peak eluting at 150 min in both the presence and absence of βH-crystallin; results not shown). SDS/PAGE confirmed that this peak contained α-lactalbumin only. Thus the ability to prevent the aggregation of α-lactalbumin appears to be a specific property of α-crystallin. Further experiments were also performed in which a 2-fold mass excess of α-crystallin was added to reduced α-lactalbumin in the absence of added salt 2 h after the addition of DTT, i.e. by which time α-lactalbumin had aggregated. The sample was then incubated for an additional 2 h at 37 °C before analysis by size-exclusion chromatography. Under these conditions, aggregated α-lactalbumin was still observed (results not shown), i.e. α-crystallin can prevent aggregation but has no capacity to de-aggregate α-lactalbumin. It should also be noted that under conditions of no salt, i.e. 50 mM sodium phosphate buffer, pH 7.2, α-crystallin is still capable of forming an α-crystallin–substrate complex with γ-crystallin upon heating [13]. That is, the reduction in ionic strength does not prevent α-crystallin from forming such complexes under heat stress.

Table 1 ANS fluorescence of α-lactalbumin (10 μM) or α-crystallin (20 μM) in the presence of various salts prior to and after the addition of 20 mM DTT

Excitation was at 387 nm and emission was monitored at 479 nm.

	Fluorescence (arbitrary units)		
	α-Lactalbumin	α-Lactalbumin + DTT	% Change
No salt	20.9 ± 2.8	29.8 ± 1.8	43% increase
0.1 M NaCl	17.0 ± 0.6	47.1 ± 4.4	177% increase
0.1 M Na_2SO_4	14.2 ± 0.6	62.9 ± 6.7	343% increase
	α-Crystallin	α-Crystallin + DTT	% Change
No salt	171.9 ± 4.1	174.2 ± 7.6	1.3% increase
0.1 M NaCl	173.4 ± 4.7	179.1 ± 7.6	3.3% increase
0.1 M Na_2SO_4	239.3 ± 5.5	251.8 ± 6.0	5.2% increase

α-Crystallin stabilizes and then complexes with a monomeric disordered molten-globule form of α-lactalbumin

Reduced α-lactalbumin forms a molten-globule-like species [14]. In agreement with this, far- and near-UV CD analysis of the reduction of α-lactalbumin by DTT in the absence of salt showed a loss of tertiary structure but maintenance of native secondary structure in the protein (results not shown, similar to that in [14]). Further indication of formation of a molten-globule state for reduced α-lactalbumin in the absence of salt was apparent from increased fluorescence of the hydrophobic probe, ANS, when bound to reduced α-lactalbumin compared with the native protein, indicating an increased exposure of clustered hydrophobicity (Table 1).

Using real-time NMR spectroscopy, we have shown previously that, in the presence of 0.1 M NaCl, α-crystallin stabilizes the disordered molten-globule state of reduced α-lactalbumin, i.e. one that lacks tertiary structure but retains some elements of secondary structure and resembles the molten-globule 'A' state of α-lactalbumin observed at low pH [4,15]. In order to determine the conformation of reduced α-lactalbumin which is stabilized by α-crystallin in the absence of added salt, the reduction of α-lactalbumin in the presence and absence of α-crystallin was

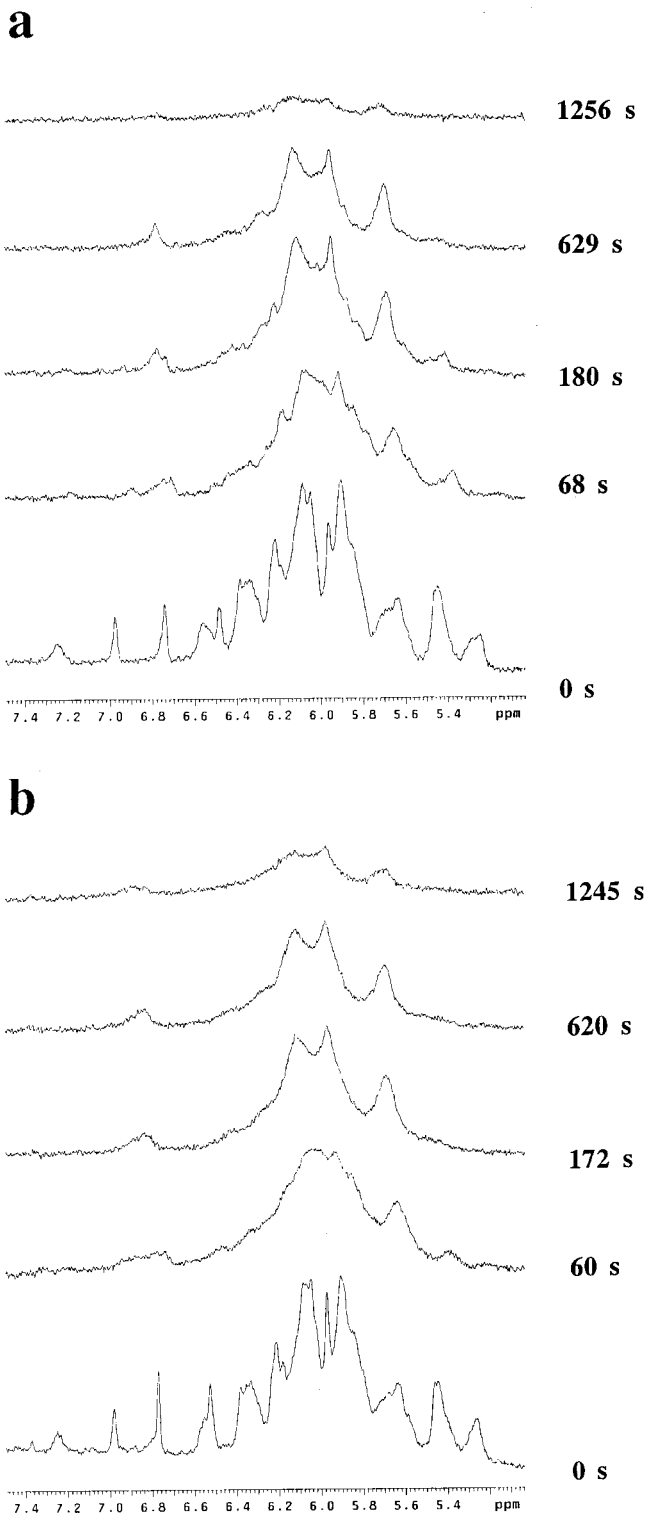


Figure 4 Aromatic region of time-course 1D ^{1}H-NMR spectra of α-lactalbumin (10 mg) in the presence of a 2-fold mass excess of α-crystallin following the addition of 20 mM DTT

Spectra were acquired at 400 MHz and 37 °C in 0.7 ml of 50 mM sodium phosphate, pH 7.2/0.02% NaN_3 (**a**) in the absence of α-crystallin or (**b**) in the presence of 20 mg of α-crystallin. Spectra were acquired at the times indicated following addition of DTT.

monitored by time-course 1D ^{1}H-NMR spectroscopy. This technique enables the observation, in real time, of the various folding intermediates of α-lactalbumin along its pathway to aggregation and precipitation or interaction with α-crystallin.

Figure 4 shows the aromatic region of a series of 1D ^{1}H-NMR experiments acquired with time after the addition of DTT. As α-crystallin has no aromatic residues in its flexible C-terminal extensions [16], only well-resolved resonances from α-lactalbumin are present in this region of the spectrum of a mixture of these two proteins. Figure 4(a) shows that following addition of DTT, α-lactalbumin forms an ordered and then disordered molten-globule species (68 and 180 s, respectively, following addition of DTT), prior to aggregation. No fully unfolded species is detected, supporting the notion that aggregation of α-lactalbumin and other proteins occurs via the association of partially folded, molten-globule intermediates [4,5,17,18]. Comparison of Figures 4(a) and 4(b) (i.e. spectra in the absence and presence of α-crystallin) shows that reduced α-lactalbumin was stabilized for a significantly longer period of time in the disordered molten-globule state by the presence of α-crystallin. Thus in the absence of α-crystallin signal is virtually lost after 1256 s (Figure 4a). However, in the presence of α-crystallin, significant signal is still observed at an equivalent time point (1245 s) arising from a disordered molten-globule species of α-lactalbumin (Figure 4b). Complete loss of signal did not occur in the presence of α-crystallin until approx. 3000 s after addition of DTT (results not shown). Similar behaviour was observed for the reduction of α-lactalbumin in the presence of 0.1 M NaCl and α-crystallin [4] and another sHsp, murine Hsp25 (R. A. Lindner, J. A. Carver, A. Kotlyarov and M. Gaestel, unpublished work). However, in the absence of salt, no precipitation of α-lactalbumin occurred upon addition of DTT. Resonances were eventually lost from the NMR spectrum in Figure 4(a) as α-lactalbumin aggregated, but did not precipitate. In the presence of α-crystallin (Figure 4b), loss of signal occurred as α-lactalbumin became incorporated into a sHsp–substrate complex, since size-exclusion chromatography revealed that under the high protein concentrations required for NMR spectroscopy, formation of a high-molecular-mass complex between α-crystallin and α-lactalbumin occurred (results not shown). SDS/PAGE and ESI-MS showed that this species was comprised of α-crystallin and α-lactalbumin (results not shown).

^{1}H-NMR experiments identical to those performed above in the absence of added salt were performed in which α-crystallin was replaced by βH-crystallin. In the presence of a 2:1 (w/w) ratio of βH-crystallin/α-lactalbumin, no stabilization of the disordered molten globule of α-lactalbumin occurred. Thus at equivalent time points after the addition of DTT, the NMR spectra were essentially identical for α-lactalbumin on its own and in the presence of βH-crystallin, i.e. the spectra were very similar to those in Figure 4(a). Size-exclusion chromatography of the solution of βH-crystallin and α-lactalbumin after acquisition of the NMR spectra showed a peak due to massive aggregation of α-lactalbumin with no discernable stabilization of the monomeric molten-globule peak of reduced α-lactalbumin (results not shown). Thus in contrast to α-crystallin, addition of βH-crystallin had no effect on the reduction of α-lactalbumin. In fact, addition of βH-crystallin may enhance the aggregation of α-lactalbumin via macromolecular crowding since some precipitated α-lactalbumin was present following completion of this experiment.

Does the interaction between α-crystallin and α-lactalbumin depend upon the rate of aggregation and precipitation?

The observation that SO_4^{2-} accelerated the precipitation of α-lactalbumin, which resulted in a significant reduction in the ability of α-crystallin to prevent precipitation (Figure 1b), supports previous observations that α-crystallin cannot prevent substrate proteins from precipitating out of solution if they are

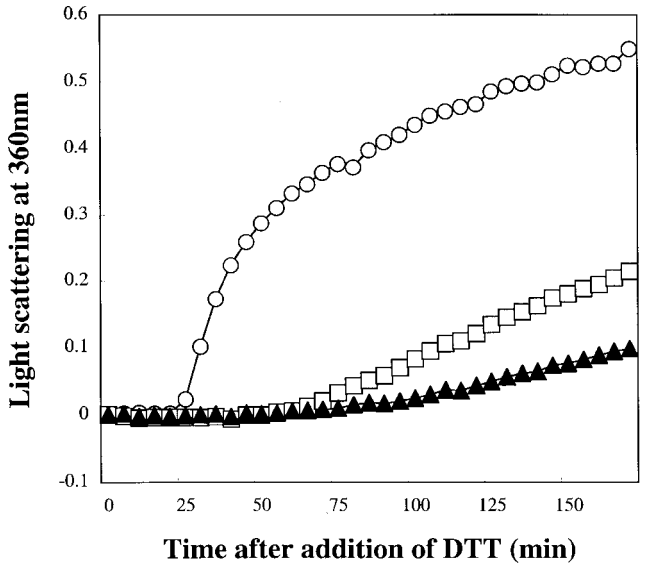


Figure 5 Protein aggregation and precipitation monitored by increasing light scattering at 360 nm with time for mixtures of α-lactalbumin (1 mg/ml) and increasing concentrations of α-crystallin following the addition of 20 mM DTT, in the presence of 50 mM sodium phosphate, pH 7.2/0.02% NaN_3/0.1 M Na_2SO_4

Ratios (w/w) of α-crystallin/α-lactalbumin used were 0:1 (○), 2:1 (□) and 4:1 (▲). Data were acquired at 37 °C.

aggregating quickly [13,19]. In an attempt to clarify this, experiments using visible absorption spectroscopy to monitor the precipitation of α-lactalbumin in the presence of 0.1 M Na_2SO_4 were repeated using 1 mg/ml α-lactalbumin rather than 2 mg/ml (as used in Figure 1b). Under these conditions, the time to onset of precipitation was increased from approx. 18 to 28 min (Figure 5). In addition, the rate of aggregation and precipitation was approximately halved compared with the higher concentration of α-lactalbumin (compare with Figure 1b). Consequently, almost complete protection from precipitation by α-crystallin was observed at a 4:1 (w/w) ratio of α-crystalllin/α-lactalbumin, i.e. α-crystallin was a much more efficient chaperone at a lower concentration of α-lactalbumin. Thus lowering the substrate concentration reduced the rate of precipitation and therefore α-crystallin was able to interact and complex with destabilized α-lactalbumin, to prevent its aggregation and precipitation.

Conformation of reduced α-lactalbumin and α-crystallin in the presence of Hofmeister salts, as monitored by ANS fluorescence and CD and NMR spectroscopy

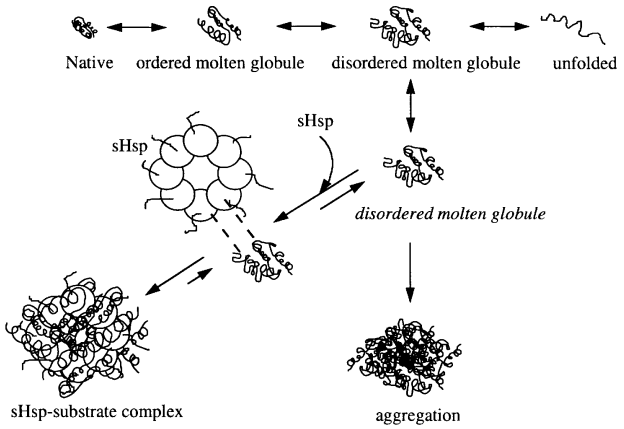
One reason for the differences in the chaperone interaction of α-crystallin with α-lactalbumin under the various salt conditions could be that the conformation of one, or both, of these proteins was altered significantly in the different buffer conditions. Accordingly, a variety of spectroscopic techniques were used to monitor conformational changes occurring in α-crystallin and α-lactalbumin under the various buffer conditions. ANS fluorescence at 37 °C of solutions of α-crystallin before and after the addition of DTT, in the presence of the various salts, was measured. In the presence of NaCl and no-salt conditions, ANS fluorescence varied little ($< 4\%$) upon addition of DTT (Table 1), implying that no gross conformational changes occur in α-crystallin under these conditions. Whereas ANS fluorescence of non-reduced α-crystallin in Na_2SO_4 was greater than that in the other buffer conditions, upon addition of DTT, ANS fluorescence increased to a similar extent (approx. 5%) compared with the other buffer conditions. CD experiments performed in the far- and near-UV regions did not detect any gross conformational changes in secondary and tertiary structure of α-crystallin in the various salt conditions both before, and up to 2 h after, addition of DTT (results not shown). It is concluded that the conformation of α-crystallin does not change significantly upon addition of DTT in the presence of a range of different salts. Consistent with these observations is that bovine α-crystallin does not contain any disulphide bonds. Thus the inability of α-crystallin to prevent the precipitation of α-lactalbumin in the presence of Na_2SO_4 upon reduction with DTT does not arise from any significant secondary or tertiary structural changes in α-crystallin.

Similar experiments also showed that native α-lactalbumin does not undergo any large-scale structural alteration in the various buffer conditions used in these experiments, i.e. 1D ^{1}H-NMR spectra in the various salt conditions were similar to those observed previously in the presence of NaCl [4]. In addition, far- and near-UV CD spectra were very similar in the presence of the various buffer conditions. CD spectra acquired over time following addition of DTT indicated the formation of a molten-globule-like intermediate (as judged by a loss of tertiary structure but retention of secondary structure) as α-lactalbumin was reduced and aggregated. Similar CD spectra were obtained in the presence of NaCl and Na_2SO_4. However, eventually signal was lost in these latter two instances as the protein aggregated and precipitated out of solution. During this aggregation and precipitation, no fully unfolded conformation was detected, i.e. elements of secondary structure were retained due to the formation of molten-globule intermediates that associate and ultimately aggregate [4,5,17,18].

An increase in fluorescence of ANS bound to reduced α-lactalbumin was observed under all salt conditions, indicating increased exposure of hydrophobicity, as is expected for a molten-globule state (Table 1). A larger increase in exposed hydrophobicity for α-lactalbumin in the presence of Na_2SO_4 (compared with other conditions) was observed after addition of DTT. This is consistent with greater clustered hydrophobicity arising from the tendency of SO_4^{2-} to favour hydrophobic interactions [9]. In turn, this increased exposed hydrophobicity results in a greater propensity for α-lactalbumin to aggregate and precipitate in the presence of SO_4^{2-} (as shown in Figure 1b). Overall, however, it is concluded that the variety of salt conditions used in this study did not result in gross structural changes in either α-crystallin or α-lactalbumin.

DISCUSSION

In this study, altering the rate and extent of aggregation and precipitation of α-lactalbumin has enabled the α-crystallin–substrate interaction to be examined in detail. In the absence of added salt, the precipitation of reduced α-lactalbumin does not occur, although α-lactalbumin still aggregates. α-Crystallin is able to prevent this aggregation by stabilizing a disordered molten-globule state of α-lactalbumin without forming a sHsp–substrate complex (Figure 2c). However, the formation of the complex does occur after prolonged incubation (Figure 3). α-Crystallin prevents aggregation of α-lactalbumin under conditions where no precipitation occurs, suggesting that α-crystallin interacts with a disordered molten-globule state of α-lactalbumin prior to aggregation. Thus the propensity for substrate proteins to aggregate, rather than to precipitate, may



Scheme 1 Proposed mechanism of action of sHsps

sHsps such as α-crystallin stabilize an aggregation-prone, disordered molten-globule state (denoted by italics). sHsps do not interact with stable (i.e. non-aggregation-prone) molten-globule states that lie along the reversible and relatively fast protein-folding/-unfolding pathway. Substrate stabilization by sHsps occurs initially through weak, transient interactions (denoted by dotted lines) that favour the non-aggregated, disordered monomeric molten-globule state shown in italics. Ultimately, the substrate becomes stabilized by complexation with the sHsp to form a soluble sHsp–substrate complex.

be the necessary trigger for interaction to form a sHsp–substrate complex.

The presence of SO_4^{2-} (which promotes hydrophobic interactions) leads to large-scale, rapid aggregation and precipitation of α-lactalbumin upon reduction with DTT (Figure 1b). The inability of α-crystallin to prevent efficiently the precipitation from solution of α-lactalbumin (at 2 mg/ml, Figure 1b), yet its ability to do so at half the concentration of α-lactalbumin (1 mg/ml, Figure 5) suggests that kinetic 'competition' exists between aggregation of α-lactalbumin and the chaperone action of α-crystallin. This competition would be similar to the kinetic competition that is proposed to exist between protein aggregation and correct protein folding [17].

The native, folded state of a protein represents one of minimum potential energy. Folding intermediates must therefore be less stable than the native state. This results in potential for misfolding and reshuffling on to 'off-pathway' reactions such as aggregation (reviewed by Jaenicke in [20]) since the formation of aggregates is also highly energetically favourable, representing an energy minimum or 'kinetic trap' [18,20]. When aggregation is sufficiently slow (e.g. for α-lactalbumin in the presence of 0.1 M NaCl), interaction of unfolding proteins with α-crystallin outruns aggregation and thus precipitation is prevented. However, when the rate of aggregation is rapid (as occurs in the presence of SO_4^{2-} at higher concentrations of α-lactalbumin), α-crystallin is unable to compete with aggregation for the unfolding proteins. Presumably, *in vivo*, interaction of unfolding proteins with α-crystallin and other sHsps outruns aggregation under normal physiological conditions. Thus in the lens where protein aggregation and precipitation occur very slowly [21], α-crystallin is ideally suited to prevent this precipitation via formation of a sHsp–substrate complex. Under conditions of stress *in vivo*, when the concentration of partly folded intermediates increases significantly resulting in an increase in the rate of aggregation over correct protein folding, expression of sHsps also increases, allowing sHsps to compete successfully with aggregation, thereby preventing large-scale precipitation.

Protein folding and its off-folding pathway, leading to aggregation and precipitation, compete kinetically, as shown in Scheme 1. sHsps such as α-crystallin alter the kinetic competition between refolding and aggregation by competing with aggregation. They do this by interacting with aggregation-prone intermediates (denoted by italics in Scheme 1) thereby preventing aggregation and precipitation. As a result, these intermediates are maintained in a potentially refolding-competent form in complexation with the sHsp where they may be rescued and refolded by other Hsps, in particular Hsp70 [22–24]. In addition, α-crystallin can stabilize a non-aggregated, disordered molten-globule-like intermediate of α-lactalbumin without complexing with it (Figure 2c). This 'free', stabilized, aggregation-prone, disordered molten globule can potentially return to the productive folding pathway [18] (Scheme 1). Consequently, there is the possibility that sHsps such as α-crystallin 'redirect' aggregation-prone intermediately folded forms of proteins back towards the productive, folding pathway. In agreement with this proposal, partial reactivation of heat-denatured citrate synthase bound to sHsps occurs upon a reduction in temperature in the absence of additional chaperones [25,26]. It is therefore possible for some sHsp-stabilized proteins to return from the 'off-folding' pathway to the productive folding pathway without the assistance of other chaperones. In addition, this ability to redirect proteins back on to the refolding pathway may also serve to increase the likelihood of these species being 'picked up' by other classes of chaperone, e.g. Hsp70 [22–24].

Note that distinction is made in Scheme 1 between aggregation-prone (italics) and non-aggregation-prone disordered molten-globule states. In reality, this distinction may only exist in terms of the time scale over which these species are present. We have shown previously that α-crystallin cannot interact efficiently with rapidly refolding proteins [13], i.e. under conditions in which the life time of these intermediates is short. We have also shown in this paper that α-crystallin cannot interact efficiently with rapidly aggregating and precipitating proteins. Thus it may be simply longevity of the disordered molten-globule state of a substrate protein that enables it to interact with α-crystallin, rather than any distinguishing structural characteristic(s). It should also be noted that Mitraki and King [18] make a similar distinction between aggregation- and non-aggregation-prone partially folded intermediates in their model describing aggregation of proteins at elevated temperatures.

In contrast to α-crystallin, Hayer-Hartl et al. [27] observed that the binding of molten-globule states of α-lactalbumin to GroEL was enhanced significantly in the presence of SO_4^{2-} compared with in the presence of Cl^-. The difference in the interaction between these two chaperones and α-lactalbumin in the presence of SO_4^{2-} can be rationalized on a kinetic basis, i.e. GroEL binds rapidly to the molten-globule states of α-lactalbumin (i.e. those of similar structure to the short-lived intermediates that form along the protein-folding pathway), whereas for sHsp–substrate complex formation with α-crystallin the molten-globule state of α-lactalbumin has to be present for a long period. Consistent with these data, GroEL binds to rapidly refolding rhodanese upon dilution of unfolded rhodanase from denaturant, whereas α-crystallin does not [19].

Our results provide an insight into the possible mechanism by which α-crystallin and other members of the sHsp family accomplish their task of stabilizing proteins. Initially, weak transient interactions occur which stabilize the monomeric, disordered molten-globule state of an unfolding substrate protein, allowing the possibility for retrieval by other chaperones such as Hsp70 or unassisted re-entry on to the protein-folding pathway. Ultimate complexation of this substrate protein with

sHsps ensures protection against aggregation and precipitation and retains the substrate in a folding-competent state which may be rescued by other chaperones, e.g. Hsp70, once native conditions have been restored. Under conditions of stress, the concentration of unfolding proteins increases, resulting in an increase in both the rate and extent of aggregation. Over-expression of sHsps under such conditions of stress (as occurs *in vivo*) is essential for successful competition with aggregation for these unfolding protein intermediates.

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