The small heat-shock protein αB-crystallin uses different mechanisms of chaperone action to prevent the amorphous versus fibrillar aggregation of α-lactalbumin

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Abstract
Stress conditions can destabilize proteins, promoting them to unfold and adopt intermediately folded states. Partially folded protein intermediates are unstable and prone to aggregation down off-folding pathways leading to the formation of either amorphous or amyloid fibril aggregates. The sHsp (small heat-shock protein) αB-crystallin acts as a molecular chaperone to prevent both amorphous and fibrillar protein aggregation; however, the precise molecular mechanisms behind its chaperone action are incompletely understood. To investigate whether the chaperone activity of αB-crystallin is dependent upon the form of aggregation (amorphous compared with fibrillar), bovine α-lactalbumin was developed as a model target protein that could be induced to aggregate down either off-folding pathway using comparable buffer conditions. Thus when α-lactalbumin was reduced it aggregated amorphously, whereas a reduced and carboxymethylated form aggregated to form amyloid fibrils. Using this model, αB-crystallin was shown to be a more efficient chaperone against amorphously aggregating α-lactalbumin than when it aggregated to form fibrils. Moreover, αB-crystallin forms high molecular mass complexes with α-lactalbumin to prevent its amorphous aggregation, but prevents fibril formation via weak transient interactions. Thus, the conformational stability of the protein intermediate, which is a precursor to aggregation, plays a critical role in modulating the chaperone mechanism of αB-crystallin.

Keywords
aggregation, lactalbumin, shock, protein, b, crystallin, uses, different, mechanisms, chaperone, action, small, prevent, heat, amorphous, versus, fibrillar, CMMB

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The small heat shock protein αB-crystallin uses different mechanisms of chaperone action to prevent the amorphous versus fibrillar aggregation of α-lactalbumin.

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Running title: Chaperone action of αB-crystallin in preventing amorphous and fibrillar aggregation.

Keywords: protein aggregation, amyloid fibrils, amorphous aggregates, chaperone, holdase, protein complex.

Abbreviations; αB-C, αB-crystallin; α-LA, α-lactalbumin; Bis-ANS, 4,4’-Dianilino-1,1’-binaphthyl-5,5’-disulfonic acid; DTT, Dithiothreitol; RCMα-LA, reduced and carboxymethylated α-lactalbumin; RCM, reduced and carboxymethylated; sHsp(s), small Heat shock protein(s); TEM, transmission electron microscopy; ThT, thioflavin T.
ABSTRACT
Stress conditions can destabilise proteins, promoting them to unfold and adopt intermediately folded states. Partially-folded protein intermediates are unstable and prone to aggregation down off-folding pathways leading to the formation of either amorphous or amyloid fibril aggregates. The small heat shock protein (sHsp), αB-crystallin, acts as a molecular chaperone to prevent both amorphous and fibrillar protein aggregation; however, the precise molecular mechanisms behind its chaperone action are incompletely understood. To investigate whether the chaperone activity of αB-crystallin is dependent upon the form of aggregation (amorphous versus fibrillar), bovine α-lactalbumin was developed as a model target protein that could be induced to aggregate down either off-folding pathway using comparable buffer conditions. Thus, when α-lactalbumin was reduced it aggregated amorphously, whereas a reduced and carboxymethylated form aggregated to form amyloid fibrils. Using this model, αB-crystallin was shown to be a more efficient chaperone against amorphously aggregating α-lactalbumin than when it aggregated to form fibrils. Moreover, αB-crystallin forms high molecular mass complexes with α-lactalbumin to prevent its amorphous aggregation, but prevents fibril formation via weak, transient interactions. Thus, the conformational stability of the protein intermediate which is a precursor to aggregation plays a critical role in modulating the chaperone mechanism of αB-crystallin.
INTRODUCTION

As proteins are exposed to stress conditions throughout their life, they are faced with the continuous threat of destabilisation. Stressors (e.g., heat stress and oxidative stress) can disrupt the hydrogen bonds and hydrophobic interactions that stabilise their native state, causing proteins to unfold and populate partially-folded intermediate states [1-3]. Protein intermediates (produced by unfolding) transiently expose hydrophobic residues at their surface which renders them thermodynamically unstable and prone to mutual associations that lead to protein aggregation and irreversible precipitation. Aggregating intermediates leave the preferred folding/unfolding pathway and enter slower off-folding pathways, forming either disordered or ordered aggregates [1-2]. Disordered aggregation occurs when intermediates randomly associate, through a three-dimensional process, producing unstructured (amorphous) aggregates which become insoluble when a critical size is reached [4]. Alternatively, intermediates that aggregate via ordered association (mediated through β-sheet interactions) form soluble protofibrils, which then laterally associate to form highly-structured, amyloid fibrils. The factors that determine which off-folding pathway a particular protein intermediate enters have not been fully elucidated but are thought to include the amino acid sequence, rate of aggregation and degree of exposed hydrophobicity [2].

In vitro studies indicate that, under specified solution conditions, protein intermediates typically favour aggregation down one particular off-folding pathway. Thus, very few proteins have been shown to be capable of forming both amorphous aggregates and amyloid fibrils when incubated under similar solution conditions. Insulin [5], bovine serum albumin [6] and wild-type β2-microglobulin [7] have the capacity to undergo either amorphous aggregation or amyloid fibril formation in vitro, however, high temperatures (often greater than 50°C), low pH, and/or the presence of organic agents (such as trifluoroethanol) are required to induce fibril formation. For example, insulin is capable of aggregating amorphously (upon reduction of its disulfide bonds) and to form amyloid fibrils, however, the latter typically requires temperatures of ~60°C and a solution pH of 2.0 [5]. Thus, such models do not allow comparisons of aggregation (or its prevention) under physiologically relevant conditions. One protein that is capable of aggregating to form amorphous or amyloid fibril aggregates under conditions of physiological temperature and pH (i.e. 37°C and pH 7.0-7.4) is the small acidic, calcium-binding milk protein, α-lactalbumin (α-LA) [8-10]. The native conformation of α-LA is stabilised by four disulfide bonds [11-13]. It is well documented that α-LA can be induced to undergo non-specific amorphous aggregation by reduction of all four disulfide bonds [9-10]. More recent work has suggested that a partially reduced form (designated 1SSα-LA as one of the four disulfide bonds remains intact) is capable of forming amyloid fibrils [8, 14]. However, the precise nature of this amyloidogenic form of α-LA was not addressed in this work.

As protein aggregation can have deleterious effects on cell viability and function, the cell has evolved systems to prevent protein aggregation and maintain protein homeostasis (proteostasis). A key component of these systems is a ubiquitous family of molecular chaperones, collectively titled the small heat shock proteins (sHsps) [15-16]. Members of the small heat shock protein family have a central α-crystallin domain (80-100 amino acid residues) and small monomeric molecular masses typically ranging from 12-42 kDa [17-19]. Mammalian sHsps (such as αB-crystallin; αB-c) form large polydisperse oligomeric complexes in solution with masses up to 1 MDa [20-21] and function as molecular chaperones through their ability to recognise and interact with partially-folded protein intermediates that have entered the off-folding aggregation pathways [1-2]. Their broad substrate specificity, high binding capacity and ATP-independent chaperone action means
that sHsps are efficient at preventing protein aggregation, even under stress conditions [1, 22]. However, the specific molecular mechanisms responsible for their chaperone action remain enigmatic. This is due, at least in part, to the polydisperse nature of sHsps [20], which makes their larger oligomeric assemblies refractory to structural investigation. Moreover, a diverse range of target proteins and buffer conditions have been used to study the chaperone action of sHsps, making definitive conclusions about their chaperone action difficult.

While no consensus has been reached, it is widely accepted that the predominant mechanism of sHsp chaperone action involves sHps binding exposed hydrophobic residues on partially-folded protein intermediates to sequester them into soluble, high molecular mass complexes, typically described as a ‘holdase’ activity [1-2]. Previous work has demonstrated that sHsps have no capacity to refold bound substrates [22]. Thus, the complex is thought to maintain the intermediates in a refolding-competent state, until cellular conditions are permissive for ATP-dependent chaperones (e.g. Hsp70) to restore the intermediates back to their native conformation [23]. Alternative mechanisms of action, however, have also been proposed. For example, it has been suggested that sHsps can act through weak transient interactions to stabilise protein intermediates, such that they are capable of re-entering the folding pathway and thus re-attaining their native conformation [24-25]. Moreover, a recent study indicates that αB-c can redirect the aggregation of α-synuclein from the fibril-forming pathway to the amorphous aggregation pathway [26].

A major limitation to examining whether sHsps use different mechanisms to prevent the aggregation of target proteins undergoing amorphous aggregation compared to amyloid fibril formation is that no study has used the same target protein and comparable, physiologically relevant solution conditions. Different solution conditions have been shown to affect the thermostability, oligomeric distribution and chaperone activity of αB-c [27]. Therefore, this study aimed to establish bovine α-LA as a model target protein amenable to both amorphous and fibrillar aggregation under buffer conditions that were comparable and physiologically relevant (i.e. pH 7.0 and temperature of 37°C). The α-LA model was then used as a tool to characterise the precise molecular mechanisms deployed by αB-c to prevent amorphous versus fibrillar aggregation. This work provides evidence that αB-c can mediate its chaperone activity through at least two distinct mechanisms. The mechanism used depends upon differences in the conformational stability and exposed hydrophobicity of the intermediate precursor to either form of aggregation.

MATERIALS AND METHODS

Materials
The pET24d(+) expression vector (Novagen, Darmstadt, Germany) containing human αB-c was a gift from Professor W. Boelens (University of Nijmegen, Netherlands). The α-LA from bovine milk (Ca²⁺-depleted; >85% pure), urea, iodoacetic acid, and thioflavin T (ThT) were obtained from Sigma Aldrich (St. Louis, MO, U.S.A). Dithiothreitol (DTT) was obtained from Amresco (Solon, OH, U.S.A) and uranyl acetate purchased from Agar Scientific (Essex, UK). Protein molecular weight standards used in electrophoresis were purchased from Bio-Rad (Hercules, CA, U.S.A.). BL21(DE3) Escherichia coli cells transformed with the pET24d(+)-human αB-c vector were used for protein expression, in accordance with methods previously described [28]. Reduction and carboxymethylation of α-lactalbumin (RCMα-LA) was performed according to the method outlined by Shechter et al. [29] Concentrations of proteins in solution were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A) and an absorbance (A280 nm) value for a 0.1%
(w/v) solution of the proteins of 0.83 for αB-c [28] and 2.0 for α-LA [30]. The goat polyclonal anti-α-LA antibody was purchased from Bethyl laboratories Inc. (Montgomery, TX, U.S.A), the monoclonal anti-αB-c (clone 1B6.1-3G4) was obtained from Abcam (Cambridge, MA, U.S.A.) and both were used at a 1:5,000 dilution. The HRP-conjugated secondary antibodies were from Sigma Aldrich and used at a 1:5,000 dilution.

**Aggregation assays**
The relative chaperone activity of αB-c was evaluated at the end of the respective assays by calculating the protection afforded by αB-c at various molar ratios using the formula:

\[
\% \text{ Protection} = \left( \frac{\Delta I - \Delta I_{\text{ch}}}{\Delta I} \right) \times 100
\]

Where \(\Delta I\) and \(\Delta I_{\text{ch}}\) signify the change in light scatter or ThT fluorescence of α-LA in the absence and presence of the chaperone αB-c, respectively. Percent protection is reported as mean ± SEM.

**Amorphous aggregation** - The change in light scattering at 360 nm was used to monitor the reduction-induced amorphous aggregation of α-LA (100 µM). Assay conditions were based on a well described protocol published by Horwitz et al. [28] but modified slightly to mimic (as closely as possible) conditions in the fibril formation assay. Thus, the solution conditions used to perform the amorphous aggregation assays was 50 mM phosphate buffer, (pH 7.0), 100 mM KCl and 5 mM EDTA. The aggregation of α-LA was induced by the addition of DTT to a final concentration of 20 mM. The final volume of solution in each well was 100 µL. The assays were conducted in µclear 96-microwell plates (Greiner Bio-One, Stonehouse, UK), sealed with transparency film and incubated at 37°C in a FLUOstar OPTIMA microplate reader (BMG Technologies, Melbourne, Australia). Amorphous aggregation was monitored by light scattering at 360 nm for 4 h with readings taken every 3 min, following an initial period of double orbital shaking for 30 sec. All samples were assayed in duplicate and the assay was repeated three times.

**Fibrillar aggregation** - The fibrillar aggregation of RCMα-LA (100 µM) was monitored using a previously described ThT fluorescence assay [27, 31]. Buffer conditions used in this assay was 50 mM phosphate buffer (pH 7.0), 100 mM KCl and 10 mM MgCl₂, unless otherwise stated. Assays were conducted in black µclear 96-microwell plates (Greiner Bio-One, Stonehouse, UK). All wells contained 20 µM ThT to permit the detection of fibril formation and were prepared to a final volume of 100 µL. Assay plates were sealed to prevent evaporation and were incubated in a POLARstar OPTIMA microplate reader (BMG Labtechnologies, Melbourne, Australia) at 37°C. The ThT fluorescence was measured using excitation and emission wavelengths of 440 nm and 490 nm, respectively. Readings were taken every 5 min over a period of 12 h, with double orbital shaking after each cycle for 4 min. All samples were assayed in duplicate and the assay was repeated three times.

In some assays, following incubation of RCMα-LA (100 µM) in the presence of αB-c (50 µM) for 12 h under the assay conditions described above, these samples were collected directly from the plate and the αB-c removed from solution by centrifuging the sample through a Amicon centrifugal filter unit (Millipore, Billerica, MA, U.S.A.) containing a membrane with a nominal molecular weight cut-off of 100 kDa. The eluate, containing the RCMα-LA, was then re-incubated using the same conditions described above.

**Transmission Electron Microscopy (TEM)**
To confirm RCMα-LA fibril formation (and the amorphous aggregation of DTT-reduced α-LA) TEM analyses were conducted. Carbon-coated nickel square mesh grids (ProSciTech,
Thuringowa, Australia) were coated with 2 µL of sample from wells in the respective assays. The grids were washed three times with 10 µL aliquots of milli-Q and stained with 10 µL of uranyl acetate (2%, w/v). Samples were viewed with a Philips CM100 transmission electron microscope (Philips, Eindhoven, Netherlands).

**Analytical size-exclusion chromatography**

Interactions between αB-c and α-LA were analysed by size exclusion chromatography. Samples from the respective aggregation assays were centrifuged (14,200 x g for 10 min at 4°C) and the resulting supernatants loaded onto a Superose 6 10/300 GL size exclusion column (GE Healthcare, Stockholm, Sweden) previously equilibrated with the buffer used in the corresponding aggregation assay. The flow rate was set to 0.3 mL/min and protein elution monitored by an in line UV-detector, with eluate collected in 0.5 mL fractions. The Superose 6 column was calibrated with standards from Bio-Rad (Hercules, CA, U.S.A.) which contained bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B12 (1.35 kDa). Size-exclusion chromatographic experiments were conducted twice using samples from two independent amorphous and fibrillar aggregation assays.

**SDS-PAGE and immunoblotting**

Eluate fractions from the analytical size-exclusion chromatographic separation were subjected to SDS-PAGE (15% v/v acrylamide gels) and immunoblotting using standard techniques. Membranes were blocked with 3% w/v BSA in Tris-buffered saline (TBS) for 1 h at room temperature. The membranes were rinsed in TBS-T (TBS containing 0.1% Tween-20) and incubated in TBS-T containing 1% w/v BSA and primary antibody (anti- α-LA and anti- αB-c: 1/5,000) overnight at 4°C. The blots were washed 4 times (10 min) with TBS-T, incubated in the corresponding secondary antibody (goat anti-rabbit 1/5,000, Sigma) conjugated to peroxidase and then washed again in TBS-T. The labeled proteins were detected using a chemiluminescent substrate according to the manufacturer’s instructions (West-dura, Pierce, Rockford, IL).

**Intrinsic and extrinsic fluorescence**

Fluorescence studies were conducted at room temperature using a Cary Eclipse fluorescence spectrophotometer (Varian Inc, Mulgrave, Victoria, Australia). All samples were equilibrated at room temperature for approximately 15 min before intrinsic tryptophan fluorescence and 4,4’-Dianilino-1,1’-binphthyl-5,5’-disulfonic acid dipotassium salt (bis-ANS) binding spectra were acquired. Excitation and emission slit widths were set to 5 nm and the scan speed set to 600 nm/min for all readings. Both α-LA and RCMα-LA (2 µM) were prepared in 50 mM phosphate buffer (pH 7.0) containing 100 mM KCl with or without 10 mM MgCl2 or 5 mM EDTA. In addition, α-LA (in 50 mM phosphate buffer (pH 7.0) with 100 mM KCl and 5 mM EDTA) was studied following 5 min of exposure to 20 mM DTT. This time point was selected as a previous study has indicated that all native α-LA is converted to partially- or fully-reduced forms after 5 min, such that 1SSα-LA (1 disulphide bond intact) and fully-reduced α-LA are the predominate species in solution [10]. All protein solutions were prepared to a final volume of 3 mL in a quartz fluorescence cuvette (Starna, Essex, England). Intrinsic tryptophan fluorescence spectra were acquired by exciting at a wavelength of 295 nm and monitoring the emission from 300-400 nm. Bis-ANS was added to samples to a final concentration of 20 µM and fluorescence emissions monitored between 400-600 nm following excitation at 350 nm. Each tryptophan and bis-ANS spectrum represents the average of 3 scans with the final spectra smoothed (Savitzky-Golay, 5-point smoothing) and
standardised for protein concentration. These experiments were repeated three times and the results shown are representative from one of these experiments.

*Far UV-circular dichroism*

The samples studied by far UV-circular dichroism (CD) were prepared under the same solution conditions as used for the intrinsic and extrinsic fluorescence experiments. Far-UV CD spectra for the $\alpha$-LA samples were acquired on a Jasco J-810 CD spectropolarimeter (Jasco, Tokyo, Japan) at room temperature. All protein samples were prepared to a final concentration of 7 $\mu$M and spectra recorded using a 0.1 cm quartz cuvette (Starna, Essex, England). Measurements were taken from 195-255 nm with continuous scanning at 100 nm/min, a bandwidth of 1 nm, response time of 1 sec and data pitch of 0.2 nm. All spectra were corrected for buffer contributions and are presented in units of mean residue ellipticity (MRE; deg.cm².dmoll⁻¹). Spectra were smoothed using Savotsky-Golay smoothing (convolution width 25) and are the average of 6 scans. These experiments were repeated three times and the results shown are representative from one of these experiments.

**RESULTS**

*Fibrillar aggregation of RCM$\alpha$-LA* – In order to establish $\alpha$-LA as a model to study the chaperone activity of $\alpha$B-c against amorphous and fibrillar aggregation, conditions were sought in which the fibrillar aggregation of the protein was comparable to that used for the amorphous aggregation assay and physiologically relevant (i.e. pH 7.0 - 7.4 and temperature of 37°C). Since the structure and function of $\alpha$B-c and assays for the amorphous aggregation of $\alpha$-LA are well established in phosphate-based buffers [9-10, 27, 32], the Tris-HCl buffer conditions described by Bomhoff *et al.* [8] for the fibrillar aggregation of partially reduced (1SS) $\alpha$-LA were adapted to a phosphate-based buffer system.

There was an increase in ThT fluorescence in the RCM$\alpha$-LA samples incubated in both Tris- and phosphate-based buffers when MgCl$_2$ was present (Figure 1a). This increase in fluorescence followed sigmoidal kinetics typical of fibril formation. Thus, there was a distinct lag phase of 4 h in the Tris-based buffer, followed by a gradual increase in ThT fluorescence, indicative of fibril elongation. ThT fluorescence reached a maximum after 9 h of incubation in the Tris-based buffer and then remained constant for the remainder of the assay. Comparatively, the lag phase was shorter (3 h) and the rate of increase in ThT fluorescence greater when RCM$\alpha$-LA was incubated in the phosphate-based buffer. The ThT fluorescence reached a maximum at 7 h in phosphate buffer and this maximum was 4-fold higher than the maximum fluorescence observed in the Tris-based buffer. Moreover, fibril formation was specific for the RCM form of $\alpha$-LA as there was no increase in ThT fluorescence when native $\alpha$-LA was incubated (either in the absence or presence of DTT) under these conditions. The Tris-based buffer condition used by Bomhoff *et al.* [8] included both MgCl$_2$ (10 mM) and EDTA (5 mM). Whilst EDTA did not affect the length of the lag phase in the phosphate buffer, it did affect the aggregation kinetics, such that the removal of EDTA increased the rate and maximum ThT fluorescence. There was no increase in ThT fluorescence associated with RCM$\alpha$-LA fibril formation when MgCl$_2$ was absent from either buffer (Figure 1a). The Tris-based and phosphate-based buffer alone samples showed no increase in ThT fluorescence over the time-course of the assay (data not shown), confirming that the change in ThT fluorescence was associated with RCM$\alpha$-LA fibril formation. To verify the formation of RCM$\alpha$-LA fibrils in the phosphate-based buffer, samples were collected at the end of the assay and analysed by TEM (Figure 1b). Electron micrographs of negatively stained RCM$\alpha$-LA showed the presence of mature, thread-like fibrils that were between 65 and 620 nm in approximate length in these samples.
The chaperone activity of αB-c against the amorphous aggregation of α-LA – αB-c was highly efficient at preventing the reduction-induced amorphous aggregation of α-LA, such that it offered 47 ± 10% protection against aggregation even at the lowest molar ratio tested (i.e. 1:0.05, α-LA:αB-c) (Figure 2a). Consistent with previous findings [9-10], higher molar ratios of αB-c afforded greater protection against α-LA amorphous aggregation and complete inhibition was achieved at a molar ratio of 1:0.25 (α-LA:αB-c). As there was no change in light scatter when αB-c was incubated alone (Figure 2a) or when neither protein was present (data not shown), increases in light scattering were attributable to the amorphous aggregation of α-LA. The mechanism by which αB-c prevents the amorphous aggregation of α-LA was investigated via analytical size exclusion chromatography using the 1:0.5 molar ratio of α-LA:αB-c (i.e. a concentration of αB-c that afforded complete inhibition of amorphous aggregation of α-LA). Chromatographic analysis of native α-LA showed that it elutes as a major symmetrical peak at a volume of 18.8 mL (Figure 2b). This elution volume corresponds to an average mass of 17 kDa which is consistent with the globular monomeric form of α-LA (typically sized at 14.2 kDa). The native α-LA sample also contained a minor peak that eluted in the void of the column (i.e. at 7.8 mL), suggesting that some of the α-LA was present as soluble aggregates prior to incubation at 37°C, as seen previously [33-35]. Reduction of α-LA by DTT in the absence of αB-c resulted in no soluble monomeric α-LA being present in the sample following incubation (i.e. most of the α-LA had precipitated as insoluble aggregates).

Consistent with αB-c existing as a large multimeric, polydisperse oligomer, under the solution conditions used for the amorphous aggregation assay [20-21], αB-c eluted as a broad, symmetrical peak centred at 13.5 mL, corresponding to an average molecular mass of 595 kDa (Figure 2b). The 1:0.5 (α-LA:αB-c) molar ratio sample, which completely inhibited α-LA amorphous aggregation, showed a substantial increase in the size of this peak. The maximum of this peak was shifted to 13.2 mL, which correlates to a 180 kDa increase in size compared to the peak corresponding to αB-c alone. The presence of high molecular mass complexes between αB-c and α-LA in this peak was confirmed by immunoblotting analysis which resolved both α-LA and αB-c in these eluate fractions (Figure 2c). Some monomeric α-LA remained in this sample as evidenced by a peak at ~19.2 mL (Figure 2b) and the presence of α-LA (and not αB-c) in eluate fractions from this peak (Figure 2c). This indicates that some reduced α-LA was maintained in a soluble, stable form, without being incorporated into the high molecular mass complexes and is consistent with previous findings [33-35].

The chaperone activity of αB-c against RCMα-LA fibril formation – The relative ability of αB-c to inhibit the fibrillar aggregation of RCMα-LA was determined using a ThT fluorescence assays. In the absence of αB-c, RCMα-LA showed similar aggregation kinetics to those previously described (Figure 1a). The addition of αB-c at low molar ratios (i.e. 1:0.05 and 1:0.1, RCMα-LA:αB-c) delayed the onset of fibril formation by increasing the length of the lag phase (to 4 h and 6 h, respectively) (Figure 3a). These molar ratios did not completely inhibit the increase in ThT fluorescence associated with RCMα-LA fibril formation: they reduced it by 14 ± 5% (1:0.05) and 30 ± 8% (1:0.1). Higher molar ratios of αB-c (at 1:0.25 and 1:0.5, RCMα-LA:αB-c) mitigated RCMα-LA fibril formation markedly, such that at a molar ratio of 1:0.5 (RCMα-LA:αB-c), αB-c suppressed RCMα-LA fibril formation by 88 ± 4%. Thus, the 1:0.5 (RCMα-LA:αB-c) molar ratio was used in subsequent
analytical size exclusion chromatography experiments to characterise the mechanism by which αB-c inhibits RCMα-LA fibril formation.

Size exclusion chromatography showed that prior to incubation RCMα-LA elutes as a symmetrical peak at ~18.1 mL, corresponding to a molecular mass of ~30kDa (Figure 3b). Analysis of the soluble fraction following fibril formation in the absence of αB-c showed that there was little monomeric RCMα-LA left in solution: the RCMα-LA that did remain soluble eluted as a broad peak at ~18.3 mL. Under these solution conditions αB-c eluted as a broad symmetrical peak at ~13.7 mL, corresponding to an average molecular mass of 540 kDa. Incubation of RCMα-LA with αB-c at a molar ratio of 1:0.5 (RCMα-LA:αB-c), i.e. the molar ratio in which fibril formation was suppressed by >90% (see Figure 3a), had no effect on the height or width of the αB-c peak. Thus, no stable high molecular mass complex was detected between αB-c and RCMα-LA following incubation. Instead RCMα-LA was found in the peak at ~18.4 mL suggesting that at least the vast majority of it remains in a soluble monomeric form in the presence of αB-c.

No evidence for a stable high molecular mass complex between αB-c and RCMα-LA was found by immunoblotting of the fractions collected from the 13.7 mL peak. Only αB-c was detected in these fractions and RCMα-LA was detected in the 18.4 mL peak (Figure 3c). No high molecular mass complex was detected by size exclusion chromatography and western blotting even when a crosslinker (Bis[sulfo)succinimidyl] suberate) was added to the sample containing RCMα-LA and αB-c at the end of the incubation (data not shown). Thus, there was no evidence for a stable complex formed between RCMα-LA and αB-c during the chaperone inhibition of RCMα-LA fibril formation (at least within the detection limit of the immunoblotting procedure used here which was ~0.5 nM for both proteins).

In order to further explore the mechanism by which αB-c prevents RCMα-LA fibril formation, samples containing αB-c and RCMα-LA that had been incubated under conditions that promote RCMα-LA fibril formation were recovered after 10 h and centrifuged through a filter unit with a 100 kDa molecular mass cut-off membrane (Figure 4a). After centrifugation, RCMα-LA was found in the eluate, however, αB-c (which exists as a large polydisperse oligomer which has a mean molecular mass of 580 kDa under these solution conditions [30]) had been retained by the membrane and was therefore absent from this solution. Upon re-incubation of this eluate, there was an increase in ThT fluorescence indicative of RCMα-LA fibril formation demonstrating that, following transient interactions with αB-c, RCMα-LA remains in a conformation capable of aggregation if the chaperone is removed.

Biophysical characterisation of DTT-reduced and RCM forms of α-LA - The findings presented above demonstrates that αB-c can inhibit both the amorphous and fibrillar aggregation of α-LA, however, the mechanisms it uses to do so are different for the two forms of protein aggregation. Thus, the chaperone forms stable high molecular mass complexes with reduced α-LA to prevent its amorphous aggregation (see Figure 2), but does not form stable high molecular mass complexes with RCMα-LA to prevent its fibril formation (see Figure 3). In order to examine the factors that may have attributed to these differences in mechanism, structural characterisation was undertaken on both these forms of α-LA (i.e. DTT-reduced and RCM, the precursors to amorphous and fibrillar aggregation respectively) to see if this could account for such differences. Studies on the DTT-reduced form of α-LA were conducted 5 min after the addition of DTT. This time point was selected as the disordered molten globule state of α-LA is the predominate species in solution after 5
min, and it is this disordered state which is associated with the precipitation of DTT-reduced α-LA [9, 12].

Bovine α-LA contains four tryptophan residues (residues 26, 60, 104, 118) and, when incubated under the conditions used in the amorphous aggregation assay, native α-LA showed maximum tryptophan fluorescence at 329.0 ± 0.6 nm, indicative of the tryptophan residues being buried in the core of the folded protein (Figure 5a). The tryptophan fluorescence spectrum of RCMα-LA was red-shifted compared to that of native α-LA such that the maximum tryptophan fluorescence for RCMα-LA under the solution conditions used in the fibrillar aggregation assay was 353.7 ± 1.2 nm and the intensity of fluorescence ~2.5 fold higher than that associated with native (folded) α-LA. This shift in the wavelength of maximum fluorescence between native α-LA and RCMα-LA suggests that the tryptophan residues of RCMα-LA are more solvent exposed or in a more polar environment compared to those in the native form of the protein (i.e. RCMα-LA is more unfolded under these conditions). DTT-reduced α-LA displayed a tryptophan fluorescence spectrum in which the wavelength of maximum fluorescence (350 ± 0.4 nm) and fluorescent intensity (46.3 ± 1.0) were midway between the native and RCM forms of the protein. This suggests that DTT-reduced α-LA exists in a state between the native (folded) and RCM states of α-LA, consistent with its molten globule characteristics that have been previously described [12]. There were no significant differences in the tryptophan fluorescence spectra of native α-LA or RCMα-LA forms in the absence or presence of MgCl₂ and the spectrum of RCMα-LA did not change upon addition of DTT (see Supplementary Figure 1). Thus, structural differences between these α-LA forms are not due to slight differences in the buffers used to prepare them.

Structural differences between DTT-reduced and RCM forms of α-LA were further investigated through the use of the extrinsic fluorescent dye, bis-ANS (Figure 5b). There was no significant change in the wavelength of maximum bis-ANS fluorescence between the forms of α-LA investigated, however, there were significant differences in the fluorescent emission intensities between different α-LA samples. Compared to either native α-LA or RCMα-LA, DTT-reduced α-LA was defined by higher bis-ANS fluorescence intensity: it had a fluorescence emission intensity that was ~3-fold higher than RCMα-LA (Figure 5b). These results are again consistent with DTT-reduced α-LA existing in a molten globule-like state which exposes significant amounts of hydrophobicity to solution [12].

The far-UV CD spectra of native α-LA was typical of a predominately α-helical protein, with minima at 208 nm and 222 nm, which is in good agreement with previous studies [12]. The addition of MgCl₂ had little effect on the far-UV CD spectra of this form of the protein (data not shown). The far-UV CD spectra of RCMα-LA had a major minimum in negative ellipticity at 200 nm, a feature characteristic of random coil (unordered) proteins [36] and, again, consistent with previously published far-UV CD spectra for this form of α-LA [12]. The absence of MgCl₂ did not change the far-UV spectra of RCMα-LA indicating that it did not significantly alter its global secondary structure (data not shown). Analysis of the spectra for DTT-reduced α-LA revealed a decrease in negative ellipticity at 222 nm, and shift of the minima to ~205 nm compared to native (non-reduced) α-LA. This indicates that DTT-reduced α-LA has begun the helix-to coil transition, however, it retains a considerable degree of native-like secondary structure when compared to RCMα-LA.

Taken together the data from these biophysical studies are consistent with reduction and carboxymethylation of α-LA inducing the protein to go from a fully-folded state to a
predominately unfolded state that exposes little hydrophobicity to solution. This form of α-LA is distinct from the DTT-reduced form which exists in a molten globule-like state with high amounts of surface exposed hydrophobicity.

DISCUSSION

Ascertaining the nature of the interaction between αB-c and aggregating target proteins is fundamental to illuminating the molecular mechanisms of its chaperone activity [10]. To determine whether αB-c uses different mechanisms of action in order to prevent target proteins aggregating amorphously or forming amyloid fibrils, this study has developed bovine α-LA as a model target protein amenable to both forms of aggregation under comparable (physiologically relevant) solution conditions. Moreover, the findings of this study extend earlier work on 1SS α-LA fibrillar aggregation [8, 14] to show that the RCM form is capable of forming fibrillar aggregates and does so under solution conditions comparable to those used to induce amorphous aggregation of α-LA by disulfide bond reduction. Changes in ThT fluorescence over time indicated that the fibrillar aggregation of RCMα-LA follows sigmoidal curve kinetics typical of amyloid fibril formation and therefore provides a useful model system to study generic aspects of this process and its inhibition (e.g. by chaperones) under physiologically relevant conditions.

Taking these results together, a revised model for the chaperone activity of sHsps against protein aggregation can be proposed. As shown in this model (Figure 6) the folding/unfolding pathway of a protein is populated by a number of conformationally distinct partially-folded protein intermediates: some are closer to the native state of the protein and others more similar to the unfolded protein state. Intermediates in a native-like molten globule state are more rigid in solution and expose significant amounts of hydrophobicity to solution (similar to the DTT-reduced form of α-LA) thereby leading to their amorphous aggregation. In contrast, stable, predominately unfolded protein intermediates with little clustered hydrophobicity exposed to solution (similar to RCMα-LA) lead to amyloid fibril formation. Acting as stability sensors, sHsps (such as αB-c) preferentially recognise disordered intermediates with high levels of surface hydrophobicity, (analogous to the DTT-reduced form of α-LA) to sequester such forms into stable high molecular mass complexes. A high affinity interaction between unstable intermediates and sHsps can be supported, as their association is energetically favourable [37]. Alternatively, relatively stable intermediates, with fewer clusters of exposed hydrophobicity (i.e. similar to RCMα-LA) are less vulnerable to an immediate threat of large-scale aggregation. Thus, sHsps use low affinity interactions with these forms maintaining them in a state resistant to aggregation through transient interactions. Our results show that these low affinity interactions redirect the protein intermediates back onto the folding pathway so that they can spontaneously reattain their native conformation. However, as such, they are still prone to aggregation should the chaperone levels or activity be depleted.

It was demonstrated that αB-c is more effective at inhibiting the amorphous aggregation of α-LA then its fibril formation. Thus, at the lowest molar ratio tested (1:0.05, α-LA:αB-c) the amorphous aggregation of α-LA was suppressed by 47 ± 10% compared to 14 ± 5% for the fibrillar aggregation. This difference in efficiency may be attributable to differences in the nature of the intermediate species which are the precursors to aggregation down either unfolding pathway. RCMα-LA adopts an extended, highly flexible conformation in solution which is attributed to the introduction of 8 negative charges upon carboxymethylation causing an increase in repulsive forces between different regions of the protein [12]. Previous studies have shown that RCMα-LA is relatively stable in solution [11], which is confirmed by
our findings that show that it has a low amount of surface exposed hydrophobic clusters comparable to the native state of the protein (Figure 5b). In contrast to this, DTT-reduced α-LA, the precursor to amorphous aggregation, is unstable and exposes significant amounts of hydrophobicity to solution (Figure 5), hence rationalising its tendency to undergo mass aggregation [9-10, 34]. The data presented here demonstrates that αB-c forms high molecular mass complexes with highly disordered, precipitation bound, intermediates that expose a high degree of hydrophobicity to solution. Alternatively, αB-c employs weak, transient interactions to suppress the aggregation of more stable, structured intermediates which expose less hydrophobicity to solution. Lindner et al. [9] and Carver et al. [10] investigated the interaction of α-crystallin with intermediately folded states of α-LA through a range of spectroscopic techniques and showed that α-crystallin was more effective at preventing the amorphous aggregation of highly disordered, slowly aggregating forms of α-LA than relatively stable forms of this protein. This work agrees with these findings and extends them to show that this effect is independent of the form of aggregation.

The work demonstrates that the description of αB-c (and other sHsps) as ‘holdase’ type chaperones does not fully encompass their chaperone activities [38]. It is clear that the weak, transient interactions of αB-c with target proteins described here, and previously [24, 26, 39] are effective in preventing aggregation since, in most cases, only sub-stoichiometric amounts of chaperone are required. In the context of the cell, this is clearly of benefit as it enables the levels of sHsps to be maintained at a low level. Upon exposure to a stress, activation of the heat shock response leads to dramatic up-regulation of sHsp expression, which would then facilitate complex formation (and stabilisation) between the sHsps and compromised target proteins.

The finding that αB-c forms complexes with more destabilised forms of α-LA supports the work of Koteiche and Mchaourab [40] who analysed the chaperone activity of αB-c towards T4 lysozyme mutants. These workers suggested that αB-c recognises destabilised mutants more readily than stable mutants based on their free energy of unfolding and that stable complexes were formed when the free energy of binding to the chaperone was comparable to the free energy of refolding [40]. In agreement with these findings, these data indicate that the conformational stability of protein intermediates plays a key role in modulating the mechanism of action used by αB-c. This is rationalised by αB-c’s physiological role in the ocular lens, in which αB-c associates with αA-crystallin to prevent other soluble lens proteins from precipitating (and hence leading to cataract formation). In this context, αB-c is required to bind unstable, highly compromised protein intermediates, in preference to stable intermediates, as the latter have the potential to independently reattain their native conformation [9].

The results here provide a greater understanding of the molecular basis for aggregation and the specific molecular mechanisms by which αB-c prevents amorphous aggregation as opposed to amyloid fibril formation in vitro. The work is consistent with the concept of distinct conformational states giving rise to different aggregate morphologies. Moreover, while complex formation is clearly an important aspect of sHsp chaperone action, this study demonstrates that complex formation is not the only mechanism used by sHsps to prevent protein aggregation. Considering the instrumental role these (and other) molecular chaperones play in preserving proteostasis and suppressing protein aggregation in vivo, ongoing research to further elucidate the mechanisms by which they interact with aggregating target proteins is essential. This is particularly true if the full potential of chaperone proteins
is to be exploited as an avenue for developing novel therapeutics against protein aggregation based diseases.

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FIGURE LEGENDS

Figure 1 RCMα-LA fibril formation. (a) ThT binding curves of RCMα-LA or native α-LA (100 µM) incubated at 37°C in Tris-based (20 mM Tris-HCl, (pH 7.4), 100 mM KCl, 10 mM MgCl₂, 5 mM EDTA) or phosphate-based (50 mM phosphate buffer, (pH 7.0), 100 mM KCl, 10 mM MgCl₂ either with (+) 5mM or without (-) EDTA) buffers. Fibril formation was monitored by an increase in ThT fluorescence at 490 nm and the change in ThT fluorescence is reported in arbitrary units (a.u.). The assay was performed three times and the data from one experiment is shown as a representative. (b) An electron micrograph of RCMα-LA after incubation at 37°C for 12 h in 50 mM phosphate buffer containing 100 mM KCl and 10 mM MgCl₂. Scale bar = 1 µm.

Figure 2 The chaperone activity of αB-c against the amorphous aggregation of α-LA. (a) α-LA (100 µM) was incubated at 37°C in 50 mM phosphate buffer (pH 7.0) containing 100 mM KCl and 5 mM EDTA. Aggregation was induced by the addition of 20 mM DTT and monitored by an increase in relative light scatter at 360 nm in the absence or presence of αB-c. The change in light scattering for each molar ratio tested (reported as α-LA: αB-c on the right) was monitored for 4 h and is shown in arbitrary units (a.u.). The results shown are representative of three independent experiments. Inset: The percentage protection afforded by αB-c against α-LA amorphous aggregation at various molar ratios (α-LA:αB-c), was determined using the calculation previously described in the methods and materials section. The results are reported as mean ± SEM (n = 3). (b) Size exclusion profiles of α-LA (100 µM), αB-c (50 µM) and soluble post aggregation samples of α-LA (100 µM) in the absence and presence of αB-c (50 µM). The elution volume for standards (kDa) used during calibration are shown at the top of the graph. (c) Immunoblot of the eluate fractions collected from the size exclusion column. A sample from every second fraction (fraction size was 0.5 mL) collected between 7 – 25 mL was loaded onto a SDS-PAGE gel, transferred to nitrocellulose and blotted with an anti-αB-c or anti-α-LA antibody.

Figure 3 The chaperone activity of αB-c against RCMα-LA fibril formation. (a) RCMα-LA (100 µM) was incubated at 37°C in 50 mM phosphate buffer containing 100 mM KCl and 10 mM MgCl₂ in the absence or presence of αB-c. The change in ThT fluorescence at 490 nm for each molar ratio tested (reported as RCMα-LA: αB-c on the right) was monitored over 12 h and is shown in arbitrary units (a.u.). The results shown are representative of three independent experiments. Inset: The percentage protection afforded by αB-c against RCMα-LA fibril formation at various molar ratios, as determined by the calculation described in the methods and materials section. Results are mean ± SEM (n = 3). (b) Size exclusion profiles of RCMα-LA (100 µM), αB-c (50 µM) and soluble post aggregation samples of RCMα-LA (100 µM) in the absence and presence of αB-c (50 µM). The elution volume for standards (kDa) used during calibration are shown at the top of the
Figure 4 Removing αB-c from solution facilitates RCMα-LA fibril formation. (a) RCMα-LA (100 µM) was incubated at 37°C in 50 mM phosphate buffer containing 100 mM KCl and 10 mM MgCl₂ in the absence or presence of αB-c (50 µM). After 10 h of incubation (indicated by the arrow), the sample containing RCMα-LA and αB-c was collected from the microtitre plate and centrifuged through a membrane with a nominal molecular weight cut-off of 100 kDa. The eluate was then re-incubated under the same conditions for a further 10 h. The change in ThT fluorescence at 490 nm for each sample is shown and reported in arbitrary units (a.u.). The results shown are representative of three independent experiments. **Inset:** SDS-PAGE gel of the sample containing RCMα-LA and αB-c collected from the microtitre plate before (Lane 1) and after (Lane 2) centrifugation through the 100 kDa cut-off membrane. The αB-c is retained by the membrane and therefore not present in the eluate following centrifugation.

Figure 5 Fluorescence and far UV circular dichroism spectroscopy reveals differences in the structure of the precursors to amorphous versus fibrillar aggregation of α-LA. (a) Intrinsic tryptophan fluorescence spectra, (b) bis-ANS spectra and (c) far UV CD spectra of native, DTT-reduced and RCM forms of α-LA. The reduction of α-LA was achieved by addition of DTT (20 mM) 5 min prior to analysis. Each experiment was independently replicated three times and the data shown are representative of one of these experiments. MRE – mean residue ellipticity.

Figure 6 A revised model for the chaperone activity of sHsps. Multiple partially-folded protein intermediate states populate the folding/unfolding pathway of a protein. Intermediates which retain elements of native-like structure, but have significant amounts of hydrophobicity exposed to solution (i.e. DTT-reduced α-LA) form the precursors to amorphous aggregation, while stable, predominately unfolded intermediate states with fewer clusters of exposed hydrophobicity (i.e. RCMα-LA) form the precursors to amyloid fibril formation. The mechanism by which sHsps, such as αB-c prevent these two forms of protein aggregation is determined by the conformational stability and exposed hydrophobicity of the precursor protein intermediates. High affinity interactions occur with highly disordered intermediates (which exceed the threshold of binding) and these are sequestered into stable high molecular mass complexes. Alternatively, weak, transient interactions occur with more stable protein intermediates, re-directed them back to the folding pathway so as to facilitate re-folding.

REFERENCES
Figure 1
Figure 2
Figure 3
Figure 4

![Graph showing ThT fluorescence over time with different conditions: RCMα-LA alone, RCMα-LA + αB-c, αB-c alone. Each condition is represented by a different line on the graph, with time in hours on the x-axis and Δ ThT fluorescence (a.u.) on the y-axis. An inset image shows a gel analysis with lanes labeled αB-c and α-LA.]
Figure 5
Supplementary data for manuscript

‘The small heat shock protein αB-crystallin uses different mechanisms of chaperone action to prevent the amorphous versus fibrillar aggregation of α-lactalbumin.’

**Supp Figure 1** Intrinsic tryptophan fluorescence spectra of native, DTT-reduced and RCM forms of α-LA. The proteins were prepared in the presence or absence of MgCl₂ (10 mM) and/or DTT (20 mM) as outlined in the methods section of the manuscript. Each experiment was independently replicated three times and the data shown are representative of one of these experiments.