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Mildly Acidic pH Activates the Extracellular Molecular Chaperone Clusterin*

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Many features of the chaperone action of clusterin are similar to those of the intracellular small heat shock proteins (sHSPs) that, like clusterin, exist in solution as heterogeneous aggregates. Increased temperature induces dissociation of some sHSP aggregates and an enhanced chaperone action, suggesting that a dissociated form is the active chaperone species. We recently reported that clusterin aggregates dissociate at mildly acidic pH. To further explore the similarities between clusterin and the sHSPs, we tested the effects of temperature and pH on the structure of clusterin and its chaperone action. Our results demonstrate that increased temperature does not induce dissociation of clusterin aggregates, or other major structural changes, and has little effect on its chaperone action. However, we show that the chaperone action of clusterin is enhanced at mildly acidic pH. Clusterin is the first chaperone shown to be activated by reduced pH. This unique mode of activation appears to result from an increase in regions of solvent-exposed hydrophobicity, which is independent of any major changes in secondary or tertiary structure. We propose a model in which low pH-induced dissociation of clusterin aggregates increases the abundance of the heterodimeric chaperone-active species, which has greater hydrophobicity exposed to solution.

Clusterin is a heterodimer of disulfide-linked polypeptide chains that bear *N*-linked carbohydrates comprising about 30% of the total mass of the molecule. Although it migrates at about 70–80 kDa on SDS-PAGE, mass spectrometry analyses have shown that the human serum form of clusterin comprises two major glycoforms, one of 58.5 kDa and the other of 63.5 kDa (1). Clusterin is normally secreted from mammalian cells and is both widely distributed and highly conserved; between different mammals, about 70–80% of the amino acid sequence of clusterin is identical (2). Clusterin expression is increased during pathological stresses (*e.g.* hydrostatic pressure insult or ischemic injury in the kidney) and certain disease states (*e.g.* gliomas) (3). A variety of independent studies have suggested that clusterin protects cells from stresses such as tumor necrosis factor α, heat, and oxidative stress (2). Furthermore, recent studies of clusterin knock-out mice have suggested that clusterin protects mice from (i) the pathological consequences of inflammation associated with experimentally induced autoimmune myocarditis (4) and ischemia (5), although the latter claim has been disputed (6), and (ii) age-dependent deposition of antibody-containing aggregates in the kidney (7). The clusterin promoter contains a highly conserved 14-bp element, which is recognized by the transcriptional regulator heat shock factor 1 (8). Heat shock factor 1 activates expression of heat shock proteins (which protect cells from stresses) and clusterin (8). An emerging theme is that clusterin is a protective molecule that is up-regulated during times of physiological stress.

We recently demonstrated that clusterin is a molecular chaperone that potently inhibits the stress-induced precipitation of many different proteins *in vitro* (9, 10). When present at physiological concentrations in undiluted human serum, clusterin is able to inhibit protein precipitation induced by heat or reduction (10). Thus, the chaperone action of clusterin might be physiologically "protective" by reducing the rate or extent of progression of diseases associated with abnormally high levels of protein precipitation. The recent demonstration that aging clusterin knock-out mice develop insoluble protein deposits containing antibody (7) suggests that clusterin may inhibit antibody aggregation and precipitation *in vivo*. Furthermore, by complexing with partly unfolded proteins or other hydrophobic molecules at sites of inflammation, clusterin might solubilize these molecules and facilitate their recycling or disposal, in either case contributing toward resolution of inflammation.

Many features of the chaperone action of clusterin are similar to that of the intracellular small heat shock proteins (sHSPs).[1] For example, both types of chaperone interact specifically with stressed proteins that are slowly aggregating on the off-folding pathway toward a precipitated state (11) to form stable, solubilized high molecular weight complexes (2, 9). They do not themselves refold stressed proteins but, by binding to them, create a refolding-competent reservoir from which other ATP-dependent chaperones may retrieve functional proteins (10). The quaternary structure and chaperone action of at least some of the sHSPs is affected by increased temperature. For

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[1] The abbreviations used are: sHSP, small heat shock protein; bisANS, 4,4′-bis(1-anilino-8-naphthalene sulfonate); BSA, bovine serum albumin; OPD, *o*-phenylenediamine dihydrochloride; DTT, dithiothreitol; GST, glutathione *S*-transferase; MES, 4-morpholineethanesulfonic acid; MRE, mean residue ellipticity; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

example, elevated temperature induces an increased rate of subunit exchange in mammalian αA-crystallin and a concomitant enhancement of its chaperone action (12). Similarly, at low to physiological temperatures, yeast HSP26 exists in solution as oligomers. However, elevated temperature induces partial dissociation of these oligomers and an enhancement of the chaperone activity of the protein, implying that a non-aggregated, possibly dimeric form of HSP26 is responsible for its chaperone action (13).

The heterodimer form of clusterin is known to reversibly aggregate; this process is pH-dependent with dissociation of the aggregates being favored at mildly acidic pH (14). This transition is associated with increased binding of clusterin to its native ligands, heparin (15), immunoglobulins, glutathione *S*-transferase, and polymeric C9 (14). However, these studies did not show whether low pH induces any changes in the chaperone activity of clusterin concomitant with dissociation of its aggregated forms nor whether the aggregation state and chaperone activities of clusterin were temperature-dependent. Therefore, we tested whether elevated temperature had any effects on the aggregation state or chaperone action of clusterin. In light of our recent results (14, 15), we also tested whether low pH affected the chaperone action of clusterin *in vitro*. In addition, we used circular dichroism spectroscopy to investigate structural changes induced in clusterin by changes in temperature and pH and binding studies with 4,4′-bis(1-anilino-8-naphthalene sulfonate) (bisANS) to test whether hydrophobic interactions were important in the chaperone action of clusterin. The results demonstrate that, unlike the sHSPs, increased temperature does not induce dissociation of aggregated forms of clusterin or major changes in its secondary or tertiary structure and gave only a minor increase in the efficiency of its chaperone action. In contrast, the results show that (i) mildly acidic pH enhances the ability of clusterin to bind to stressed proteins and to inhibit their precipitation, (ii) this transition is accomplished with little measurable changes in the secondary or tertiary structure of clusterin but is accompanied by an increase in regions of solvent-exposed clusterin hydrophobicity, and (iii) the exposed regions of hydrophobicity on clusterin probably (at least partly) mediate its interactions with stressed proteins.

EXPERIMENTAL PROCEDURES

Materials

Alcohol dehydrogenase, α-lactalbumin, 4,4′-bis(1-anilino-8-naphthalene sulfonate) (bisANS), bovine serum albumin (BSA), catalase, casein, insulin, iodoacetamide, *o*-phenylenediamine dihydrochloride (OPD), and ovotransferrin were all obtained from Sigma. Dithiothreitol (DTT) was obtained from Roche Molecular Biochemicals (Sydney, Australia). All buffer salts and H_2O_2 were obtained from Ajax Chemical Co. (Sydney, Australia). Clusterin was purified from human serum (obtained from the Red Cross Blood Bank, Sydney, Australia) by immunoaffinity chromatography as previously described (16). Glutathione *S*-transferase (GST) was prepared by thrombin cleavage of GST fusion proteins as described (17). α-Crystallin was purified from bovine lenses as previously described (18).

Circular Dichroism Analyses

A Jasco J-720 spectropolarimeter, linked to a Neslab RTE-111 cooling system, was used to acquire circular dichroism data. Far-UV (195–260 nm) CD studies were performed using a 1-mm cell with clusterin at 200 μg/ml in 10 mM sodium phosphate, pH 7.4. Spectra were acquired at 50-millidegree sensitivity with a step resolution of 0.5 nm and a bandwidth of 1 nm. Estimates of the percentages of α-helical, β-sheet, β-turn, and unordered secondary structure were made by deconvolution of these data using the programs CDSSTR (19) and CONTIN-LL (20). The means of the values calculated using these two programs are presented in Fig. 1*B*. Near-UV (260–320 nm) spectra were obtained using a 1-cm cell with clusterin at 1.2–1.4 mg/ml in either 20 mM sodium phosphate, pH 7.4, or 20 mM MES, pH 5.5. Spectra were acquired at 10-millidegree sensitivity with a resolution and bandwidth both of 0.2 nm. The data shown are mean residue ellipticities ($[\theta]_{MRE}$), calculated from an average of three scans, using values of 6.1×10^4 g/mol for the average molecular weight of serum clusterin (1) and 117 for its mean residue weight.

Measurements of bisANS Fluorescence

All fluorometric analyses were performed using an LS 50B luminometer controlled by FL Winlab software (PerkinElmer Life Sciences). All samples were degassed and filtered through 0.45-μm filters (Millipore, Sydney, Australia) prior to analysis. Clusterin (100 μg/ml) in PBS containing 10 μM bisANS was incubated at temperatures between 25 and 60 °C, and the fluorescence of bisANS at 480 nm (5 nm bandwidth) was measured with an excitation wavelength of 385 nm (5-nm bandwidth). Control samples contained 40% (v/v) ethanol instead of clusterin.

Photochemical Labeling of Clusterin with bisANS

This was done using the method of a previous study (21). Clusterin (0.8 mg/ml) was mixed with 100 μM bisANS in PBS and allowed to incubate at 25 °C for 20 min. Excess bisANS was removed by dialysis against PBS. The sample was then placed in a 3.5-cm Petri dish and illuminated at 254 nm for 20 min using a UV lamp, placed ~2 cm above the sample. Control samples were treated similarly, except that they did not receive bisANS. Covalent labeling of the clusterin with bisANS was confirmed by SDS-PAGE of aliquots of these samples; a fluorescent band corresponding to the migration position of clusterin was observed (results not shown). A bisANS:clusterin molar ratio of 7:1 was calculated from absorbance measurements, assuming an extinction coefficient for bisANS at 385 nm of 16.7 $mM^{-1}cm^{-1}$.

ELISA

Temperature and pH Dependence of the Binding of Clusterin to Stressed Proteins—GST and ovotransferrin, at 20 μg/ml in 50 mM Na_2HPO_4, pH 7.0, were adsorbed onto ELISA plates (Sarstedt Australia, Adelaide, Australia) by incubating at 37 °C (non-stress) or 60 °C (stress) for 1 h. BSA (1 mg/ml) was adsorbed onto ELISA plates by incubation in the same buffer for 5 h in the presence or absence of 20 mM DTT. The plates were then blocked with 1% (w/v) heat-denatured casein prepared in PBS. To exclude any possible formation of disulfide bonds between clusterin and BSA, DTT-treated BSA was then incubated with 5 mM iodoacetamide at 37 °C for 1 h. Clusterin, initially at 10 μg/ml in 50 mM Na_2HPO_4, pH 7.0, was then serially diluted in the same buffer in binary steps down each ELISA plate and incubated for 1 h at 37 °C (or other temperature as indicated). In other experiments, the procedure was the same, except that the 50 mM Na_2HPO_4 buffer was adjusted to pH 6.0–7.5; in some cases, instead of purified clusterin, normal human serum (initially undiluted, adjusted to pH 6.0–7.5) was applied to the plate and serially diluted in buffer of corresponding pH. An equi-volume mixture of hybridoma culture supernatants containing G7, 41D, and 78E anti-clusterin monoclonal antibodies was used to detect bound clusterin (9). Bound primary antibodies were detected with sheep anti-mouse Ig-horseradish peroxidase (Silenus, Sydney, Australia) using *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) as substrate.

Inhibition of the Binding of Clusterin to Stressed Proteins by bisANS—Insulin (100 μg/ml) and lysozyme (20 μg/ml) in 0.1 M $NaHCO_3$, pH 9.5, were adsorbed to ELISA plates for 1 h at 37 °C. The plates were then incubated in the presence of 25 mM DTT in PBS for 1 h at 37 °C and then 10 mM iodoacetamide in PBS for 30 min at 37 °C. Alcohol dehydrogenase (20 μg/ml in 0.1 M $NaHCO_3$, pH 9.5) was adsorbed to ELISA plates for 1 h at 60 °C. In all cases the plates were then blocked with 10 mg/ml BSA in 10 mM NaH_2PO_4, 10 mM MES, 0.15 M NaCl, pH 6 (PBS/MES/BSA). In some experiments, stressed lysozyme adsorbed to ELISA wells was incubated with 0–100 μM bisANS in PBS/MES/BSA for 1 h at 37 °C before the binding of clusterin to it was measured. PBS/MES/BSA containing clusterin (10 μg/ml) and bisANS (at concentrations indicated in the figure legends) was then added to the wells and incubated for 1 h at 37 °C. Subsequently, bound clusterin was detected using undiluted tissue culture supernatant from G7 hybridoma cells and, finally, sheep anti-mouse Ig-horseradish peroxidase and OPD substrate as described above.

Protein Precipitation Assays

The effects of clusterin on stress-induced precipitation of target proteins was monitored by measuring, using an automated diode-array

spectrophotometer (Hewlett-Packard GMBH, Germany), the turbidity associated with target protein precipitation as absorbance at 360 nm. In some experiments, the effects of different temperatures on the extent of DTT-induced protein precipitation in the presence and absence of clusterin were tested. Solutions of BSA (750 μg/ml), or a mixture of BSA (750 μg/ml) with clusterin (at 300 μg/ml), were prepared in 50 mM Na_2HPO_4, pH 7.0, containing 20 mM DTT. These solutions were then incubated at 37 or 50 °C, and turbidity was measured every 15 min for 2.5–5 h. In one set of experiments, individual solutions of GST (250 μg/ml) or mixtures of GST (at the same final concentration) with 100 μg/ml of either unmodified clusterin or bisANS-modified clusterin (made as described above) in PBS were heated at 60 °C, and turbidity was measured as described every 30 s for 20 min. In other experiments, the extent of protein precipitation at pH 7.5 was compared with that at lower pH values, in the presence and absence of clusterin or the sHSP α-crystallin. Individual solutions of ovotransferrin (2 mg/ml) or mixtures of ovotransferrin at the same final concentration with clusterin (at 75 μg/ml) were prepared in 50 mM Na_2HPO_4 (pH 6.0, 6.5, or 7.5) and heated at 60 °C. Related experiments were performed identically, except that ovotransferrin was used at 1 mg/ml and α-crystallin (0.65 mg/ml) was used instead of clusterin. Measurements of turbidity were taken every 1 min for a total of 30 min and were performed as described above. Lastly, normal human serum or clusterin-depleted human serum (prepared by immunoaffinity chromatography) was diluted 1 in 10 in 50 mM Na_2HPO_4 (pH 6.0–7.5) and heated at 60 °C, and measurements of turbidity were taken as previously described.

Quantitative Data Analysis and Plotting

Kaleidagraph Version 3.51 (Synergy Software, Reading, PA) was used for quantitative data analysis and plotting. The CD spectra shown were subjected to Stineman smoothing, which reduces the noise without perturbing the appearance of the data (22). Apparent inhibition constants for the inhibition of binding of clusterin to stressed proteins by bisANS were obtained by non-linear regression analysis of equation (1) onto the data:

$$B = \frac{B_{max}}{1 + [\text{bisANS}]/K_i} \quad \text{(Eq. 1)}$$

where B_{max} and K_i represent the ELISA signal in the absence of bisANS and the apparent inhibition constant, respectively, and B is the measured ELISA signal. Statistical comparisons between different apparent inhibition constants were made using Student's t test; t values with $p < 0.05$ were considered significant.

RESULTS

Effects of Temperature on the CD Spectra of Clusterin—To assess the extent to which the overall secondary structure of clusterin changes with increasing temperature, we measured the far UV circular dichroism spectra of clusterin over the temperature range 25–85 °C. The results of calculation of the secondary structural content of clusterin from these data are shown in Fig. 1*A*. These analyses showed that, as clusterin was heated from 25 °C to 85 °C, its calculated α-helical content decreased from about 62% to about 35%, with a concomitant increase in calculated disordered structure from about 17% to about 28% and smaller increases in calculated β-sheet and β-turn content. The midpoint for these changes was about 55 °C. These results indicate that there was progressive loss of secondary structure in clusterin over this temperature range. Upon cooling these changes were largely reversible, with recovery of calculated α-helical content reaching 57% and disordered structure of 21% (Fig. 1*A*). It is worth noting that clusterin retains significant secondary structure even at elevated temperatures, because a minimum in the spectra at about 200 nm, characteristic of disordered structure, was not observed at any temperature tested (data not shown). Similar analyses of near-UV CD spectra for clusterin indicated that there was also a progressive loss of tertiary structure as the temperature increased from 24 °C to 56 °C but that the changes induced at 56 °C were only partly reversible (data not shown).

Effects of Temperature on the Extent of Exposed Clusterin Hydrophobicity—We assessed whether there were temperature-dependent changes in the level of exposed hydrophobic regions on clusterin by measuring the fluorescence of bisANS bound to clusterin at different temperatures. bisANS is a polarity-sensitive probe that has been used to monitor changes in exposed hydrophobic regions in sHSPs (23, 24). There was a progressive decrease in the fluorescence of bisANS bound to clusterin with temperature over the range 20–50 °C (Fig. 1*B*). This change was reversible, because there was overlap of the heating and cooling traces (Fig. 1*B*, compare *open* and *closed squares*). A number of factors could contribute to this decrease: changed collisional quenching, increased non-radiative decay and

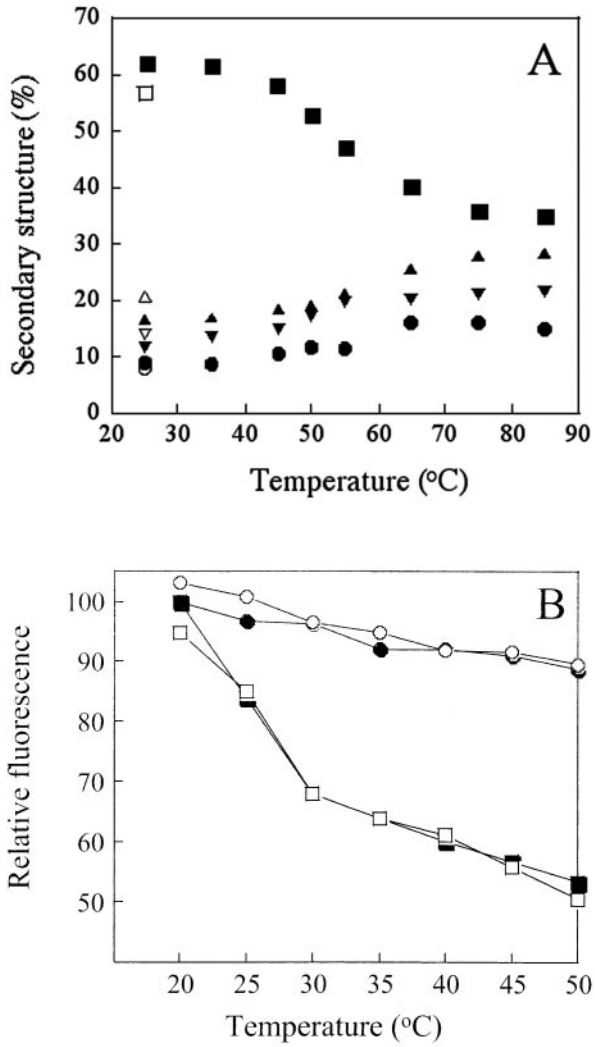


FIG. 1. **Temperature dependence of the far-UV CD spectrum of clusterin and solvent-exposed clusterin hydrophobicity.** *A*, far-UV CD. The percentages of α-helical (■), β-sheet (●), β-turn (▼), and disordered secondary structure (▲) in clusterin were calculated from data acquired as described under "Experimental Procedures." The corresponding *open symbols* at 25 °C represent the calculated secondary structural elements in clusterin after cooling from 85 to 25 °C. *B*, bisANS fluorescence. Samples of 10 μM bisANS in PBS containing 100 μg/ml clusterin (■, □) and 10 μM bisANS in 40% (v/v) ethanol (●, ○) were progressively heated to the indicated temperatures between 20 and 50 °C (heating cycle). The samples were held at each temperature for 1–2 min, and their bisANS fluorescence (*closed symbols*) was measured as under "Experimental Procedures." During the subsequent cooling cycle, the samples were progressively cooled back to 20 °C and were held for 1–2 min at the indicated temperatures while their bisANS fluorescence (*open symbols*) was measured. The data for both samples have been normalized to their 20 °C fluorescence at the start of the heating cycle and are means ± S.D. of five measurements; in most cases the *error bars* are smaller than the data symbols, and in some cases the data symbols at particular temperatures in the heating and cooling cycles overlap.

temperature-dependent changes in exposed hydrophobic regions on clusterin. To assess the relative contributions of these, we also measured the temperature dependence of the fluorescence of bisANS in 40% ethanol (the relative quantum yield and emission maxima of bisANS in this solvent were similar to those of bisANS bound to clusterin). The fluorescence of bisANS in 40% ethanol also decreased with temperature over the range 20–50 °C, and this change was also reversible (Fig. 1*B*, compare *open* and *closed circles*); this decrease can be attributed to the combined effects of changes in the collisional quenching and non-radiative decay of the excited state of bisANS with temperature. However, the finding that the decrease in the fluorescence of bisANS bound to clusterin over this temperature range was greater than the decrease in the fluorescence of bisANS in 40% ethanol suggests that there is a decrease in the extent of exposed hydrophobicity on clusterin with increasing temperature.

Effects of Temperature on the Aggregation State of Clusterin—When clusterin was analyzed by size exclusion chromatography at either 37 or 50 °C, there were no significant differences in the distribution of molecular species fractionated (data not shown). This indicates that variation in temperature over the range 37–50 °C had little effect on the distribution of clusterin between its 80-kDa heterodimeric form and larger oligomeric species.

Effects of Temperature on Interactions of Clusterin With Stressed Proteins—In ELISA, the binding of clusterin to stressed BSA was only slightly enhanced when the temperature was increased above 37 °C (Fig. 2). Similar results were obtained in other ELISAs, which indicated that the binding of clusterin to stressed GST or lysozyme was also only marginally affected by increased temperature (data not shown). Furthermore, temperature had only a small effect on the ability of clusterin to inhibit stress-induced protein precipitation. In the absence of clusterin, precipitation of BSA induced by reduction was more rapid at 50 °C than at 37 °C, but the final level of precipitation obtained in the absence of clusterin was similar at both temperatures (Fig. 3). At 50 °C, at the end of the time course (300 min), clusterin had decreased precipitation of BSA to a level about 13.5% less than that achieved at 37 °C (Fig. 3). Similar results were obtained by testing the effects of increased temperature on the ability of clusterin to inhibit the reductive stress-induced precipitation of α-lactalbumin, lysozyme, and insulin (data not shown). Small effects of temperature on the chaperone action of clusterin were also confirmed in similar experiments comparing the ability of clusterin to inhibit precipitation of stressed proteins at 37 and 42 °C (data not shown). Collectively, the results indicate that (i) an increase in temperature from 37 to 50 °C causes only a small increase in the ability of clusterin to bind to stressed proteins and to inhibit their precipitation from solution, and (ii) the structural

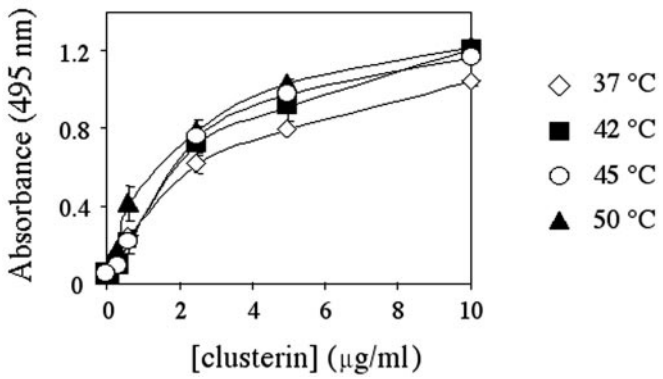


FIG. 2. **Effects of temperature on the binding of clusterin to stressed BSA in ELISA.** Reductively stressed BSA was adsorbed to ELISA plates and subsequently incubated with clusterin at the temperatures indicated in the key. Bound clusterin was detected as described under "Experimental Procedures." Individual data points are the mean of triplicate measurements. In each case, the *error bars* shown represent standard deviations of the mean and in many cases are too small to be visible. The binding of clusterin to unstressed BSA in ELISA was negligible (data not shown; see Ref. 9). The results shown are representative of two independent experiments.

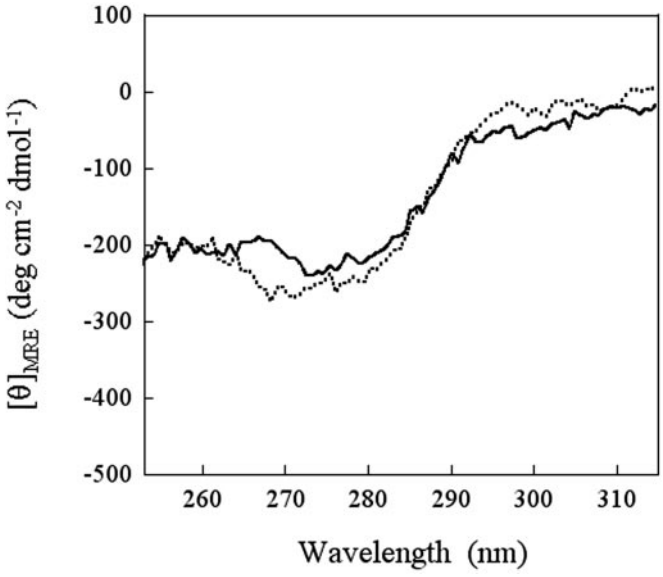
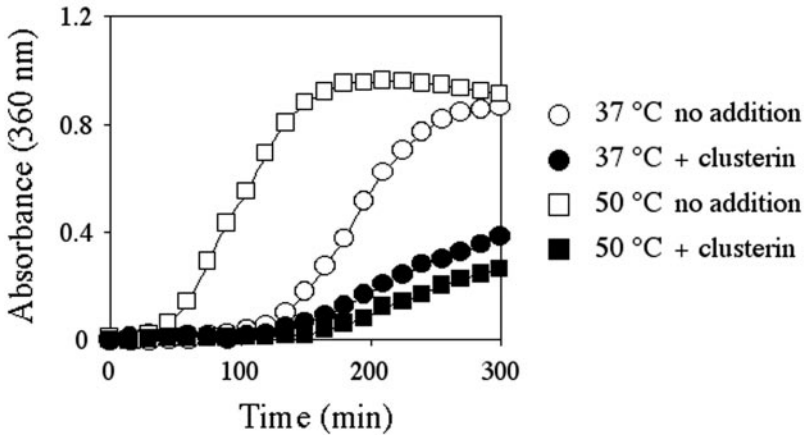


FIG. 4. **pH dependence of the near-UV CD spectrum of clusterin.** Near-UV spectra of clusterin in 20 mM sodium phosphate, pH 7.4 (*dashed line*) or in 20 mM MES, pH 5.5 (*solid line*) were obtained as described under "Experimental Procedures." The data shown are means of three scans.

FIG. 3. **Effects of temperature on the *in vitro* chaperone action of clusterin.** BSA was stressed by reduction with DTT as described under "Experimental Procedures" and protein precipitation at either 37 °C (*circular symbols*) or 50 °C (*square symbols*) measured as absorbance at 360 nm as a function of time. These experiments were performed either in the absence of clusterin (*empty symbols*) or in the presence of clusterin (*solid symbols*) (see key). Each experiment was performed a minimum of three times, and the individual traces shown are representative.



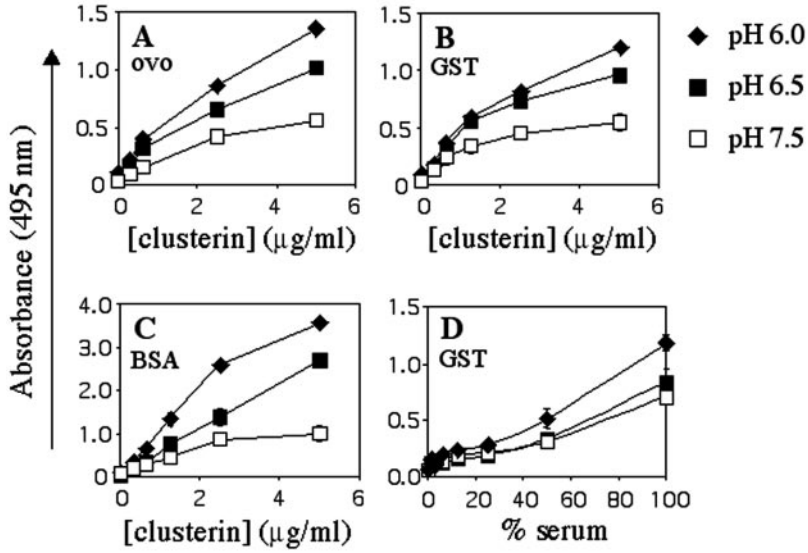


FIG. 5. **Plots showing results from ELISA measuring the concentration-dependent binding of clusterin to stressed proteins at different pH values (indicated in the key).** Stressed proteins tested were heat-stressed (*A*) ovotransferrin (*ovo*) and (*B*, *D*) GST, and reduced (*C*) BSA. Clusterin was provided either as a purified protein (*A–C*) or in unfractionated human serum (*D*), as described under "Experimental Procedures." Data points shown represent means of triplicate measurements. In each case, the *error bars* shown represent standard deviations of the mean, and in many cases are too small to be visible. The binding of clusterin to unstressed proteins in ELISA was negligible (data not shown; see Ref. 9).

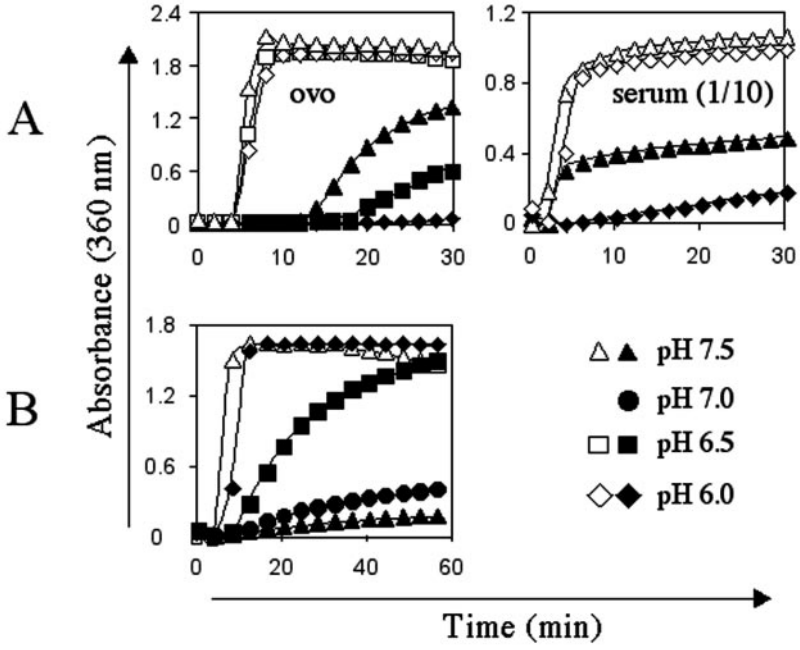


FIG. 6. **Effects of mildly acidic pH on the *in vitro* chaperone action of clusterin and α-crystallin.** *A*, mildly acidic pH enhances the chaperone action of clusterin. Ovotransferrin (*ovo*) or unfractionated human serum (diluted 1 in 10) were stressed as described under "Experimental Procedures," and protein precipitation at pH 7.5 (*triangular symbols*), 6.5 (*square symbols*), or 6.0 (*diamond symbols*) was measured as absorbance at 360 nm as a function of time. These experiments were performed either in the absence of clusterin (*empty symbols*) or in the presence of clusterin (*solid symbols*) (see key). Clusterin-depleted serum (*CDS*) was prepared from normal human serum (*NHS*) by immunoaffinity chromatography, as described under "Experimental Procedures." *B*, mildly acidic pH inhibits the chaperone action of α-crystallin. The same type of experiment was performed, using ovotransferrin as the stressed protein target, in the absence (*empty symbols*) or presence of α-crystallin (*solid symbols*). The different pH values tested are represented by the same symbols as in *A*, pH 7.0, is represented by *circles*. For clarity, results in the absence of α-crystallin at pH 6.5 and 7.0 are not shown. Each experiment was performed a minimum of three times and the individual traces shown are representative.

changes induced in clusterin by heating have only a limited effect on its binding to stressed protein.

Effects of Low pH on the Structure of Clusterin—We previously demonstrated that reduction of pH from 7.4 to 5.5 induced in clusterin (i) no apparent changes in the predicted contents of α-helical, β-sheet, and β-turn regions (detected by far-UV CD spectroscopy); (ii) significant dissociation of clusterin oligomers to form the 80-kDa clusterin heterodimer (detected by size exclusion chromatography); and (iii) an increase in the exposed hydrophobic surface (detected by measurements of bisANS fluorescence) (14). To further examine the effects of mildly acidic pH on clusterin structure, we measured near-UV CD spectra for clusterin at pH 7.5 and 5.5. The results show that reducing the pH from 7.4 to 5.5 did not induce any major changes in the broad negative amplitude peak in $[\theta]_{MRE}$ in the near-UV region (Fig. 4). This suggests that mildly acidic pH does not cause major changes in the tertiary structure of those regions of clusterin containing its aromatic residues.

Effects of Low pH on Interactions of Clusterin with Stressed Proteins—In ELISA, purified clusterin bound most strongly to all three stressed proteins at pH 6.0, with progressively less binding detected at pH 6.5 and 7.5 (Fig. 5, *A–C*). Similarly, enhanced binding of purified clusterin at mildly acidic pH was observed when insulin, alcohol dehydrogenase and lysozyme were used as target proteins (data not shown). This trend was the same, regardless of whether the target protein was stressed

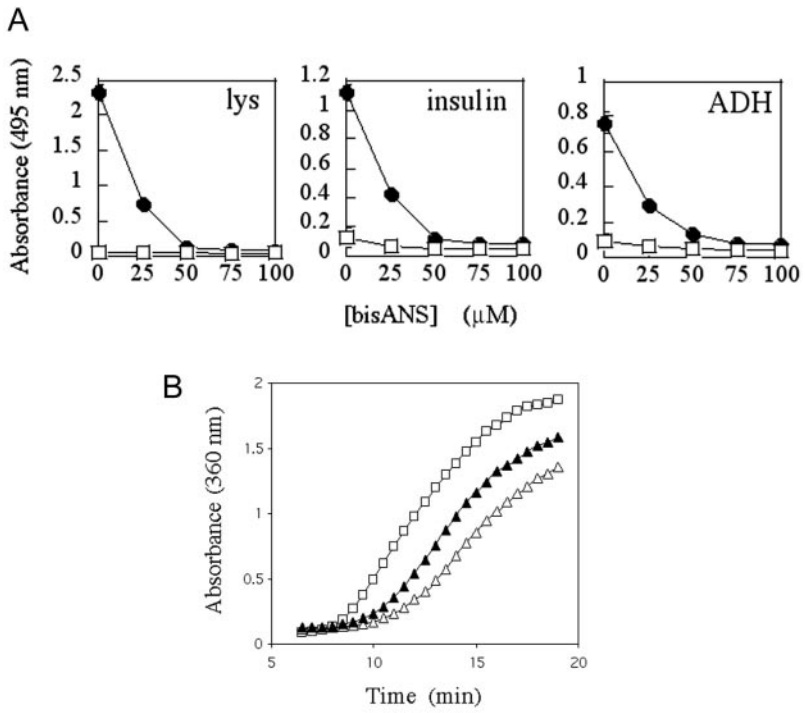


FIG. 7. **Effects of bisANS on the interaction between clusterin and stressed proteins.** *A*, bisANS inhibits the binding of clusterin to stressed proteins in ELISA. The binding of clusterin (10 μg/ml in PBS/MES/BSA) to stressed lysozyme (*lys*), insulin, and alcohol dehydrogenase (*ADH*) in the presence of the indicated concentrations of bisANS was measured as described under "Experimental Procedures." The binding of clusterin to the stressed proteins (●) and to the BSA blocker only (□) is shown. The data shown are means ± S.D. of triplicate determinations. In many cases, the *error bars* are too small to be visible. *B*, photochemical cross-linking of bisANS to clusterin reduces the ability of clusterin to inhibit stressed protein precipitation. Solutions containing GST alone (□) or GST and either unmodified clusterin (△) or bisANS-modified clusterin (▲) were heated at 60 °C, and protein precipitation was measured as described under "Experimental Procedures." The data shown are representative of two independent experiments.

by heat or reduction. Increased binding at reduced pH was also obtained in ELISA experiments in which clusterin, present in pH-adjusted unfractionated human serum, bound to stressed GST (Fig. 5*D*) or ovotransferrin or lysozyme (data not shown) adsorbed to ELISA wells. The increase in binding of clusterin to stressed proteins at low pH was greater for purified clusterin than for clusterin in human serum (Fig. 5, compare *A–C* to *D*). This may result from some serum protein(s), partly unfolded at pH 6, competing with plate-bound stressed protein for clusterin binding. The level of binding of purified clusterin to stressed proteins at pH 5.0 and 4.0 was comparable to that obtained at pH 6.0 (data not shown). In control experiments, we verified that incubating stressed proteins coated onto ELISA wells with any of the different pH buffers used did not affect the amount of stressed protein bound to the wells (data not shown).

In heat-induced protein precipitation assays, in the absence of clusterin, changes in pH between 7.5 and 6.0 had little effect on the time course or extent of precipitation of purified ovotransferrin in solution (Fig. 6*A*) or proteins in unfractionated human serum (Fig. 6*B*). In contrast, the extent of protein precipitation in the presence of clusterin was significantly reduced at lower pH, both for purified proteins and unfractionated human serum. Under the conditions tested, the ability of clusterin to inhibit stress-induced precipitation of ovotransferrin was least at pH 7.5 (~33% inhibition), increased at pH 6.5 (~71% inhibition) and was greatest at pH 6.0 (~97% inhibition) (Fig. 6*A*). Similar results were obtained using catalase and GST as stressed protein targets (data not shown). The corresponding levels of clusterin-mediated inhibition of protein precipitation in unfractionated human serum were 53% at pH 7.5 and 83% at pH 6.0 (Fig. 6*A*). Further decreasing the pH to 5.0 and 4.0 only marginally increased the ability of clusterin to inhibit heat-induced precipitation of proteins (data not shown). Thus, at mildly acidic pH, clusterin binds more strongly to stressed proteins and is more efficient at inhibiting their precipitation from solution. To the best of our knowledge, the effects of mildly acidic pH on the action of other chaperones have not previously been reported. Our results demonstrate that the ability of the cytosolic sHSP α-crystallin to inhibit heat-induced precipitation of ovotransferrin decreases as the pH is changed from pH 7.5 to 7.0 and undergoes further more substantial decreases as the pH is dropped to pH 6.5 and then 6.0 (Fig. 6*B*). Similar results were obtained when catalase was used as a stressed protein target (data not shown). Thus, in stark contrast to the effects of mildly acidic pH on clusterin, the chaperone activity of α-crystallin is progressively reduced as the pH is decreased below that normally found in cytosol.

bisANS Inhibits the Interaction of Clusterin with Stressed Proteins—We have previously shown that extra hydrophobic surfaces, detected using bisANS, are exposed on clusterin at mildly acidic pH (14). We hypothesized that these region(s) may provide additional site(s) for binding of clusterin to stressed proteins. We tested this hypothesis by examining the effects of bisANS on the binding of clusterin to stressed proteins in ELISA under mildly acidic conditions. The results of this showed that, at pH 6.0, bisANS gave dose-dependent inhibition of the binding of clusterin to stressed lysozyme, insulin and alcohol dehydrogenase (Fig. 7*A*). At a concentration of 50–100 μM, bisANS gave near-complete inhibition of binding in all cases. These data indicate that hydrophobic interactions predominate in the binding of clusterin to stressed proteins. Non-linear regression analysis of Equation 1 onto these data gave apparent inhibition constants for bisANS of 7.5 ± 2.6, 10.6 ± 2.6, and 12.6 ± 2.0 μM for stressed lysozyme, insulin, and alcohol dehydrogenase, respectively. *t* test analyses showed that there was no significant difference between any of these values. In addition, we showed that, relative to unmodified clusterin, clusterin, which had been photochemically labeled with bisANS, showed an impaired ability to prevent stress-induced precipitation of both GST (Fig. 7*B*) and alcohol dehydrogenase (data not shown).

DISCUSSION

One of the similarities between clusterin and the mammalian sHSPs is that both exist in solution as heterogeneous aggregates of high molecular mass (14, 25). The sHSPs undergo structural alterations with increasing temperature, leading to the exposure of hydrophobic sites that are then available to bind to target proteins (12, 13, 25, 26). Furthermore, at higher temperatures, sHSPs show increased subunit exchange and

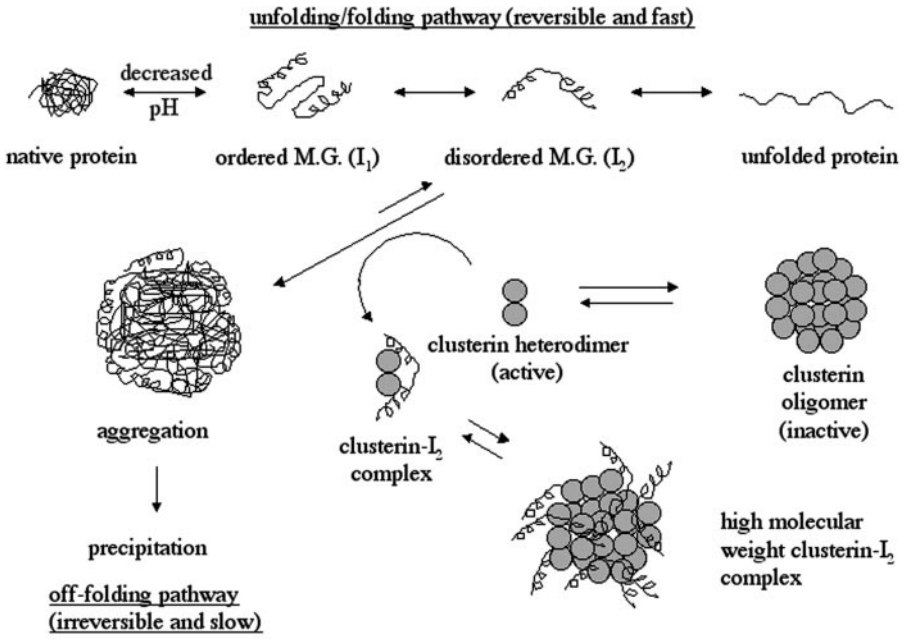


FIG. 8. **A schematic representation of a possible mechanism for the chaperone action of clusterin.** Under conditions of stress, in this case decreased pH, a native protein will start to unfold and adopt a series of partly folded intermediate (molten globule) states (*e.g.* I_1 and I_2). The I_1 state has more residual structure than I_2. These intermediates, particularly I_2, expose significant hydrophobicity to solution. I_2 may aggregate and enter the slow off-folding pathway that can potentially lead to irreversible aggregation. Along this pathway, the chaperone-active heterodimeric form of clusterin interacts and binds to I_2, which is then sequestered into a large clusterin-I_2 complex. The concentration of heterodimeric clusterin is enhanced at acidic pH due to a shift in the equilibrium from the oligomeric form and is an important factor influencing the efficiency of clusterin chaperone action. The mechanism requires no input of energy.

enhanced chaperone ability. This has led to a model in which temperature-induced dissociation of sHSP aggregates yields a chaperone-active species in which the hydrophobic surfaces previously located at the interfaces between the subunits of the aggregate are now available to bind to target proteins (13, 26, 27). In contrast, the distribution of clusterin between different aggregated forms is independent of temperature over the range 37–50 °C (data not shown). Moreover, the ability of clusterin to bind to stressed proteins in ELISA (Fig. 2) and its capacity to prevent the precipitation of these proteins (Fig. 3) also show little variation over this temperature range.

Despite the above findings, far-UV (Fig. 1*A*) and near UV (data not shown) CD spectra showed that clusterin undergoes a progressive and incompletely reversible loss of secondary and tertiary structure with increasing temperature, with a transition at about 50 °C. In addition, the finding that the fluorescence of bisANS bound to clusterin decreased to a greater extent with temperature than did the fluorescence of bisANS in 40% ethanol (Fig. 1*B*) suggests that there is a decrease in exposed hydrophobic regions on clusterin with increasing temperature. How can these data be reconciled with the finding that increased temperature has little effect on the chaperone action of clusterin? One inherent limitation of CD and fluorescence measurements is that they do not identify the specific locations of induced structural changes in a protein molecule, rather, they only provide overall structural information. Therefore, it is possible that the structural changes detected in the experiments represented in Fig. 1 do not affect the target protein-binding region(s) in clusterin. Accepting this hypothesis, the observations that (i) quantitative analysis of the far-UV CD data (Fig. 1*A*) showed that there was an appreciable loss of α-helical content of clusterin at 50 °C compared with 37 °C, and (ii) the chaperone action of clusterin was largely unaffected at 50 °C compared with 37 °C (Figs. 2 and 3), imply that the temperature-sensitive α-helical regions do not contribute to the binding site(s) on clusterin for stressed proteins. Furthermore, because there is no change in the aggregation state of clusterin with increasing temperature, we can deduce that the hydrophobic regions of clusterin that become less exposed to solution at elevated temperatures are probably discrete from those regions that associate during clusterin aggregation and that may form parts of the target protein-binding site(s).

We have previously shown that the aggregation state of clusterin is sensitive to pH, with the 80-kDa form being favored at mildly acidic pH. This was correlated with increased exposure of hydrophobic regions on clusterin (probed by bisANS fluorescence) and increased binding of clusterin to native protein and polysaccharide ligands but very little, if any, change in overall secondary structure (14, 15). Here we show that mildly acidic pH has little effect on the environment of the aromatic residues of clusterin (probed by near-UV CD; Fig. 4) but that these conditions induce a substantial increase in the ability of clusterin to bind to stressed proteins and to prevent their precipitation (Figs. 5 and 6). The pH-dependent enhancement of the chaperone action of clusterin did not appear to reflect the effects of pH on the target proteins, because, using the same target proteins, there was a progressive decrease in the chaperone activity of α-crystallin with reducing pH (Fig. 6*B*). This indicates that mildly acidic pH is probably directly affecting clusterin to enhance its chaperone activity.

The ability of bisANS to inhibit the interaction between clusterin and stressed proteins (Fig. 7*A*) could be explained by it binding to exposed hydrophobic regions on either or both binding partners. If the binding of bisANS predominantly occurred to the stressed protein, it is likely that different K_i values would be obtained for the different targets (*e.g.* bisANS binds to tubulin with about 20-fold higher affinity than to stromelysin-1 (28, 29)), particularly considering the large difference in size and structure of insulin (5.8 kDa), lysozyme (14 kDa), and alcohol dehydrogenase (147 kDa). Thus, the fact that the apparent inhibition constants for bisANS were (within experimental error) the same suggests that its interaction with clusterin, rather than the stressed proteins, was primarily responsible for the inhibition of binding. The hypothesis that hydrophobic regions of clusterin are important in its chaperone action is further supported by our demonstration that photochemical cross-linking of bisANS to clusterin reduces its ability to inhibit the precipitation of stressed proteins (Fig. 7*B*). bisANS has been similarly used to label hydrophobic regions important in the action of other chaperones (30). Thus, although we cannot exclude the possibility that bisANS-mediated inhibition of clusterin binding to stressed proteins may be partly due to bisANS binding to hydrophobic regions on stressed proteins, the results strongly suggest that (i) a large component of the bisANS inhibitory effect is due to its binding, in each case, to the same region(s) of exposed hydrophobicity on

clusterin, and (ii) these regions are important in the chaperone action of clusterin.

Collectively, our results indicate that dissociation of clusterin aggregates is favored at mildly acidic pH and, despite no major changes in overall secondary or tertiary structure detected by CD spectroscopy, more hydrophobic surfaces are exposed to solution under these conditions. These surfaces are probably responsible for the enhanced ability of clusterin to bind to stressed proteins and to inhibit their precipitation at mildly acidic pH. The response of clusterin to mildly acidic pH is thus strikingly similar to that of the sHSPs to increased temperature. By analogy with the sHSPs, we propose that, at physiological pH, hydrophobic chaperone binding surfaces on clusterin are buried in interfaces between the 80-kDa heterodimers in the aggregated forms of the protein. However, at mildly acidic pH, the equilibrium between the heterodimer and aggregated forms of clusterin shifts in favor of the heterodimer, leading to enhanced exposure of these hydrophobic surfaces, which are then available for binding to target proteins. The finding that these effects occur at mildly acidic pH suggests that they may be linked to protonation of histidine residues. A comparison of the sequences of clusterin from eight different mammals shows that there are five highly conserved histidine residues within residues 241 and 290 of the protein (human clusterin numbering). Thus, His-252 and His-263 are found in all of the available sequences, whereas His-290, His-241, and His-261 are also highly conserved, being found in a minimum of five of the eight sequences. It is therefore possible that this region of clusterin represents an "electrostatic switch"; pH-dependent protonation of its histidine residues may lead to disruption of the interfaces between heterodimers within clusterin aggregates, favoring dissociation of the aggregates.

A possible mechanism for the chaperone action of clusterin is schematically represented in Fig. 8. This scheme is analogous to that proposed for the sHSPs (25) and draws on the conclusions of recent work demonstrating that clusterin binds preferentially to slowly aggregating proteins on the off-folding pathway (11). The unfolding of a target protein under stress conditions occurs via a series of partly structured intermediates or molten globule states that are present along the normal protein folding pathway. These intermediates are relatively long-lived and potentially unstable, because they expose significant hydrophobicity to solution, which may facilitate their aggregation and precipitation via the irreversible off-folding pathway. A protein that is present on the off-folding pathway undergoes dynamic processes of association and dissociation. In solution, clusterin also exhibits reversible association to form aggregates of various sizes (14). According to our model, the chaperone-active form of clusterin is the 80-kDa heterodimer. The proportion of clusterin present in solution in this form at pH 6 is about 3-fold greater than that present at pH 7.5 (estimated by deconvolution analysis of the areas under the size exclusion chromatographic peaks shown in Fig. 4 in Ref. 14). Consistent with our model, this increase is comparable in magnitude to the corresponding pH-dependent increases measured for (i) the binding of clusterin to stressed ovotransferrin, GST, and BSA in ELISA (2.4-, 2.2-, and 3.5-fold enhanced binding, respectively, estimated from the change in absorbance measured at a clusterin concentration of 5 μg/ml, see Fig. 5, *A–C*) and (ii) clusterin-mediated inhibition of heat-stressed ovotransferrin (2.9-fold reduced precipitation, estimated from the change in absorbance at 30 min, see Fig. 6*A*). The interaction of clusterin with a stressed protein target during chaperone action may involve the target protein binding to the clusterin heterodimer, which is then incorporated into a highly aggregated complex of two proteins (Fig. 8). We have previously demonstrated that clusterin forms high molecular weight complexes with stressed proteins (9). This proposed mechanism does not require major conformational changes within the protein, and thus an input of energy is unnecessary. It is also consistent with the lack of effect of ATP on the chaperone action of clusterin (10).

The extent to which clusterin undergoes pH-dependent structural and functional changes *in vivo* is an important question that remains to be completely addressed. Owing to the known associations of clusterin in plasma with high density lipoprotein particles and apoA-I (31), and IgG (16), and the enhanced interaction of clusterin with apoA-I and IgG in serum at mildly acidic pH (14), it is very difficult to assess the extent to which clusterin exhibits pH-dependent changes in its aggregation state *in vivo*. However, three pieces of evidence support the hypothesis that clusterin undergoes pH-dependent functional changes *in vivo*: (i) Clusterin purified from serum at pH 6 is associated with 4–6 times as much of its native protein ligands, apoA-I and IgG, than clusterin purified at pH 7.4 (14); (ii) The binding of clusterin in serum to stressed proteins is enhanced at mildly acidic pH (Fig. 5*D*); and (iii) Mildly acidic pH also enhances the ability of clusterin in serum to inhibit the heat-induced precipitation of serum proteins (Fig. 6*A*).

The enhanced ligand-binding and chaperone actions of clusterin at low pH may have important physiological relevance. A phenomenon known as local acidosis occurs at sites of tissue damage or inflammation where the local pH falls to <6. This phenomenon has been reported to occur at sites of inflammation (32), cardiac ischemia (33), and infarcted brain (34) and in the brains of Alzheimer's sufferers (35). Under these conditions, clusterin oligomers may dissociate, and the enhanced binding/chaperone actions of the 80-kDa species could help to inhibit the aggregation and deposition of inflammatory and/or toxic insoluble protein deposits, which would otherwise exacerbate pathology.

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