Clusterin Is an ATP−Independent Chaperone with Very Broad Substrate Specificity that Stabilizes Stressed Proteins in a Folding-Competent State†

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ABSTRACT: We recently reported that the ubiquitous, secreted protein clusterin has chaperone activity in vitro [Humphreys et al. (1999) *J. Biol. Chem. 274*, 6875−6881]. In this study, we demonstrate that clusterin (i) inhibits stress-induced precipitation of a very broad range of structurally divergent protein substrates, (ii) binds irreversibly via an ATP-independent mechanism to stressed proteins to form solubilized high molecular weight complexes, (iii) lacks detectable ATPase activity, (iv) when acting alone, does not effect refolding of stressed proteins in vitro, and (v) stabilizes stressed proteins in a state competent for refolding by heat shock protein 70 (HSP70). Furthermore, we show that, at physiological levels, clusterin inhibits stress-induced precipitation of proteins in undiluted human serum. Clusterin represents the first identified *secreted* mammalian chaperone. However, reports from others suggest that, at least under stress conditions, clusterin may be retained within cells to exert a protective effect. Regardless of the topological site(s) of action, the demonstration that clusterin can stabilize stressed proteins in a refolding-competent state suggests that, during stresses, the action of clusterin may inhibit rapid and irreversible protein precipitation and produce a reservoir of inactive but stabilized molecules from which other refolding chaperones can subsequently salvage functional proteins.

One of the most striking things about clusterin is the breadth of its biological distribution. In animal tissues, clusterin mRNA is near ubiquitous, with reports describing its occurrence in locales as diverse as the rat prostate gland, the velvet antler from red deer and quail neuroretinal cells. Across this broad species range clusterin maintains a remarkably high level of sequence conservation, comparisons between mammalian species typically being in the range of 70−80% (*1*). The broad distribution and high sequence conservation of clusterin suggest that it performs a function or functions of fundamental biological importance. A genuine biological function for clusterin has yet to be firmly established. Structurally, clusterin is a 75−80 kDa disulfide-linked heterodimeric protein with about 30% of the mass of the molecule comprised of N-linked carbohydrate which is complex (*2*). The protein is transcribed from a single structural gene as a full-length mRNA of about 1.6 kb, and the translated product is internally cleaved to produce the two subunits, which are held together by five disulfide bonds. Although mammalian cells generally secrete clusterin, it is apparently retained within chicken cells as an uncleaved single-chain polypeptide (*3*). Mammalian clusterin is translated with a typical hydrophobic signal peptide, 21 amino acids in length, which is proteolytically removed during translocation of the protein to the ER lumen (*2*).

There is extensive evidence of a correlation between clusterin expression and different types of disease (e.g., Alzheimer's disease, gliomas) or pathological stress (e.g., hydrostatic pressure insult or ischemic injury in the kidney) (*2*). This correlation has led in the past to repeated suggestions that clusterin is a stress-response protein. This notion has recently been strengthened by the finding that heat shock factor 1 (a transcriptional activator of heat shock protein genes) binds to a highly conserved region of the clusterin promoter, which is virtually identical to a corresponding element in many heat shock protein promoters, and upregulates clusterin gene expression (*4*). Furthermore, we recently reported that clusterin can protect proteins in vitro from either heat or reduction-induced precipitation by forming a solubilized high molecular weight complex (*5*). Taken together, these findings raise the possibility that clusterin is a novel, stress-induced heat shock protein with cytoprotective abilities.

In vivo, different heat shock proteins (HSPs) are thought to cooperate in stabilizing partially folded proteins; for example, the correct folding of proteins after their synthesis on the ribosome often requires the sequential action of HSP70 and HSP60. The combined action of the heat shock proteins probably protects cells from stress by preserving the integrity of critical proteins (*6*). A number of different chaperones known to be directly or indirectly involved in catalyzing correct protein folding have ATP binding sites and some have ATPase activity. For example, HSP70 binds in an ATP-dependent manner to unfolded proteins and HSP60 hydroly-

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ses ATP as part of a mechanism to facilitate protein folding (*7*, *8*).

Our previous work indicates that, like the small heat shock proteins (sHSPs), clusterin is a chaperone with the ability to stabilize partly unfolded stressed proteins (*5*). The sHSPs are able to stabilize a very broad range of substrate proteins against stress-induced precipitation (*9*). Previously, we showed that clusterin could stabilize four different proteins against stress-induced precipitation (*5*). In this report, we first extend our previous work to demonstrate that, like the sHSPs, clusterin has a chaperone action with extremely broad substrate specificity. Furthermore, we demonstrate that, at physiological levels, clusterin inhibits stress-induced precipitation of proteins in undiluted human serum. Although sequence analysis predicts that clusterin contains a possible nucleotide binding motif (*10*), the effects of ATP on clusterin's chaperone action are unknown. In addition, although we previously reported that clusterin was unable to protect two enzymes from heat-induced loss of activity (*5*), its ability to catalyze recovery of enzyme activity following stress is unknown. We investigated both these issues. We report that for all proteins tested, ATP had no effect on clusterin's ability to inhibit stress-induced precipitation. We demonstrate that clusterin lacks detectable ATPase activity and, when acting alone, regardless of the presence or absence of ATP, does not promote recovery of enzyme activity following heat stress. However, importantly, we show that clusterin stabilizes heat-stressed enzymes in a state competent for HSP70-mediated protein refolding, which results in recovery of enzyme activity.

MATERIALS AND METHODS

Materials. ATP, ADP, alcohol dehydrogenase (ADH), bovine serum albumin (BSA), catalase, 1-chloro-2,4-dinitrobenzene, glutathione, lactate dehydrogenase, lysozyme, nicotinamide adenine dinucleotide (NAD^+), NADH, ovotransferrin, 3-phosphoglycerate, 3-phosphoglycerate kinase, and pyruvate kinase were all obtained from Sigma (St. Louis, MO). Bovine Hsc70 (a constitutively expressed form of HSP70) was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Dithiothreitol (DTT) was obtained from Boehringer Mannheim (Sydney, Australia). *N*-(2-Hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (Hepes) was purchased from United States Biochemical (Cleveland, OH). All buffer salts, and H_2O_2, were obtained from Ajax (Sydney, Australia). Clusterin was purified from human serum by immunoaffinity chromatography as previously described (*11*). Glutathione-*S*-transferase (GST) was prepared by thrombin cleavage of GST-fusion proteins as described (*12*). Human serum was obtained from the Red Cross Blood Bank, Sydney, Australia.

Protein Precipitation Assays. In all cases, the effects of clusterin on stress-induced precipitation of target proteins was monitored by measuring the turbidity associated with target protein precipitation as absorbance at 360 nm (A^{360}). Unless otherwise indicated, this was measured using a diode array spectrophotometer (Hewlett-Packard GMBH, Germany). In one series of experiments, the extent of precipitation in the absence of added ATP was compared to that in the presence of 2 mM ATP. The conditions used to induce precipitation of purified target proteins were as follows: (i) ovotransferrin (2 mg/mL in 50 mM phosphate, 50 mM KCl, 5 mM $MgCl_2$, pH 7.0) was heated at 60 or 70 °C for up to 40 min or incubated with 20 mM DTT at 42 °C for up to 6.5 h; (ii) lysozyme (0.3 mg/mL in 10 mM phosphate, 150 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, pH 7.4) was heated at 42 °C in the presence of 20 mM DTT for up to 5 h; (iii) ADH (0.6 mg/mL in either 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 (PBS) or 50 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) was heated at 55 °C for up to 20 min; (iv) GST (0.42 mg/mL in either PBS or 50 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) was heated at 57 °C for up to 10 min; (v) catalase (0.2 mg/mL in 50 mM phosphate, 50 mM KCl, 5 mM $MgCl_2$, pH 7.0) was heated at 55 °C for up to 40 min. For GST and ADH, similar results were obtained using either PBS or 50 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4; only results obtained using PBS are shown. In a second series of experiments, unfractionated normal human serum (NHS) or aliquots of the same batch of serum depleted of clusterin by immunoaffinity chromatography [i.e., clusterin-depleted serum, CDS (*11*)] were diluted 1 in 20 in PBS and heated at 60 °C. Immunoaffinity depletion of clusterin produced negligible dilution of the total protein concentration in NHS. Prior to heating, 1 mL aliquots of diluted serum were supplemented by the addition of 50 μL of either PBS or purified clusterin or control proteins (at 2 mg/mL in PBS; to give a final concentration of 100 μg/mL). In a third series of experiments, whole NHS and CDS supplemented with 20 mM DTT (to induce protein precipitation) and, in some cases, with 100 μg/mL of clusterin or control proteins (as above), were incubated at 37 °C. These experiments were performed in 96 well trays and the turbidity (A^{360}) measured using a Spectramax 250 plate reader (Molecular Devices, Sunnyvale, CA).

Assays of Heat-Induced Loss of Enzyme Activity. Loss of GST or ADH enzyme activity was assayed by removing aliquots from samples of enzyme undergoing heat-induced precipitation as above. Prior to assay, aliquots of GST were mixed with an equal volume of ice-cold 50 mM phosphate and 1 mM EDTA, pH 7.4, while aliquots of ADH were held undiluted on ice. Precipitated protein was removed by centrifugation (30 s at 10000*g* in a benchtop microfuge) and aliquots of the supernatant added to a reaction buffer. For GST, the reaction buffer was 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene, in 50 mM phosphate, 1 mM EDTA, pH 7.4. Formation of 1-chloro-2,4-dinitrobenzene: glutathione conjugate was measured by monitoring the increase in absorbance of the solutions at 350 nm for 60 s. For ADH, the reaction buffer was 2.5 mM NAD^+, 100 mM ethanol in 50 mM TrisCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4, and formation of NADH was measured by monitoring the increase in absorbance of the solutions at 340 nm for 30 s. In control experiments (not shown), it was established that plots of absorbance vs time were linear for at least 60 s in these assays and that there was a linear relationship between the rate of change of absorbance and the amount of GST or ADH added. Loss of catalase enzyme activity was assayed by removal of aliquots from samples heated as above and immediately adding them to 0.12% (v/v) H_2O_2 in 50 mM phosphate, pH 7.0. Catalase activity was measured as a decrease in absorbance at 210 nm. In some experiments, the enzymes were heated in the presence of 100 μg/mL clusterin and/or 2 mM ATP.

Assays for Recovery of Enzyme Activity Following Heat Stress. Solutions of catalase (0.2 mg/mL) in 100 mM Na_2HPO_4, 50 mM KCl, 5 mM $MgCl_2$, pH 7.0, with or without clusterin (100 μg/mL) or lysozyme, α-lactalbumin, or myoglobin (control proteins, each at 100 μg/mL), were heated for 30 min at 55 °C. The solutions were then cooled on ice and centrifuged for 30 s at 10000*g* to remove precipitated protein. A total of 20 μL of supernatant was added to 180 μL of refolding buffer (5 mM $MgCl_2$, 50 mM KCl, 1 mg/mL BSA, 50 mM Tris, pH 7.5), with or without the addition of 35 μg/mL Hsc70 and/or 2 mM ATP, and incubated at room temperature. At various times, aliquots were taken, and their catalase activity was measured as above. The assays for recovery of ADH activity were carried out similarly, except that ADH activity was measured as above.

ATPase Assays. Production of ADP from ATP was measured using an enzyme-coupled assay in which ADP production is linked to oxidation of NADH. The reaction mixture contained 2 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 10 μM EDTA, 170 μM ATP, 840 μM phosphoenol pyruvate, 105 μM NADH, 33.4 units/mL of lactate dehydrogenase and 21.1 units/mL of pyruvate kinase (*13*). The reaction mixture (0.7 mL) was held in a quartz cuvette, maintained at 37 °C, and NADH oxidation monitored as a decrease in absorbance at 340 nm, measured as a function of time after addition of clusterin. The validity of this assay was confirmed by showing that (i) addition of exogenous ADP (21 nmol) or (ii) generation of ADP from ATP through phosphorylation of 3-phosphoglycerate (1.1 μmol added) catalyzed by 3-phosphoglycerate kinase (0.76 units added) both led to NADH oxidation (Figure 5). To test the possibility that clusterin might exhibit ATPase activity only when complexing with stressed proteins, a 180 μL aliquot of a mixture of clusterin (75 μg/mL) and ovotransferrin (1 mg/mL) that had been heated at 60 °C for 5 min was added to the ATPase reaction mixture above and the assay performed as described.

RESULTS

Clusterin's Chaperone Action Protects Ovotransferrin, Lysozyme, and ADH from Stress-Induced Precipitation. In a recent report, we showed that clusterin formed "solubilized" high molecular weight complexes with four proteins (GST, catalase, α-lactalbumin, and BSA) when they were stressed by either heat or reduction (*5*). To demonstrate that the chaperone action of clusterin has a truly general substrate specificity, we first verified that clusterin was able to inhibit stress-induced precipitation of three previously untested proteins—ovotransferrin, lysozyme, and ADH. Heating ovotransferrin (at 60 °C), lysozyme (at 42 °C in the presence of 20 mM DTT), or ADH (at 55 °C) produced extensive protein precipitation within 15, 90, and 20 min, respectively, shown by an increase in absorbance at 360 nm (Figure 1, panels A, C, and D). The addition of 20 mM DTT to solutions of ovotransferrin at 42 °C produced extensive protein precipitation within 2−3 h (Figure 1B); the small decrease in absorbance measured commencing at about 180 min corresponded to a visible settling of large molecular aggregates from solution. In contrast, clusterin did not precipitate when heated at 60 °C for 40 min or when treated with DTT at various temperatures for up to 5 h (data not shown). When coincubated with any of the proteins subjected to heat or DTT-mediated reduction, clusterin potently inhibited protein precipitation (Figure 1). For ovotransferrin, higher concentrations of clusterin were required to inhibit precipitation induced by DTT versus heat (compare Figure 1, panels A and B). Control proteins (e.g., ovalbumin) had only a small effect on the stress-induced precipitation of any of the proteins tested (data not shown).

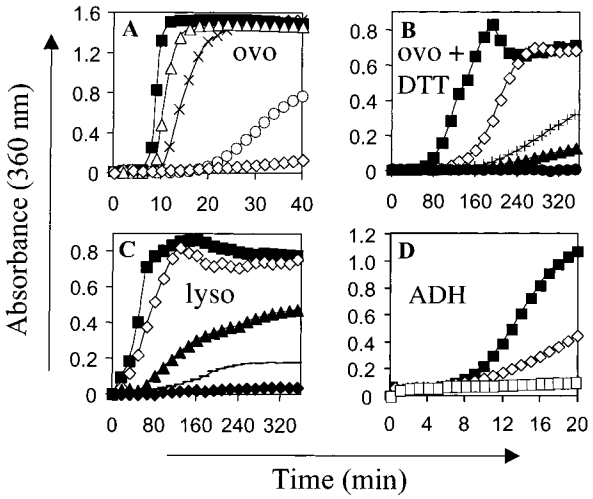

FIGURE 1: Effects of clusterin onstress-induced precipitation of ovotransferrin (ovo), lysozyme (lyso) and ADH. The turbidity associated with protein precipitation was measured as A^{360}, as described in Materials and Methods, and in each case, the results shown are representative of three independent experiments. (A) Ovotransferrin (2 mg/mL) was heated at 60 °C in the presence of different concentrations of clusterin [(■) 0 μg/mL; (△) 25 μg/mL; (×) 50 μg/mL; (○) 75 μg/mL; (◇) 100 μg/mL]. Almost identical results were obtained when ovotransferrin was heated at 70 °C (compare with Figure 2A). (B) Ovotransferrin (1 mg/mL) was heated at 42 °C with 20 mM DTT and in the presence of different concentrations of clusterin [(■) 0 μg/mL; (◇) 100 μg/mL; (+) 250 μg/mL; (▲) 400 μg/mL; (●) 550 μg/mL]. (C) Lysozyme (0.3 mg/mL) was heated at 42 °C with 20 mM DTT and in the presence of different concentrations of clusterin [(■) 0 μg/mL; (◇) 100 μg/mL; (▲) 400 μg/mL; (−) 700 μg/mL; (◆) 1000 μg/mL]. (D) ADH (0.6 mg/mL in PBS) was heated at 55 °C in the presence of different concentrations of clusterin [(■) 0 μg/mL; (◇) 100 μg/mL; (□) 200 μg/mL).

Using a similar approach to that described in ref *5*, we also verified by ELISA that clusterin bound preferentially to stressed forms of ovotransferrin, ADH, and lysozyme and used size-exclusion chromatography and native gel electrophoresis to show that clusterin binds to stress-induced conformations of ovotransferrin and lysozyme to form solubilized high molecular weight complexes (data not shown). Thus, by a mechanism similar to that previously reported for four other proteins, clusterin's chaperone action stabilizes stressed forms of ovotransferrin and lysozyme and inhibits their precipitation from solution. The same mechanism is almost certainly responsible for clusterin's ability to inhibit heat-induced precipitation of ADH.

Clusterin's Chaperone Action Inhibits Stress-Induced Precipitation of Human Serum Proteins. When heated at 60 °C, proteins contained within normal human serum (NHS), diluted 1 in 20 in PBS undergo time-dependent aggregation and precipitation (Figure 2A). We have not identified which serum proteins are responsible for the measured heat-induced precipitation. When the washed precipitate was boiled in SDS−PAGE sample buffer and loaded onto a 10% poly-

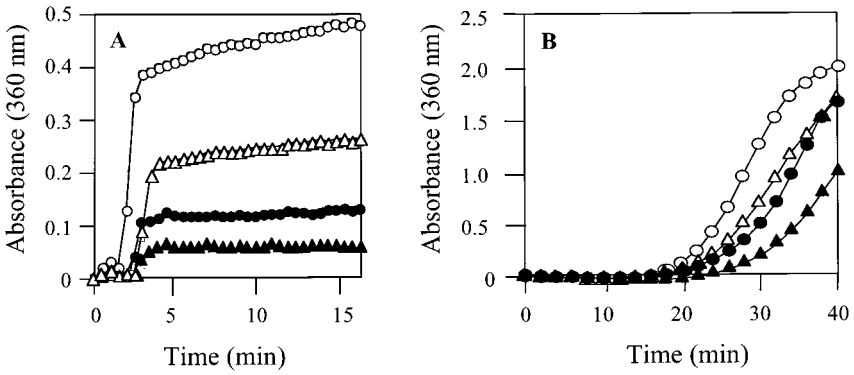

FIGURE 2: Effects of clusterin on heat-induced precipitation of human serum proteins. (A) Unfractionated normal human serum [NHS (△)] or clusterin-depleted serum [CDS (○)] was diluted 1 in 20 in PBS and heated at 60 °C. In some cases, purified clusterin (100 μg/mL) was added to diluted NHS [NHS + clusterin (▲)] or CDS [CDS + clusterin (●)] before heating. The turbidity associated with protein precipitation was measured as A^{360}, using a diode array spectrophotometer (see Materials and Methods). The data points shown are means of duplicate determinations (the mean range was 5% of the A^{360} measured in each case). (B) Samples of undiluted NHS (△) or CDS (○), supplemented with 20 mM DTT, were incubated at 37 °C in the wells of a 96-well microplate (200 μL/well). In some cases, 100 μg/mL of clusterin was also added to samples of NHS [NHS + clusterin (▲)] or CDS [CDS + clusterin (●)]. The turbidity associated with protein precipitation (at times up to 40 min, see Materials and Methods) was measured as A^{360} using a Spectramax 250 plate reader. Each data point shown represents the mean of triplicate measurements. In each case, the standard error of the mean is smaller than the symbol used to indicate the data point. In both panels A and B, the addition of 100 μg/mL of control proteins (e.g., ovalbumin) had no effect on the extent of protein precipitation measured (data not shown). For both panels A and B, the results shown are representative of several independent experiments.

acrylamide gel, the protein(s) were unable to enter the gel, suggesting that large SDS-resistant aggregates have formed (data not shown). Following immunoaffinity depletion of endogenous clusterin from serum, under the conditions tested, the extent of heat-induced protein precipitation approximately doubled (Figure 2A). This result indicates that, in 20-fold diluted human serum, endogenous levels of clusterin (present in undiluted NHS) at 50−370 μg/mL (*14*) are sufficient to significantly inhibit protein precipitation induced by heating at 60 °C. When 100 μg/mL of purified clusterin was added to clusterin-depleted serum (CDS; diluted 1 in 20 in PBS), the extent of heat-induced protein precipitation was reduced by about 75% (Figure 2A). Similarly, when 100 μg/mL of purified clusterin was added to NHS (diluted 1 in 20 in PBS), the extent of heat-induced protein precipitation was also reduced by about 75% (Figure 2A). These results indicate that, relative to the effects of endogenous clusterin and under the conditions tested, supraphysiological levels of clusterin caused greater inhibition of heat-induced protein precipitation. The addition of 100 μg/mL of control proteins (e.g., BSA, ovalbumin) had no significant effects on the extent of heat-induced protein precipitation (data not shown).

The very high level of protein precipitation induced in undiluted human serum by heating at 60 °C is such that it is not possible to measure its progress in real time by spectrophotometry. In contrast, heating undiluted serum at 42 °C (near physiological levels) induced little precipitation (data not shown). To induce a level of protein precipitation that could be measured in real time by spectrophotometry, we added 20 mM DTT to undiluted serum and incubated it at 37 °C. Between 25 and 40 min after the addition of DTT, the extent of protein precipitation in CDS was clearly greater than that measured in NHS (Figure 2B). The addition of 100 μg/mL clusterin to CDS reduced the extent of DTT-induced precipitation during this period to slightly less than that measured for NHS (Figure 2B). Furthermore, in this period, the prior addition of 100 μg/mL clusterin to NHS also caused a significant decrease in the extent of DTT-induced protein precipitation (Figure 2B). At times greater than 40 min after the addition of DTT, the type and extent of protein precipitation in each sample was such that an opalescent gel formed in the sample wells. The exact time at which this occurred varied between samples—CDS samples were observed to undergo this change most rapidly. Once this had taken place, the plate reader was incapable of measuring any further changes in turbidity in the affected wells. Therefore, in these experiments, measurements of turbidity (ie A^{360}) were confined to the period 0−40 min after the addition of DTT. The addition of 100 μg/mL of control proteins (e.g., ovalbumin) had no significant effects on the extent of DTT-induced protein precipitation (data not shown). By removing aliquots from samples of undiluted NHS and CDS undergoing heating at 60 °C, and measuring the turbidity (A^{360}) of these aliquots after dilution, we obtained results similar to those shown for DTT-induced protein precipitation (data not shown). Collectively, these results demonstrate that (i) clusterin inhibits heat- and reduction-induced precipitation of human serum protein(s), and (ii) in undiluted human serum, normal endogenous levels of clusterin are sufficient to partially inhibit protein precipitation induced either by heating at 60 °C or by incubation with 20 mM DTT.

ATP Has No Effect on the Ability of Clusterin To Inhibit Stress-Induced Protein Precipitation. The effects of ATP on the ability of clusterin to inhibit stress-induced precipitation of ovotransferrin, lysozyme, catalase, GST, and ADH are shown in panels A−E of Figure 3, respectively. The addition of ATP to any of the proteins tested had little effect on the extent of stress-induced protein precipitation, either in the presence or absence of clusterin (Figure 3). In the cases of ovotransferrin, lysozyme, catalase, and ADH, the addition of ATP slightly reduced the extent of precipitation, however, the same relative level of inhibition resulted whether clusterin was present or absent (Figure 3, panels A, B, C, and E). Therefore, for all five proteins tested, ATP had no effect on the ability of clusterin to inhibit stress-induced protein precipitation.

Clusterin Does Not Protect Enzymes Against Stress-Induced Loss of Activity or Independently Promote Protein Refolding. We previously showed that clusterin was unable to prevent a large loss of enzyme activity when catalase or

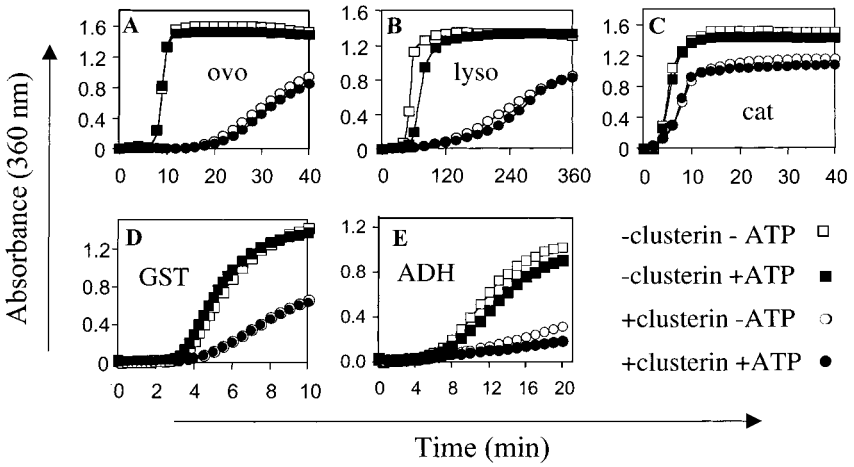

FIGURE 3: Effects of ATP on the inhibition of stress-induced protein precipitation by clusterin. Stress-induced precipitation of the target proteins only (□), target proteins in the presence of 2 mM ATP (■), target proteins in the presence of clusterin (○) and target proteins in the presence of both clusterin and 2 mM ATP (●), was monitored as A^{360} as described in Materials and Methods. In each case the results shown are representative of three independent experiments. (A) Ovotransferrin (ovo) (1 mg/mL) or mixtures of ovotransferrin (1 mg/mL) and clusterin (75 μg/mL) were heated at 70 °C. (B) Lysozyme (lyso) (0.25 mg/mL) or mixtures of lysozyme (0.25 mg/mL) and clusterin (400 μg/mL) were heated at 42 °C with 20 mM DTT. (C) Catalase (cat) (1 mg/mL) or mixtures of catalase (1 mg/mL) and clusterin (100 μg/mL) were heated at 60 °C. (D) GST (0.42 mg/mL) or mixtures of GST (0.42 mg/mL) and clusterin (100 μg/mL) were heated at 57 °C. (E) ADH (0.6 mg/mL) or mixtures of ADH (0.6 mg/mL) and clusterin (100 μg/mL) were heated at 55 °C.

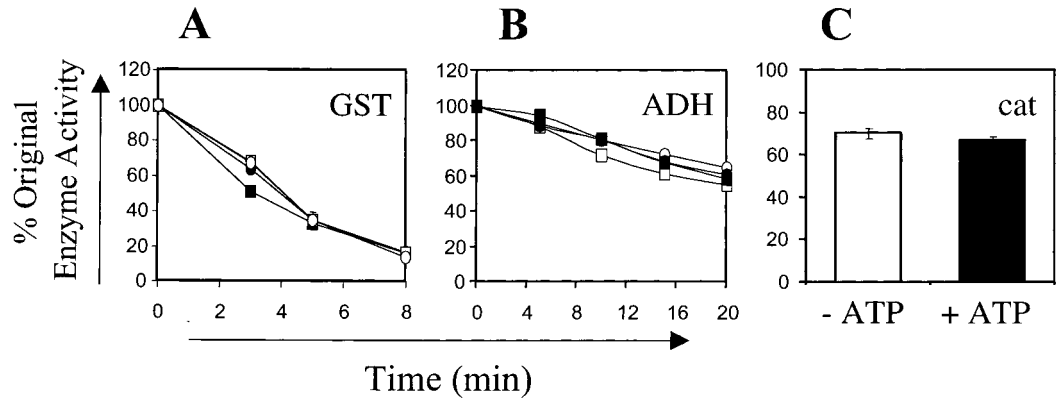

FIGURE 4: Effects of clusterin and ATP on loss of enzyme activity during heat stress. (A) GST (0.42 mg/mL) in the presence (●) or absence (○) of 2 mM ATP or mixtures of GST (0.42 mg/mL) and clusterin (100 μg/mL) in the presence (■) or absence (□) of 2 mM ATP were heated at 57 °C. At the indicated times, aliquots were removed and assayed for GST activity as described in Materials and Methods. The data (shown as percentages of the initial GST activity) are means ± ranges of duplicate measurements; in many cases the error bars are too small to be visible. (B) ADH (0.6 mg/mL) in the presence (●) or absence (○) of 2 mM ATP or mixtures of ADH (0.6 mg/mL) and clusterin (100 μg/mL) in the presence (■) or absence (□) of 2 mM ATP were heated at 55 °C. At the indicated times, aliquots were removed and assayed for ADH activity as described in Materials and Methods. The data (shown as percentages of the initial ADH activity) are means ± ranges of duplicate measurements; in many cases the error bars are too small to be visible. (C) Mixtures of catalase (200 μg/mL) and clusterin (100 μg/mL) were heated at 55 °C for 30 min in the absence (empty bar) or presence (solid bar) of 2 mM ATP and then assayed for catalase activity as described in Materials and Methods. The data (shown as percentages of the initial catalase activity) represent means of triplicate measurements, error bars represent standard deviations (SD) of the means. In all cases, the results are representative of at least two independent experiments.

GST were exposed to 50−55 °C for 30 min (*5*). However, our previous data, based on measurements of enzyme activities before and after heat stress, did not assess the possibility that clusterin might reduce the rate at which enzyme activity is lost during heat stress nor did they evaluate the effects of ATP on the ability of clusterin to protect enzymes from stress. We addressed these issues by carrying out a series of experiments, the results from which are summarized in Figure 4. Clusterin, with or without added ATP, had no effect on the rates of heat-induced loss of GST or ADH activity (Figure 4, panels A and B). After 20 min of heating, about 60% of ADH enzyme activity remained (Figure 4B), even though, under these conditions, formation of insoluble aggregates of ADH (measured as an increase in A^{360}) had reached a maximum (see Figure 3E). Measurements of A^{280} after removal of these aggregates by centrifugation showed that a similar proportion (about 60%) of ADH protein remained in solution (data not shown). Consistent with the results obtained for GST and ADH, ATP had no effect on the inability of clusterin to protect catalase from heat-induced loss of activity (Figure 4C). Collectively, these results indicate that, irrespective of the presence of ATP, clusterin is unable to protect enzymes from heat-induced loss of activity.

Clusterin Lacks ATPase Activity. Purified clusterin had no detectable ATPase activity either when tested alone or during heat-induced association with ovotransferrin (Figure 5). These assays were validated by showing that they could detect ADP, either added exogenously or generated from ATP-dependent phosphorylation of 3-phosphoglycerate cata-

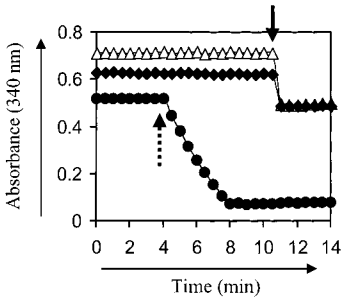

FIGURE 5: Results of spectrophotometric assays demonstrating that clusterin lacks detectable ATPase activity. The absorbance of ATPase assay buffer (see Materials and Methods) was measured as a function of time after the addition of either 195 μg/mL clusterin (◆) or a mixture of clusterin and ovotransferrin (previously heated at 60 °C for 5 min) (△) to give a final concentration of 15.3 μg/mL clusterin and 0.2 mg/mL ovotransferrin. In both experiments, 21 nmol of ADP was added to the solution at 11 min (solid arrow). In similar experiments, purified ovotransferrin alone was added to the ATPase assay buffer. No ATPase activity was measured associated with ovotransferrin (data not shown). Shown on the same plot, the absorbance of ATPase assay buffer (containing 84 μM NADH) was measured as a function of time after the addition of 0.76 units of 3-phosphoglycerate kinase (●); at 4 min, 1.1 μmol of 3-phosphoglycerate was added (dotted arrow). The data shown are representative of three independent experiments.

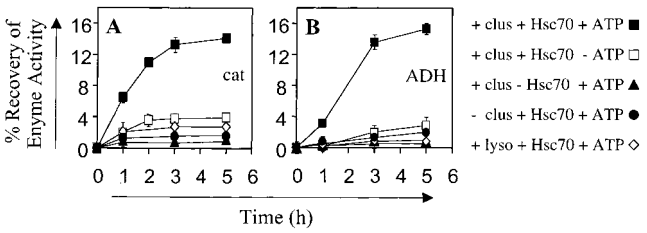

FIGURE 6: Recovery of the enzyme activities of ADH and catalase (cat) following heat-induced inactivation. Samples of catalase (A) and ADH (B) were inactivated by heating at 55 °C for 10 min in the presence or absence of clusterin (clus; 100 μg/mL) or lysozyme (lyso; 100 μg/mL). Aliquots, cleared of precipitated protein by centrifugation, were then added to refolding buffer, with or without the addition of Hsc70 (35 μg/mL) or 2 mM ATP (see key) and recovery of enzyme activity measured spectrophotometrically (see Materials and Methods for details). The recovery of enzyme activity is expressed as a percentage of the original enzyme activity (prior to heat stress). Immediately following heat stress, approximately 66% of the original catalase activity remained, the corresponding proportion for ADH was 82 %. The data are means ± standard deviations of triplicate measurements (in some cases the error bars are too small to be visible) and are representative of three independent experiments. In similar experiments, when α-lactalbumin or myoglobin were substituted for clusterin, results similar to those obtained with lysozyme were recorded (data not shown).

lyzed by phosphoglycerate kinase (Figure 5).

Clusterin Stabilizes Stressed Proteins in a State Competent for Refolding. When acting alone, clusterin was unable to promote the recovery of ADH or catalase activity following heat stress (Figure 6). We reasoned that, like the sHSPs (*15*, *16*), clusterin might be able to stabilize stressed proteins in a form suitable for subsequent refolding mediated by other chaperones. We hypothesize that clusterin exerts its chaperone action extracellularly, although an intracellular function remains possible (see Discussion). Currently, clusterin is the only identified mammalian chaperone found in the extracellular space. Thus, it was not possible to test known extracellular chaperones for their ability to refold stressed proteins stabilized by clusterin. Despite this, we reasoned that if clusterin could stabilize stressed proteins in a refolding-competent state, then it was feasible that other chaperones known to have refolding activity, even if from a different topological location, might be able to refold stressed proteins complexed with clusterin to regain protein function. We tested this hypothesis using the intracellular chaperone Hsc70.

When stressed enzyme mixtures containing clusterin were added to a buffer supplemented with ATP and Hsc70, a gradual recovery of the original enzyme activity (immediately following heat stress) was measured over a period of 5 h. For catalase, the recovery was, approximately, from an initial value of 66% to a final value of 80% of the original activity (Figure 6A). The corresponding recovery for ADH was, approximately, from an initial value of 82% to a final value of 98% of the original activity (Figure 6B). This level of recovery was not detected when enzymes were heated in the absence of clusterin, or when stressed enzyme mixtures containing clusterin were assayed in the absence of Hsc70 or ATP (Figure 6). For catalase, under the conditions used, there was about a 4% recovery of enzyme activity when the enzyme was heated with clusterin and subsequently incubated with Hsc70 in the absence of ATP (Figure 6A). In similar experiments, when lysozyme was substituted for clusterin, and the heated enzyme mixture subsequently incubated with Hsc70 and ATP, only about a 3% recovery of enzyme activity was measured (Figure 6A). A similar pattern of dependence for enzyme reactivation was seen with ADH, except the levels of reactivation in the absence of ATP or clusterin were lower (Figure 6B).

These results indicate that clusterin binds to stressed ADH and catalase to form stable complexes that preserve the incorporated enzymes in a state competent for subsequent ATP-dependent refolding by Hsc70. The level of recovery of enzyme activity obtained in these assays is very similar to that reported for recovery of citrate synthase activity following heating with the small heat shock protein HSP25 and subsequent incubation with HSP70 [about 14% recovery (*16*)]. This effect appears to be specific for clusterin because, in similar experiments, lysozyme, α-lactalbumin or myoglobin were unable to support the high level of enzyme reactivation achieved by clusterin (data not shown).

DISCUSSION

Results presented here demonstrate that clusterin inhibits stress-induced precipitation of ovotransferrin, lysozyme, and ADH (Figure 1). At physiological pH, clusterin exists as an equilibrium mixture of monomers, dimers, and higher aggregation states (*17*). As previously reported, DTT induces partial dissociation of large clusterin aggregates to form monomers (80 kDa species) or lower order clusterin oligomers but does not appear to cause substantial dissociation of the α and β chains (*5*). Therefore, the greater efficiency with which clusterin inhibited heat-versus DTT-mediated precipitation of ovotransferrin may result from effects of a reducing environment on the structure and/or aggregation state of clusterin (which has five interchain disulfide bonds) and/or from different effects of the two stresses on the structure of ovotransferrin. Table 1 summarizes the physical characteristics, and tested stress conditions for purified proteins that we have identified serve as substrates for

Table 1: Data for the Various Protein Substrates for Which Clusterin Has Been Shown To Inhibit Stress-Induced Precipitation[a]

protein	cellular location	protein mass (kDa)	no. of chains	polypeptide mass(es) (kDa)	disulfide bonds	secondary structural features	stress tested
ADH	intracellular	147	4	36.7	no	α-helix, β-sheet	heat
BSA	extracellular	66.4	1	66.4	yes	α-helix, β-sheet	reduction[b]
catalase	intracellular	230	4	57.6	yes	α-helix, β-sheet	heat[b]
GST	intracellular	51	2	25.5	no	α-helix, β-sheet	heat[b]
IgG	extracellular	150	4	25, 50	yes	β-sheet	heat[c]
insulin	extracellular	5.8	1	5.8	yes	α-helix	reduction[d]
α-lactalbumin	extracellular	14.1	1	14.1	yes	α-helix, β-sheet	reduction[b]
lysozyme	extracellular	14.3	1	14.3	yes	α-helix, β-sheet	reduction (at 42 °C)
ovotransferrin	extracellular	75.8	1	75.8	yes	α-helix, β-sheet	heat, reduction (at 42 °C)

[a] Some of the data shown are taken from information available on the SwissProt database. The molecular masses shown do not include contributions from glycosylation. [b] From ref *5*. [c] D. Humphreys (unpublished results). [d] S. Easterbrook-Smith (unpublished results).

clusterin's chaperone action. These substrates include examples (i) found in the intra- and extracellular environments, (ii) ranging in mass from 5.8 to 230 kDa, (iii) comprised of 1−4 subunits, and (iv) having a variety of secondary structural features, including disulfide bonds, α-helix and β-pleated sheets (Table 1). Thus, clusterin has a chaperone action with a very broad specificity and is able to inhibit the stress-induced precipitation of many different proteins with divergent structural features.

As a step toward investigating the role of clusterin's chaperone action in a physiological context, we showed that heat- and reduction-induced precipitation of proteins in human serum was (i) enhanced by immunoaffinity depletion of clusterin from serum and (ii) specifically inhibited by the addition of purified clusterin (Figure 2 and data not shown). Endogenous clusterin was unable to completely inhibit protein precipitation induced in undiluted human serum by incubation with 20 mM DTT (Figure 2B) or by heating at 60 °C (data not shown). However, these stresses are more extreme than any likely to be encountered in vivo. Therefore, our demonstration that endogenous clusterin in normal human serum partially inhibits experimental stress-induced protein precipitation in this fluid may have important medical implications. Alzheimer's, Creutzfeldt-Jacob and Parkinson's diseases are associated with abnormally high levels of protein precipitation (*18*). In cases such as these, our results suggest the possibility that the levels of clusterin in biological fluids such as plasma may affect the rate or extent of disease progression.

Although we previously reported some similarities in the chaperone actions of clusterin and the sHSPs (*5*), this does not imply that the mechanism of clusterin's chaperone action is similar in all respects to that of the sHSPs. We were intrigued by the facts that (i) sequence analysis predicts that clusterin contains a possible nucleotide binding motif (*10*), and (ii) many other chaperones have ATP-binding sites and/or ATPase activity which are required for protein refolding activity (*7*). Therefore, we investigated whether clusterin can promote refolding of enzymes following heat stress (measured as recovery of enzyme activity) and whether ATP has any effects on this or other aspects of clusterin's chaperone action.

Taken together, our results (Figures 3−6) indicate that ATP does not play a direct role in the in vitro chaperone action of clusterin. However, these results do not exclude the possibility that clusterin may interact with ATP-dependent chaperones in vivo. These in vitro studies also indicate that clusterin cannot itself promote refolding of stressed enzymes to recover lost enzyme activity. The fact that we have demonstrated stable associations between clusterin and stressed proteins by ELISA, size-exclusion chromatography and native gel electrophoresis (ref *5* and data not shown) suggests that the in vitro binding of clusterin to stressed proteins is of high affinity. Thus, the available evidence suggests that, in vitro, clusterin potently inhibits stress-induced protein aggregation by binding with high affinity (via an ATP-independent mechanism) to partly unfolded proteins to form high molecular weight solubilized complexes but does not itself effect protein refolding. In all these respects, the chaperone action of clusterin appears similar to that of the sHSPs.

Many sHSPs are produced only under stress conditions and they play a major role in vivo by stabilizing partly unfolded proteins during times of stress to prevent their aggregation and precipitation. Furthermore, it was recently reported that HSP18.1 (a sHSP from pea) can maintain stressed proteins in a state that is competent for subsequent refolding mediated by other chaperones (*15*). Similarly, it was shown that mammalian HSP25 binds to heat-stressed citrate synthase to form a stabilized complex that can be subsequently reactivated by HSP70-mediated, ATP-dependent refolding (*16*). These data suggest that, following stress, sHSP-stabilized complexes provide a reservoir from which other chaperones facilitate refolding of stress-inactivated proteins (*9*, *15*). Our demonstration that stress-induced formation of clusterin-ADH and clusterin-catalase complexes preserves the enzymes in a state competent for subsequent ATP-dependent refolding by Hsc70 (Figure 6) indicates another important property shared with the sHSPs, which may have an important role in vivo (see below). The clusterin/Hsc70 system was efficient, demonstrating substantial recovery of the enzyme activity lost during heat stress (Figure 6). This level of efficiency was equal to or greater than that reported for comparable models incorporating sHSPs (*15*, *16*).

If clusterin is a sHSP-like chaperone, in a physiological context, what is its site(s) of action and the purpose of its activity? Studies of clusterin biosynthesis in HepG2 (*19*) and MDCK (*20*) cell lines have shown that mammalian clusterin is generally secreted from the cell. However, it was reported that treatment of HepG2 and CCL64 cells with TGFβ induces translocation of a truncated form of clusterin to the nucleus (*21*) and that clusterin may interact within cells with a nuclear-localized DNA-binding protein known as Ku70 (*22*, *23*). Furthermore, it was recently reported that clusterin is normally intracellular in chickens (*3*). Therefore, at this stage

it is not possible to exclude an intracellular function for clusterin during cell stress. Weighed against these considerations is the fact that clusterin is constitutively secreted to accumulate at high levels in mammalian body fluids, where it presumably performs one or more functions. Thus, it is likely that clusterin plays a physiological role in (at least) the extracellular environment.

There is abundant evidence that clusterin expression is increased during cellular stresses (*24*). Previous studies have reported that overexpression of clusterin protects cells from TNFα (*25*, *26*) and that, relative to control cells, clusterin anti-sense transfectants have reduced cellular resistance to TNFα (*25*), heat shock, and oxidative stress (*27*). Furthermore, it has been reported that purified clusterin added to medium containing cultured cells protects the cells from TNFα (*28*) and from oxidative stress (*29*). In addition, in preliminary experiments, we have found that when clusterin is added at 100 μg/mL (a physiologically relevant concentration) to the culture medium of human cell lines it specifically increased cell survival following physiological-level heat stress (42 °C for 44 h; S. Poon, unpublished results). Thus, multiple lines of evidence indicate that clusterin expression is increased during cellular stresses and that clusterin exerts cytoprotective effects.

The chaperone action of clusterin could be cytoprotective in either or both the intra- or extracellular environments. If clusterin exerts an intracellular chaperone action under stress conditions, it clearly could cooperate with ATP-dependent chaperones such as Hsc70 to facilitate stabilization and refolding of stressed proteins. This would provide a mechanism of cytoprotection similar to, and operating in parallel with, that proposed for the sHSPs (*9*, *15*). There are a variety of potential mechanisms by which an extracellular chaperone action of clusterin could be cytoprotective. As one example, it is possible that extracellular clusterin might bind to and prevent aggregation of partly unfolded proteins (such as receptors) on the surface of stressed cells. This action could promote cell survival by minimizing stress-induced aberrant signaling. Furthermore, we could speculate that, in vivo, clusterin-stabilized stressed cell surface proteins may be refolded to recover normal function by the action of as yet unidentified ATP-dependent extracellular chaperones. In this context, it is relevant to note that oxidative and osmotic stresses are known to induce release of ATP from cells to the extracellular space (*30*, *31*). Thus, it is feasible that stresses might induce sufficient accumulation of ATP in local microenvironments close to stressed cell membranes to facilitate ATP-dependent refolding of stressed proteins. However, further investigations are required to clarify the biological site(s) at which the chaperone action of clusterin is physiologically active and to elucidate the molecular mechanism(s) by which clusterin protects cells from stresses.

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