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Abstract

Clusterin is an extracellular mammalian chaperone protein which inhibits stress-induced precipitation of many different proteins. The conformational state(s) of proteins that interact with clusterin and the stage(s) along the folding and off-folding (precipitation-bound) pathways where this interaction occurs were previously unknown. We investigated this by examining the interactions of clusterin with different structural forms of α-lactalbumin, γ-crystallin and lysozyme. When assessed by ELISA and native gel electrophoresis, clusterin did not bind to various stable, intermediately folded states of α-lactalbumin nor to the native form of this protein, but did bind to and inhibit the slow precipitation of reduced α-lactalbumin. Reduction-induced changes in the conformation of α-lactalbumin, in the absence and presence of clusterin, were monitored by real-time ^{1}H NMR spectroscopy. In the absence of clusterin, an intermediately folded form of α-lactalbumin, with some secondary structure but lacking tertiary structure, aggregated and precipitated. In the presence of clusterin, this form of α-lactalbumin was stabilised in a non-aggregated state, possibly via transient interactions with clusterin prior to complexation. Additional experiments demonstrated that clusterin potently inhibited the slow precipitation, but did not inhibit the rapid precipitation, of lysozyme and γ-crystallin induced by different stresses. These results suggest that clusterin interacts with and stabilises slowly aggregating proteins but is unable to stabilise rapidly aggregating proteins. Collectively, our results suggest that during its chaperone action, clusterin preferentially recognises partly folded protein intermediates that are slowly aggregating whilst venturing along their irreversible off-folding pathway towards a precipitated protein.

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Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway

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ABSTRACT

Clusterin is an extracellular mammalian chaperone protein which inhibits stress-induced precipitation of many different proteins. The conformational state(s) of proteins that interact with clusterin and the stage(s) along the folding and off-folding (precipitation-bound) pathways where this interaction occurs were previously unknown. We investigated this by examining the interactions of clusterin with a variety of different structural forms of α-lactalbumin, γ-crystallin and lysozyme. When assessed by ELISA and native gel electrophoresis, clusterin did not bind to various stable, intermediately folded states of α-lactalbumin nor to the native form of this protein, but did bind to and inhibit the slow precipitation of reduced α-lactalbumin. Reduction-induced changes in the conformation of α-lactalbumin, in the absence and presence of clusterin, were monitored by real-time ^{1}H NMR spectroscopy. In the absence of clusterin, an intermediately folded form of α-lactalbumin, with some secondary structure but lacking tertiary structure, aggregated and precipitated. In the presence of clusterin, this form of α-lactalbumin was stabilised in a non-aggregated state, possibly via transient interactions with clusterin prior to complexation. Additional experiments demonstrated that clusterin potently inhibited the slow precipitation, but did not inhibit the rapid precipitation, of lysozyme and γ-crystallin induced by different stresses. These results suggest that clusterin interacts with and stabilises slowly aggregating proteins but is unable to stabilise rapidly aggregating proteins. Collectively, our results suggest that during its chaperone action, clusterin preferentially recognises partly folded protein intermediates that are slowly aggregating whilst venturing along their irreversible off-folding pathway towards a precipitated protein.

Keywords

Molecular chaperone; Protein unfolding; Aggregation; Clusterin; Small heat-shock proteins

Abbreviations

3SS-α-lactalbumin: α-lactalbumin with Cys6-Cys120 selectively reduced and carboxymethylated; DTT: dithiothreitol; GuHCl: guanidinium hydrochloride; HDC: 1% heat-denatured casein dissolved in PBS; HMW: high-molecular-weight; PBS: phosphate-buffered saline; R-cam-α-lactalbumin: reduced and carboxyamidated α-lactalbumin; R-cm-α-lactalbumin: reduced and carboxymethylated α-lactalbumin; sHsp: small heat-shock protein.

1. INTRODUCTION

Molecular chaperones are a diverse group of proteins that interact with unfolded or partly folded proteins to stabilise them, thereby preventing them from potentially aggregating and precipitating [1,2]. Molecular chaperones are involved in a variety of cellular tasks including protein folding, trafficking and general stabilisation. As a result of their stabilising ability, the expression of many molecular chaperones is upregulated in disease states that are associated with protein misfolding and precipitation, for example, Alzheimer's, Creutzfeldt-Jakob and Parkinson's diseases [3]. Intracellularly, the well-characterised chaperone proteins, Hsp40, Hsp60 and Hsp70 (DnaJ, GroEL and DnaK in *E. coli*), are intimately involved in regulating the correct folding of some proteins in the crowded environment of the cell [4]. Another class of intracellular molecular chaperones, the small heat-shock proteins (sHsps), are not directly involved in protein folding. Instead, they are the major proteins associated with stabilising other proteins under stress conditions (e.g. heat, oxidation, infection, etc.) to prevent their large-scale precipitation [5]. In this process, the partly folded, stressed protein interacts via predominantly hydrophobic interactions with the sHsp to form a complex. Subsequently, when cellular conditions return to normal, this protein may be refolded via interaction with Hsp70 coupled to ATP hydrolysis [5,6]. Thus, the expression of sHsps and Hsp70 is significantly increased under stress conditions [5].

Recently, we described the sHsp-like chaperone ability of the ubiquitous extracellular mammalian protein, clusterin [7]. Our data indicate that clusterin shares many similarities with sHsps in its mode of interaction with stressed proteins: it is a very efficient chaperone; it forms, primarily via hydrophobic interactions, complexes (of high mass) with stressed proteins; and it has no inherent ability to refold stressed proteins after the removal of stress [7-9, unpublished data]. Furthermore, as has been shown for some of the sHSPs [5,6], we have demonstrated that stressed proteins complexed with clusterin are stabilised in a folding-competent state that can readily be recovered into a correctly folded form via an ATP-dependent interaction with Hsp70 [9]. The implication from this observation is that the stressed protein is bound to clusterin in a partly folded state that is readily accessible to molecular chaperones with refolding ability.

Following synthesis, proteins traverse rapidly along their folding pathway from unfolded to folded (and hence active) conformations. In between these two extremes, proteins adopt a variety of partly folded intermediates or molten globule states (e.g. designated I_1 and I_2) with varying degrees of structure. Along this pathway, molecular chaperones such as Hsp40, Hsp60 and Hsp70 assist the folding of proteins into their correct conformation. The protein folding pathway is reversible and, under stress conditions, proteins unfold from their native conformation along the reverse of this pathway to adopt intermediately folded states. Being partly structured, these intermediates expose significant hydrophobicity to solution. It is then possible for them to deviate from the folding/unfolding pathway along an irreversible off-folding pathway, leading to aggregation and potentially precipitation. sHsps interact with proteins along this pathway [10,11]. The conformational state(s) of proteins that interact with clusterin and the stage(s) along these pathways where this interaction occurs were previously unknown. We used an approach similar to that taken with the sHsps [10] to investigate this by examining the interactions of clusterin with a variety of structured forms of α-lactalbumin, γ-crystallin and lysozyme.

α-Lactalbumin is a small, monomeric milk protein (mass ~14 kDa) that has a bound calcium ion at the interface between its two domains [12]. It is possible to prepare chemically-modified, monomeric and stable states of α-lactalbumin with varying

degrees of partly folded structure that have structural similarities to intermediates along the folding/unfolding pathway of α-lactalbumin (Table 1) [13,14]. GroEL interacts with some of these stable α-lactalbumin intermediates [15,16] but α-crystallin, a representative sHsp, does not [10]. The folding/unfolding pathway of α-lactalbumin, and the intermediates along it, have been well characterised [17]. Reduction of the four disulphide bonds of α-lactalbumin with dithiothreitol (DTT) also leads to a partly structured intermediate (Table 1). However, this intermediate has significant exposed hydrophobicity and slowly aggregates and precipitates along the off-folding pathway [10,11]. α-Lactalbumin is an extracellular protein and is therefore a potential protein target for clusterin during its chaperone action *in vivo* under stress conditions. Reduction is not a physiologically relevant extracellular stress *in vivo*. However, since the structures of α-lactalbumin intermediates are well studied and understood, conclusions drawn from studies of their interactions with clusterin have implications for defining the conformational features of stressed proteins that act as chaperone substrates for clusterin *in vivo*.

In this study, the interaction of clusterin with intermediately folded forms of α-lactalbumin was examined by ELISA, native gel electrophoresis and real-time ^{1}H NMR spectroscopy. In addition, the interaction of clusterin with both slowly and rapidly precipitating forms of γ-crystallin and lysozyme was investigated. Overall, the results indicate that clusterin is like the sHsps in recognising only slowly aggregating, precipitation-bound forms of stressed proteins on an off-folding pathway.

2. EXPERIMENTAL

Reagents

Bovine α-lactalbumin (calcium-depleted), lysozyme, bovine serum albumin, ovotransferrin and dithiothreitol (DTT) were purchased from Sigma (Sydney, Australia). Deuterated 1,4-D,L-d_{10}-DTT was obtained from Cambridge Isotopes Laboratories (MA, USA). γ-Crystallin was purified from bovine lenses by size exclusion chromatography using standard methods [18]. Three stable, partly folded derivatives of α-lactalbumin were prepared by methods described in [13,14]: R-cam-α-lactalbumin with its four disulphide bonds reduced and subsequently blocked by carboxyamidation, R-cm-α-lactalbumin with its four disulphide bonds reduced and subsequently blocked by carboxymethylation, and 3SS-α-lactalbumin with Cys6-Cys120 selectively reduced and blocked by carboxymethylation. Their correct masses were confirmed by electrospray ionisation mass spectrometry. Apo α-lactalbumin was prepared by heating solutions of the native protein, containing 1.5 mM EDTA, for 1 h at 45 ^{o}C. Clusterin was prepared from human serum using immunoaffinity chromatography as described earlier [19]. Clusterin-specific monoclonal antibodies, G7, 41D and 78E, were as described [20]. Agarose was obtained from Promega (Sydney, Australia). All other chemicals were of reagent grade and were obtained from Ajax Chemicals (Sydney, Australia).

ELISA

The α-lactalbumin derivatives (R-cam-α-lactalbumin, R-cm-α-lactalbumin, 3SS-α-lactalbumin) at 1 mg/ml in 20 mM NaH_2PO_4, pH 7.2, were adsorbed onto ELISA trays for 1 h at 37 °C (45 °C for apo α-lactalbumin). Native α-lactalbumin was adsorbed to ELISA trays at 1 mg/ml in 20 mM NaH_2PO_4, pH 7.2, for 5 h at 37 °C. α-Lactalbumin was also adsorbed to ELISA trays during reduction-induced aggregation by incubating the protein (at 1 mg/ml) in 20 mM NaH_2PO_4, 0.1 M NaCl, pH 7.2, containing 20 mM DTT, in ELISA wells for 5 h at 37 ^{o}C. Lyzozyme (50 μg/ml in 0.1 M $NaHCO_3$, pH 9.5) was adsorbed for 1 h at 37 °C and then reduced by incubation with 15 mM DTT in PBS for 1 h at 37 °C. Following these steps, the ELISA trays were blocked for 1 h at 37 °C with HDC (10 mM phosphate, 150 mM NaCl, 1%

(w/v) heat-denatured casein, 0.04% (w/v) thymol, pH 7.4). Subsequently, purified clusterin dissolved in HDC (at 10 μg/ml or at concentrations indicated in Figures) was incubated in ELISA wells for 1 h at 37 ^{o}C. The wells were then repeatedly washed with PBS and clusterin bound to the adsorbed proteins was measured using (i) an equi-volume mixture of G7, 41D and 78E monoclonal antibody tissue culture supernatants, followed by (ii) horseradish peroxidase-conjugated sheep anti-mouse Ig antibody, as described previously [7]. The substrate used was *o*-phenylenediamine dihydrochloride (OPD; 2.5 mg/ml in 0.05 M citric acid, 0.1 M Na_2HPO_4, pH 5.0, containing 0.03% (v/v) H_2O_2).

Native gel electrophoresis and blotting

Solutions containing either α-lactalbumin (at 5 mg/ml), clusterin (at 1 mg/ml) or mixtures of both (at the same respective concentrations) in 50 mM Na_2HPO_4, pH 7.0, were incubated for 5 h at 37 ^{o}C with or without the addition of 20 mM DTT. In addition, solutions containing α-lactalbumin derivatives (at 0.67 mg/ml) or mixtures of these derivatives and clusterin (both at 0.67 mg/ml) in 50 mM Na_2HPO_4, pH 7.0, were incubated (without DTT) for 5 h at 37 ^{o}C. Precipitated protein was then removed by centrifugation for 1 min at 10,000 rpm in a benchtop microfuge. 2 μl of loading buffer (0.01 % bromophenol blue, 20% (v/v) glycerol) was added to aliquots of the supernatant in each case (containing 10 μg of total protein) before loading on to 1% (w/v) agarose gels. Proteins were electrophoresed at 60 V for approximately 3 h using 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0 as running buffer (the structures of the various forms of α-lactalbumin tested are not affected by EDTA). Proteins were subsequently transferred to polyvinylidine difluoride membrane (MSI, Westborough, MA, USA) by capillary transfer. The membranes were stained with SuperStain (Pierce, Rockford, IL, USA) for approximately 5 min, destained (in 45% (v/v) methanol, 10% (v/v) glacial acetic acid) for approximately 20 min, and then scanned using a BioRad GS670 Imaging Densitometer.

NMR spectroscopy

Real-time one-dimensional ^{1}H NMR spectra were acquired at 500 MHz on a Varian Inova-500 NMR spectrometer. A sweep width of 5500 Hz was used over 9000 data points with an acquisition time of 0.82 s per scan and a delay between scans of 1.0 s.

Sixteen scans were acquired per spectrum which, along with two dummy scans, gave a total time between successive spectra of approximately 33 s. Successive spectra were acquired following the addition of 30 μl of a 0.4 M solution of d_{10}-DTT to 0.6 ml of 50 mM phosphate, 0.1 M NaCl, in D_2O, pH 7.2 containing 3.0 mg/ml α-lactalbumin in the absence or presence of 0.7 mg/ml clusterin. Under the latter conditions, the precipitation from solution of reduced α-lactalbumin was completely prevented by the formation of a solubilised, high-molecular-mass clusterin-α-lactalbumin complex [7]. An exponential line broadening of 3 Hz was applied to all NMR spectra prior to Fourier transformation.

The effect of clusterin on slow versus rapid stress-induced precipitation of γ-crystallin and lysozyme

The effects of clusterin on the slow precipitation from solution of γ-crystallin were determined by heating γ-crystallin (at 1 mg/ml in 50 mM Na_2HPO_4, pH 7.0) at 60 ^oC with or without 50 μg/ml clusterin. Similar experiments were conducted in which lysozyme (250 μg/ml) was incubated at 42 ^oC in the same buffer as above containing 20 mM DTT, with or without 400 μg/ml clusterin. The extent of protein precipitation in each case was measured as turbidity (light scattering) at 360 nm using a model 8453 diode-array spectrophotometer (Hewlett-Packard GMBH, Germany). We have shown before that control proteins (eg ovalbumin) do not inhibit protein precipitation under these conditions [7]. In other experiments, the effects of clusterin on the rapid precipitation of the same two proteins was determined; in these cases, for each target protein, the mass ratio of clusterin:target was maintained at the same level as in the experiments measuring slow precipitation (γ-crystallin:clusterin = 20:1.0, lysozyme:clusterin = 1.0:1.6). γ-Crystallin (300 μg) was incubated for 20 min in 20 μl of denaturing buffer (50 mM Na_2HPO_4, pH 7.0, 6 M guanidine hydrochloride (GuHCl)). This solution was added to 10 μl of PBS containing 15 μg of clusterin or one of two control proteins (BSA or ovotransferrin) before, over approximately 1 min, successively adding 10-20 μl aliquots of 50 mM Na_2HPO_4, pH 7.0, to effect rapid protein precipitation. Similarly, 3 mg of lysozyme was incubated for 20 min in 30 μl of denaturing buffer before 1 μl of this solution was added to 19 μl of PBS containing 160 μg of either clusterin, BSA or ovotransferrin. These mixtures were

then diluted 5-fold, over approximately 1 min, with subsequent additions of 10 μl aliquots of 50 mM Na_2HPO_4, pH 7.0, to give a final volume of 100 μl. For both proteins, the requirement for step-wise dilutions precluded kinetic analyses of the same type as that performed in the slow precipitation experiments. Experiments measuring rapid protein precipitation were performed in the wells of microplates and the end-point turbidity (representing the final level of protein precipitation) measured as light scattering at 360 nm with a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3. RESULTS

ELISA and native gel electrophoretic analyses of the interaction of clusterin with various structural forms of α-lactalbumin

In ELISA, only negligible (probably non-specific) binding of clusterin to stable, intermediately folded or unfolded forms of α-lactalbumin (see Table 1) or to the native form of the protein was detected (Figure 1A). In contrast, clusterin bound strongly to reduced α-lactalbumin (Figure 1A), confirming our earlier report of this interaction [7]. The inability of clusterin to bind to the other structural forms of α-lactalbumin tested suggested that it is unable to complex with these forms in solution. This interpretation was confirmed by native gel electrophoretic analyses, in which pre-incubation with clusterin had no effect on the electrophoretic mobility of native α-lactalbumin or its stable derivatives (Figure 1B). When clusterin was co-incubated with α-lactalbumin prior to electrophoresis, two discrete bands corresponding to each of the individual proteins were detected (Figure 1B, lane 5). However, when these proteins were co-incubated with DTT under similar conditions, very much less protein was detected in the bands corresponding to the individual proteins and a single protein band of unique electrophoretic mobility, representing a clusterin-α-lactalbumin complex [7], was detected (Figure 1B, compare lanes 5 and 6). In a previous study [7], reduced α-lactalbumin was prepared in the same manner as this study. The precipitation of reduced α-lactalbumin was prevented by the presence of a sufficient quantity of clusterin which resulted in the formation of a solubilized, high molecular mass α-lactalbumin-clusterin complex, detected by size exclusion chromatography. SDS-PAGE was used to demonstrate that both proteins were present in this complex [7].

Real-time ^{1}H NMR analysis of the interaction between clusterin and reduced α-lactalbumin

Real-time ^{1}H NMR spectra were acquired of reduced α-lactalbumin in the absence and presence of sufficient clusterin to prevent the precipitation of reduced α-lactalbumin (a 4.3:1.0 w:w ratio of α-lactalbumin:clusterin; [7]). Figures 2A & B show the aromatic region of selected NMR spectra of α-lactalbumin without clusterin

or in the presence of clusterin at various times after the addition of DTT. Being a structured protein, α-lactalbumin has a well-dispersed NMR spectrum, as is apparent from the spectrum acquired prior to the addition of DTT (at 0 s; Figure 2A). After the addition of DTT, successive spectra reflect the loss in structure of α-lactalbumin as its four disulphide bonds were sequentially reduced (Figure 2A). The hyper-reactive disulphide bond between Cys6 and Cys120 is reduced very quickly (< 1 s) whereas the remaining disulphide bonds are reduced over a much longer timeframe [14]. In the absence of clusterin, as disulphide bond reduction occurred, the spectra changed from (i) the resolved NMR spectrum of the native species (at 0 s), to (ii) a broad spectrum with some dispersion, reflecting a partly folded intermediate containing most secondary and some tertiary structure (I_1, e.g. at 384 s), to (iii) a broad spectrum lacking dispersion (I_2, e.g. at 639 s), produced by an intermediate that has some secondary structure but that lacks any tertiary structure (Figure 2A). The broadness of both spectra reflects the dynamic nature of these intermediates that have indeterminate conformation. The spectrum of I_2 has strong similarities to that of the partly folded intermediate of α-lactalbumin observed at pH 2, the so-called molten globule "A-state" [10]. With time, this spectrum was lost as reduced α-lactalbumin aggregated and precipitated.

Spectra from the I_2 state of α-lactalbumin were completely lost after ~ 1,700 s in the absence of clusterin but, in the presence of clusterin, were still present 5400 s after the addition of DTT (Figure 2B). This suggests that clusterin stabilises the I_2 state of α-lactalbumin. Other than this difference, the real-time NMR spectra acquired following the addition of DTT to α-lactalbumin, either in the absence or presence of clusterin, were generally similar in form (compare Figures 2A and 2B). The concentration of clusterin used was too small to give rise to significant resonances in the NMR spectrum. As for spectra acquired in the absence of clusterin, in the presence of clusterin the spectrum from the I_2 state of α-lactalbumin was gradually lost (Figure 2B). However, in this case, the loss of resonances was not due to the protein precipitating but very probably arose because, under these conditions, α-lactalbumin is known to complex with clusterin to produce species of large molecular mass [7] that would tumble slowly and give rise to very broad NMR spectra.

The resonance at 6.8 ppm in the spectrum of the I_2 form arises from the (3,5) aromatic protons of the tyrosine residues of reduced α-lactalbumin and is relatively isolated from the broad envelope of other aromatic resonances (Figures 2A & B) [10,11]. Conveniently, native α-lactalbumin does not have a significant resonance at this chemical shift. Therefore, monitoring its intensity with time following addition of DTT provides a means of following the buildup and decay of this state without complications from resonance(s) arising from the native state of α-lactalbumin. Figure 2C shows a plot of the intensity of this resonance versus time after addition of DTT for α-lactalbumin in the absence and presence of clusterin. In both cases, the resonance intensity built up rapidly to a maximum at ~ 380 s, indicating that clusterin had no significant effect on the buildup of signal from the I_2 state. Mass spectrometric and stopped flow UV studies have shown that the maximum NMR resonance intensity from the I_2 state of α-lactalbumin occurs when all four of its disulphide bonds are reduced (J.A. Carver, R.A. Lindner, C. Lyon, D. Canet, H. Hernandez, C.M. Dobson and C. Redfield, submitted for publication). Therefore, the data indicate that clusterin does not significantly affect the rate of reduction of disulphide bonds in α-lactalbumin. After maximum resonance intensity was reached, the signal decayed as I_2 either (i) aggregated (in the absence of clusterin), or (ii) complexed with clusterin (Figure 2C). As a means to compare the rates of decay of the 6.8 ppm resonance for α-lactalbumin, the equation for a first–order process was fitted to the data shown in these figures. The resonance intensity decayed with time with apparent first-order rate constants of, in the absence of clusterin, $(1.81 \pm 0.09) \times 10^{-3}\ s^{-1}$ and, in the presence of clusterin, $(5.07 \pm 0.13) \times 10^{-4}\ s^{-1}$. Thus, under the conditions described, clusterin approximately tripled the lifetime of the I_2 state of α-lactalbumin, before complexing with it.

Analysis of the interaction of clusterin with slowly and rapidly precipitating forms of γ-crystallin and lysozyme

γ-Crystallin, a major lens protein, slowly aggregates and precipitates when exposed to elevated temperature [18]. Under the conditions tested (mass ratios of γ-crystallin:clusterin = 20:1.0, and lysozyme:clusterin = 1.0:1.6), clusterin significantly inhibited the slow precipitation of (i) γ-crystallin, induced by heating at 60 ^{o}C (Figure 3A), and (ii) lysozyme, induced by reduction at 42 ^{o}C (Figure 3B). Higher ratios of

clusterin:target gave dose-dependent increases in the inhibition of precipitation of either γ-crystallin or lysozyme (data not shown). When either γ-crystallin or lysozyme is unfolded in 6 M GuHCl and then diluted into phosphate buffer, they rapidly precipitate from solution. Using the same mass ratios of clusterin:target as in the experiments measuring slow protein precipitation, clusterin had no significant effect on the rapid precipitation of either γ-crystallin or lysozyme (Figures 3C and 3D). Furthermore, even when the clusterin:target ratios were doubled, clusterin still failed to inhibit the rapid precipitation of either protein (data not shown).

4. **DISCUSSION**

Clusterin is a heterodimer comprised of α and β subunits linked by five disulphide bonds that adopts an ill-defined heterogeneous aggregation state in aqueous solution. Size exclusion chromatographic analysis indicates that DTT does not cause physical dissociation of the α and β chains of clusterin [7]. Furthermore, the native gel electrophoretic mobilities for unreduced and reduced clusterin (lanes 1 and 2 in Fig. 1B) are essentially identical, indicating that reduction does not induce any gross changes in clusterin structure. Furthermore, reduction does not abrogate the chaperone action of clusterin with a variety of target proteins [7,9]. Thus, reduced clusterin is a functionally active protein.

From the results presented in Figure 1, it is apparent that clusterin does not bind to (i) native α-lactalbumin, (ii) its stable, monomeric, partly structured derivatives, or (iii) the fully unfolded form of α-lactalbumin. Previously, we also demonstrated that α-crystallin did not interact with these states of α-lactalbumin [10]. However, clusterin does bind to the reduced (stressed) form of the protein (Figure 1, and [7]). CD spectra of reduced α-lactalbumin show that it has some secondary structure but very little, if any, tertiary structure (i.e. it is I_2-like) [16]; this form slowly aggregates and precipitates [7,10]. R-cam-α-lactalbumin also has an I_2-like structure (Table 1) but, unlike reduced α-lactalbumin, is stable in solution as a monomeric species and does not interact with clusterin. Clearly, the marked variation between the two α-lactalbumin species in their affinity for clusterin cannot be explained on the basis of gross differences in structure. However, there must be subtle structural differences which, in the case of reduced α-lactalbumin, mean a greater exposure of hydrophobicity to solution and a consequent tendency for it to undergo large-scale, ill-defined aggregation [21]. Clusterin exists as a heterogeneous aggregate and undergoes subunit exchange [22], a process similar to that undergone by reduced α-lactalbumin monomers which are continuously exchanging during their aggregation to form species of a very broad mass range [21]. Thus, the similar dynamic and heterogeneously aggregated nature of clusterin and reduced α-lactalbumin may facilitate their mutual incorporation into a complex during chaperone interaction.

There are two phases to the interaction between α-crystallin and the I_2 state of reduced α-lactalbumin. Firstly, I_2 is significantly stabilised and aggregation is prevented, probably via transient interactions between the monomeric I_2 state and α-crystallin. Secondly, I_2 stably binds to α-crystallin to form a complex of large mass [10,11]. Other mammalian sHsps and plant sHsps behave in the same manner during their interaction with reduced α-lactalbumin [10,11, J.A. Carver, R.A. Lindner, M. Gaestel, E. Basha, and E. Vierling, unpublished results]. Our results suggest that clusterin interacts with the I_2 state of α-lactalbumin by a similar two-stage mechanism. Firstly, real time NMR analysis showed that, during reductive stress, clusterin extended the lifetime of the I_2 state of α-lactalbumin about three-fold (Figure 2C). This indicates that clusterin stabilised the non-aggregated (probably monomeric) form of this intermediate. Secondly, the demonstration that clusterin forms a high-molecular-mass complex with reduced α-lactalbumin [7] suggests that it, like α-crystallin, preferentially complexes with an unstable, intermediately folded state of α-lactalbumin. Furthermore, the progressive loss of the NMR spectrum of the I_2 state of α-lactalbumin during reduction in the presence of clusterin is consistent with the formation of a high-molecular-mass α-lactalbumin-clusterin complex. A broad range of other target proteins that have been stressed by either reduction or heating also form complexes with clusterin [7,9].

The aggregation and precipitation of reduced α-lactalbumin occurs relatively slowly. The ability of clusterin to interact efficiently with this form of α-lactalbumin and prevent its precipitation suggested that kinetic factors might be important in regulating the efficiency of clusterin chaperone action. To examine this hypothesis, we tested the relative ability of clusterin to inhibit (i) the slow precipitation of γ-crystallin and lysozyme (induced by heat and reduction, respectively), versus (ii) the rapid precipitation of the same proteins induced by dilution from denaturant. Under both conditions, the proteins unfold to adopt partly structured intermediates. Experimentally, it has been shown that under dilute conditions, the major γ-crystallin subunit, γB, and lysozyme both form partly folded intermediates in the presence of denaturant and when diluted from denaturant [23,24]. γB-crystallin at pH 2.0 and in 3M urea forms an intermediate that has an unfolded C-terminal domain and a folded N-terminal domain [23]. When lysozyme is diluted from denaturant, an intermediate

rapidly forms that has tyrosine residues, but not tryptophan residues, exposed to solvent [24]. This intermediate then rearranges with a $t_{1/2}$ of 350 ms to form the native state in which the tyrosine residues become buried but two tryptophan residues become exposed to solvent. It is likely that these intermediates of γB-crystallin and lysozyme resemble those present in our studies in which high concentrations of these proteins were rapidly diluted from solutions containing denaturant, resulting in their rapid aggregation and precipitation. The results herein demonstrate that clusterin efficiently inhibits the slow precipitation of γ-crystallin and lysozyme induced by heat or reduction, but is incapable of preventing the rapid precipitation of the same proteins induced by dilution from denaturant (Figure 3). Thus, the findings support the hypothesis that kinetic factors are important in the chaperone action of clusterin and suggest that it is unable to efficiently inhibit rapid protein precipitation.

In contrast to GroEL [15,16], the sHsps have no affinity for stable, partly folded intermediate states of α-lactalbumin, or unstable intermediate states of proteins that aggregate and precipitate very rapidly [10,18,26]. The sHsps only bind to long-lived, intermediate states of proteins that slowly aggregate as they progress along their off-folding pathway [10,11]. Furthermore, unlike Hsp60 and Hsp70, sHsps do not affect the rate of folding of proteins (e.g. α-lactalbumin) when they progress via a series of transient intermediates along a folding pathway from their unfolded to folded form [18,26, J.A. Carver, R.A. Lindner, C. Lyon, D. Canet, H. Hernandez, C.M. Dobson and C. Redfield, submitted for publication]. Collectively, our results suggest that clusterin shares all of these features with the sHsps. Furthermore, for α-lactalbumin, our results indicate that during reductive stress, like α-crystallin and other sHsps, clusterin stabilises the I_2 state of α-lactalbumin. Figure 4 summarises the steps in the chaperone action of clusterin with reduced α-lactalbumin.

In conclusion, the above data support the hypothesis that clusterin does not interact in a chaperone role with stable, non-aggregating intermediate states of proteins or those that are present when proteins undergo very rapid aggregation and precipitation. Furthermore, we recently demonstrated that clusterin does not affect the heat-induced loss of activity of glutathione-S-transferase or alcohol dehydrogenase or, when acting alone, promote the refolding of these proteins to regain function following the

removal of heat stress [9]. Thus, it appears likely that clusterin does not interact with proteins along their folding/unfolding pathway, a process that occurs relatively rapidly. Instead, clusterin prefers slowly aggregating, long lived and precipitation-bound intermediate states of proteins that are present on an irreversible, off-folding pathway (Figure 4). Clusterin complexes to these and thereby prevents their precipitation. Therefore, the efficiency of the chaperone action of clusterin, like that of the sHsps [10,11,26], is dependent on both conformational and kinetic factors.

Clusterin expression is increased in diseases characterised by protein misfolding, aggregation and precipitation, for example, in Alzheimer's and Creutzfeldt-Jakob diseases [27]. In these diseases, fibrous protein precipitates known as amyloid or plaque slowly form in extracellular spaces [3]. It is believed that amyloid formation occurs via the association of destabilised, intermediately folded states of proteins [28]. The observations that clusterin is an extracellular chaperone [8] and that it interacts with these forms of proteins (results presented here), suggest that it may influence the development of amyloid diseases. Indeed, Oda *et al.* [29] demonstrated that clusterin inhibited the formation of large insoluble fibrils resulting from the aggregation of amyloid β-peptide, the putative causative agent in Alzheimer's disease. However, the pathogenicity of the resultant mixture towards neuronal cells was enhanced, suggesting that the smaller fibrils formed in the presence of clusterin are the pathogenic species. Similar effects were reported for the interaction of sHsps with the amyloid β-peptide [30,31]. The association of clusterin with Alzheimer's plaques *in vivo* [27] may reflect the inability of clusterin to completely inhibit the large-scale precipitation of amyloid β-peptide under pathogenic conditions. To examine the potential role of clusterin in amyloid diseases, we are currently testing its interactions with amyloid-forming proteins.

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Table 1. Modifications and structural features of α-lactalbumin derivatives investigated for their interaction with clusterin.

α-Lactalbumin derivative	**Modifications**	**Structural features**
R-cam-α-lactalbumin	Four disulphide bonds reduced and subsequently blocked by carboxyamidation	Disordered conformation, with some secondary structure but no tertiary structure [10,13,16] (I_2-like)
R-cm-α-lactalbumin	Four disulphide bonds reduced and subsequently blocked by carboxymethylation	Unfolded conformation [13,15,18]
3SS-α-lactalbumin	Cys6-Cys120 selectively reduced in the presence of EDTA and blocked by carboxymethylation	Relatively folded conformation with most secondary and some tertiary structure. Similar in conformation to heated apo-α-lactalbumin [10,13,14,16] (I_1-like)
apo-α-lactalbumin	Heated at 45 ^{o}C in the presence of EDTA to remove bound Ca^{2+}	Ordered conformation with most secondary structure and some tertiary structure. Similar in conformation to 3SS-α-lactalbumin [10,13,16] (I_1-like)
reduced α-lactalbumin	Reduced by incubation with 20 mM DTT at 37°C	Similar to R-cam-α-lactalbumin (I_2-like) with the exception that, unlike R-cam-α-lactalbumin, this form is unstable in solution and slowly aggregates and precipitate [16]

FIGURE LEGENDS

Figure 1. (A) *Binding of clusterin to derivatives of α-lactalbumin in ELISA.* Results of ELISA testing the binding of clusterin to various structural forms of α-lactalbumin. As controls, ELISA plates were also coated with reduced (DTT-treated) α-lactalbumin to which clusterin binds [7]. The results shown are representative of several independent experiments. In each case, the error bars shown are standard errors (SE) of the mean of triplicate measurements. In some cases, the SE are too small to be visible. (B) *Native gel electrophoresis of mixtures of clusterin and various forms of α-lactalbumin.* Clusterin, or mixtures of clusterin with various forms of α-lactalbumin, were stressed as described in the Experimental section or left untreated before subjecting the proteins to native gel electrophoresis. The image shows a native gel following electrophoresis and protein staining. The key to the right of the image indicates the identity of proteins or protein mixtures loaded into each lane. The arrow indicates, in lane 6, a band of unique electrophoretic mobility corresponding to a clusterin-α-lactalbumin complex (see text). The results shown are representative of three independent experiments. Note that there are no protein bands visible in lane 4 because reduced α-lactalbumin precipitates from solution.

Figure 2. Real-time ^{1}H NMR spectroscopy and kinetics of the reduction of α-lactalbumin in the absence and presence of clusterin.

Aromatic region of the ^{1}H NMR spectra of α-lactalbumin at the times indicated after the addition of DTT. (A) α-Lactalbumin only; (B) 4.3:1.0 w:w α-lactalbumin:clusterin. Representative results are shown. (C) Plots showing the resonance intensity of the α-lactalbumin tyrosine (3,5) aromatic protons (at 6.8 ppm) as a function of time during reduction; α-Lactalbumin only (◊); 4.3:1.0 w:w α-lactalbumin:clusterin (♦).

Figure 3. The effect of clusterin on the slow and rapid precipitation of γ-crystallin and lysozyme

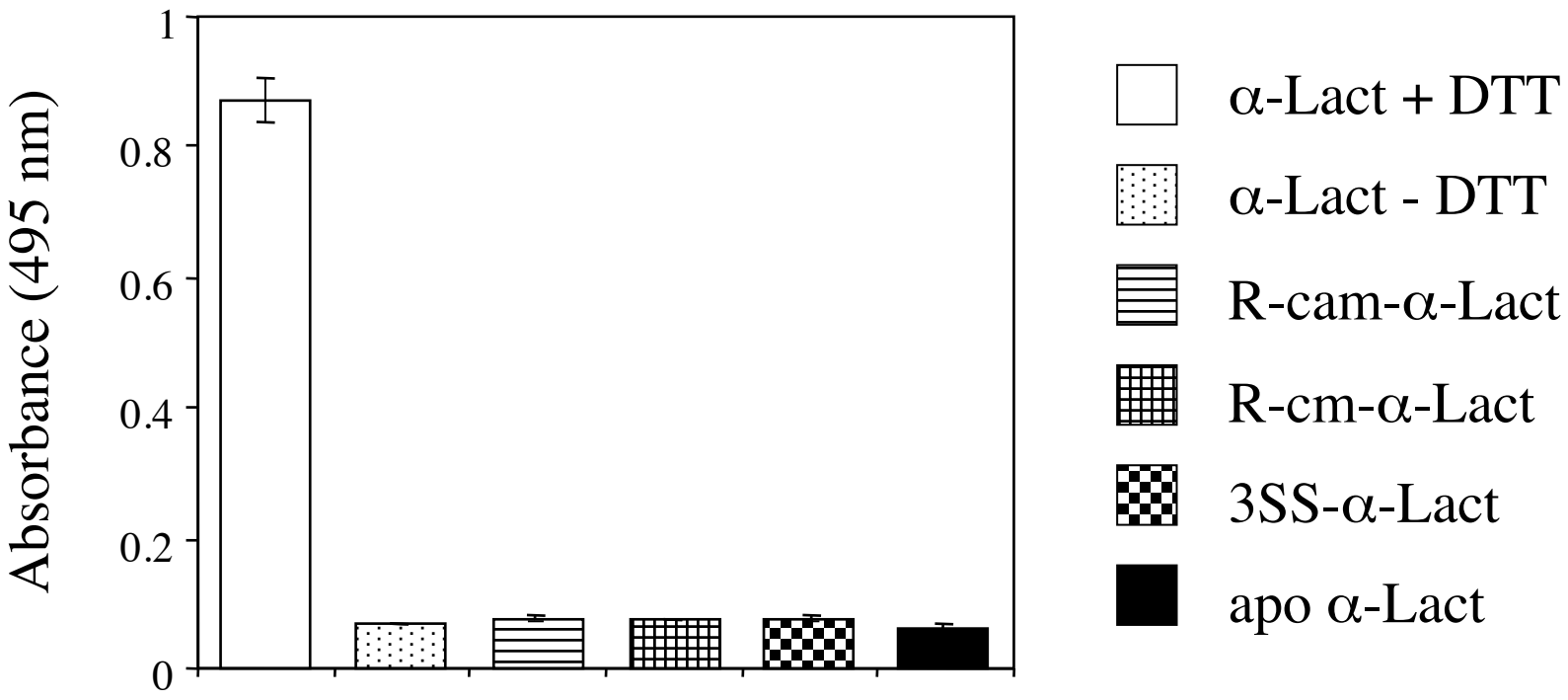
Slow precipitation: (A) γ-Crystallin (1 mg/ml in 50 mM Na_2HPO_4, pH 7.0) was heated at 60 ^{o}C in the presence (●) or absence (□) of clusterin (50 μg/ml). (B)

Lysozyme (250 μg/ml) was reduced at 42 ^{o}C with 20 mM DTT in the presence (●) or absence (□) of clusterin (400 μg/ml). The data points shown in (A) and (B) each represent the mean of triplicate measurements; the SE of the means are too small to be visible. *Rapid precipitation:* (C) 300 μg of 6 M GuHCl-denatured γ-crystallin (see Materials and Methods) was rapidly precipitated following a 10-fold dilution with 50 mM Na_2HPO_4 buffer, pH 7.0, in the presence of 15 μg of either clusterin (solid bar), BSA (shaded bar) or ovotransferrin (striped bar). (D) 100 μg of 6 M GuHCl-denatured lysozyme was rapidly precipitated following a 5-fold dilution with 50 mM Na_2HPO_4 buffer, pH 7.0, in the presence of 160 μg of either clusterin (solid bar), BSA (shaded bar) or ovotransferrin (striped bar). In (A) to (D), protein precipitation (turbidity) was measured as light scattering at 360 nm (A^{360}). In (C) and (D), only end point precipitation was measured and error bars shown are standard errors of the mean of triplicate measurements in each case; none of the proteins added had a significant effect on the extent of rapid precipitation of either γ-crystallin or lysozyme ($p>0.05$, t-test). The results shown are representative of three independent experiments.

Figure 4. A proposed mechanism for the chaperone interaction of clusterin with reduced α-lactalbumin.

I_1 and I_2 represent, respectively, more structured and less structured, partly folded intermediates of α-lactalbumin. Thus, I_1 is characterised by having most secondary structure in place and some elements of tertiary structure, whereas I_2 has some elements of secondary structure but little or no tertiary structure. Under reducing conditions, the I_2 species is long lived and is prone to entering the slow and irreversible off-folding pathway leading to aggregation and precipitation. It is this species that clusterin recognises, stabilises (probably via transient interactions with the monomeric species), and eventually complexes with, to prevent precipitation of α-lactalbumin.

Figure 1A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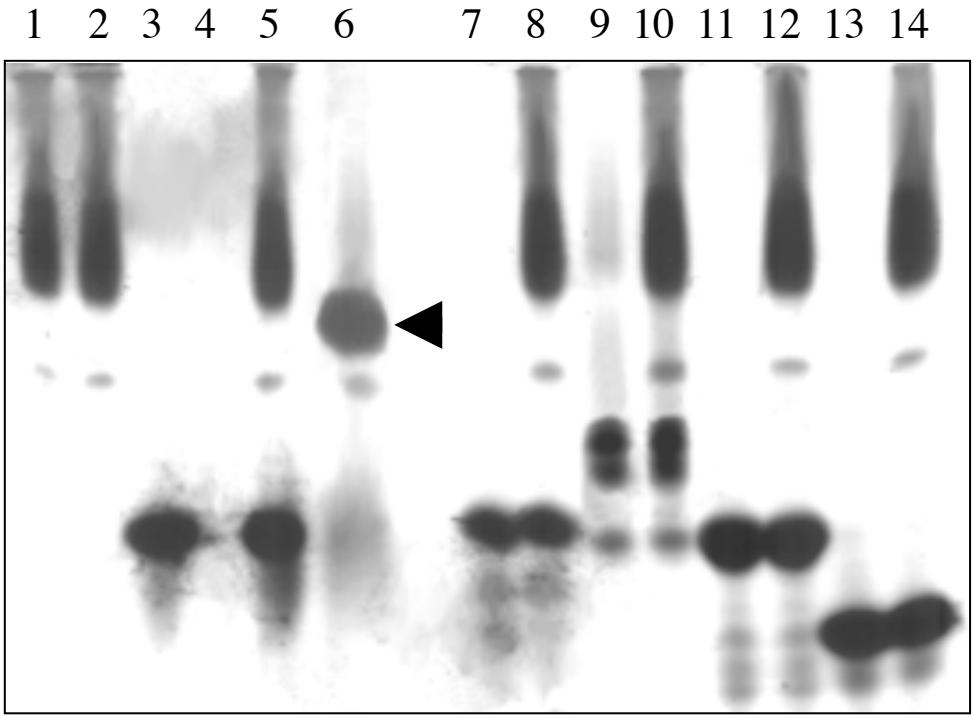
0.6
0.4
0.2
0
α-Lact + DTT
α-Lact - DTT
R-cam-α-Lact
R-cm-α-Lact
3SS-α-Lact
apo α-Lact

Figure 1B



Lane: 1 = Unstressed clusterin

2 = Reduced clusterin

3 = Unstressed α-lactalbumin

4 = Reduced α-lactalbumin

5 = Unstressed clusterin + α-lactalbumin

6 = Reduced (clusterin + α-lactalbumin) (complex)

7 = Apo α-lactalbumin

8 = Apo α-lactalbumin + clusterin

9 = CAM-α-lactalbumin

10 = CAM-α-lactalbumin + clusterin

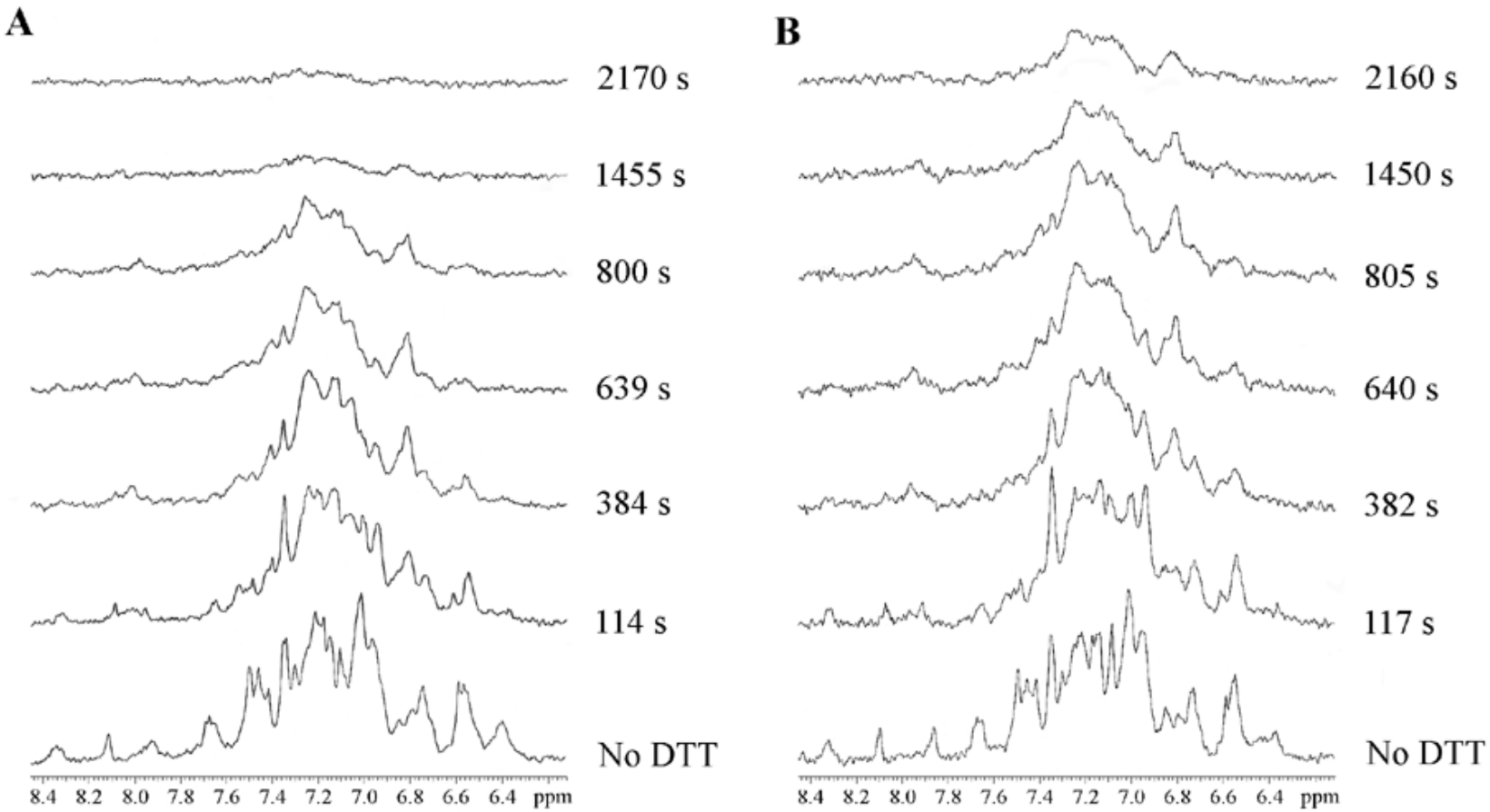
11 = CM-α-lactalbumin

12 = CM-α-lactalbumin + clusterin

13 = 3SS-α-lactalbumin

14 = 3SS-α-lactalbumin + clusterin

Figure 2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1455 s
800 s
639 s
384 s
114 s
No DTT
8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 ppm
B
2160 s
1450 s
805 s
640 s
382 s
117 s
No DTT
8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 ppm

Figure 2C

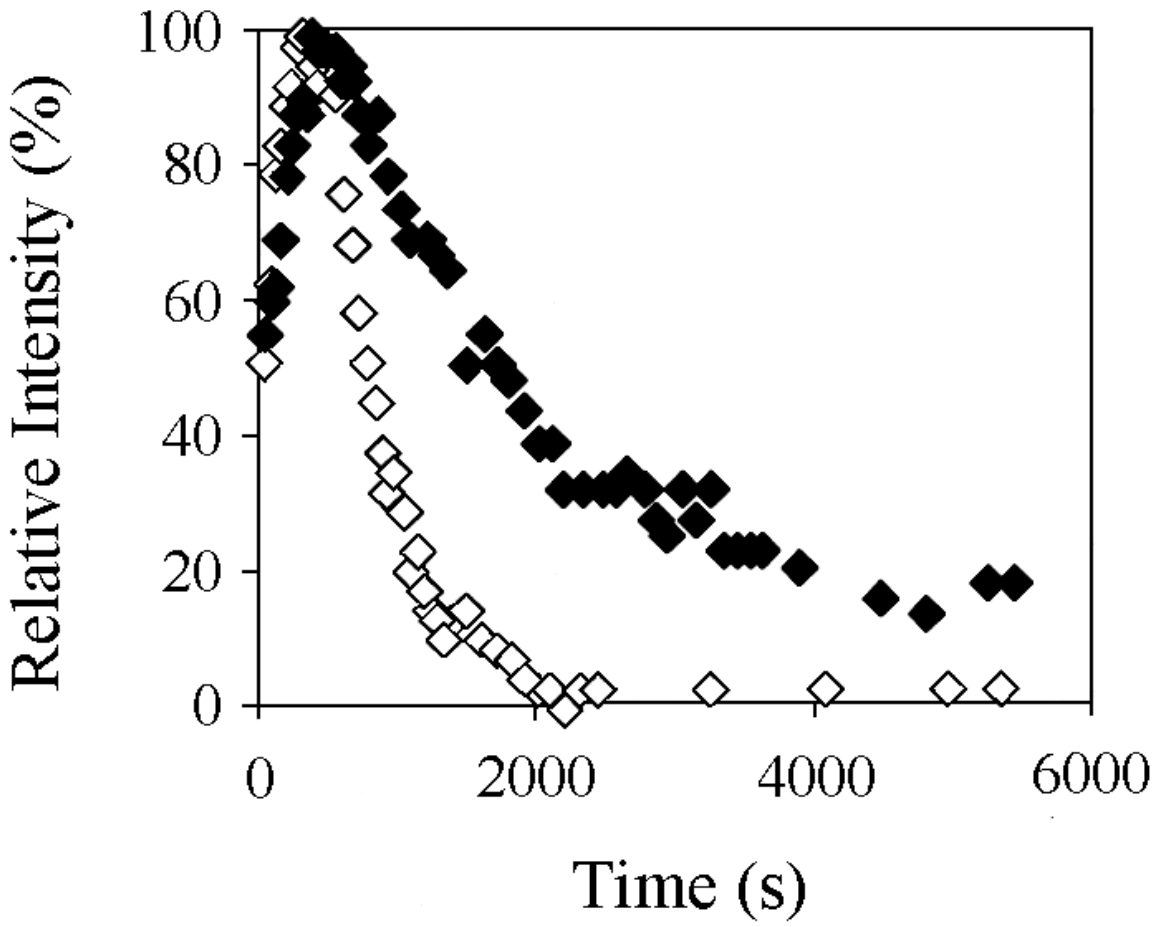
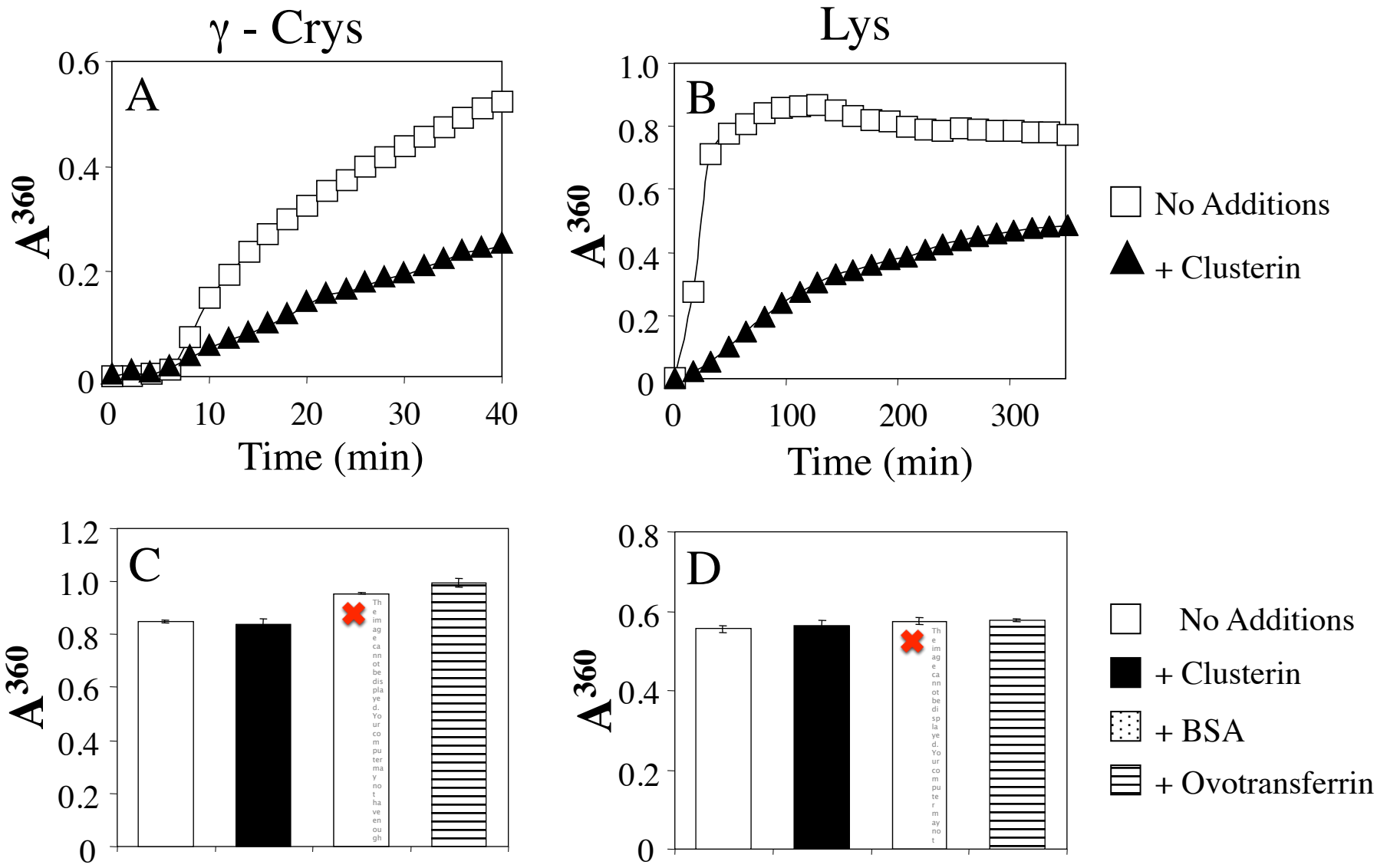


Figure 3


γ - Crys
A
0.6
0.4
0.2
0
A360
0
10
20
30
40
Time (min)
Lys
B
1.0
0.8
0.6
0.4
0.2
0
A360
0
100
200
300
Time (min)
No Additions
+ Clusterin
C
1.2
1.0
0.8
0.6
0.4
0.2
0
A360
D
0.8
0.6
0.4
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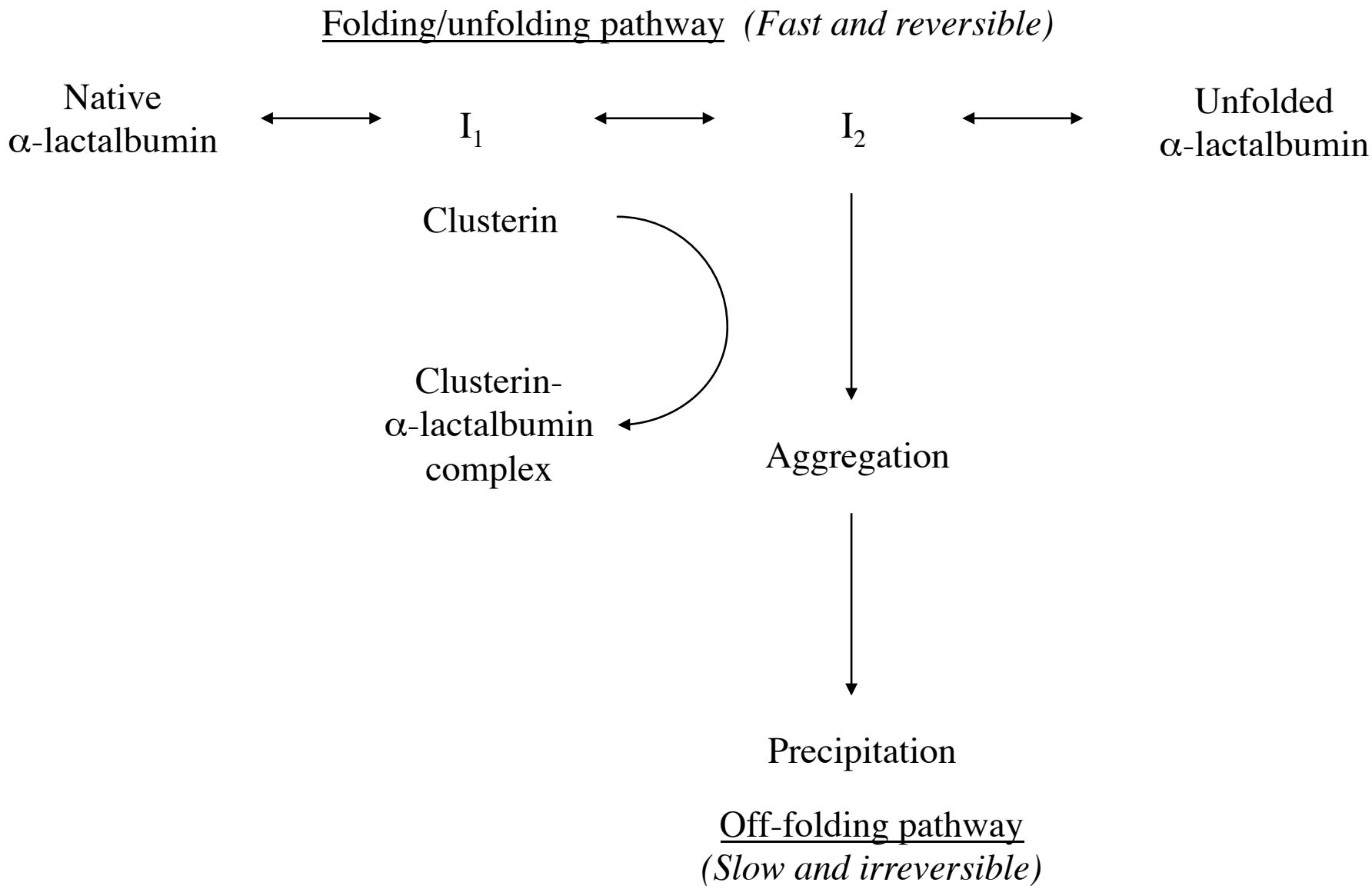
A360
No Additions
+ Clusterin
+ BSA
+ Ovotransferrin

Figure 4


Folding/unfolding pathway (Fast and reversible)
Native α-lactalbumin
I1
I2
Unfolded α-lactalbumin
Clusterin
Clusterin-α-lactalbumin complex
Aggregation
Precipitation
Off-folding pathway
(Slow and irreversible)