pH-Dependent Changes in the in Vitro Ligand-Binding Properties and Structure of Human Clusterin

Tim Hochgrebe,[‡] Greg J. Pankhurst,[‡] Jackie Wilce,[§] and Simon B. Easterbrook-Smith*

Department of Biochemistry, University of Sydney, Sydney NSW 2006, Australia

Received July 8, 1999; Revised Manuscript Received October 21, 1999

ABSTRACT: Clusterin is a glycoprotein which is locally overexpressed at sites of tissue damage or stress, leading to the proposal that it may be a cytoprotective protein. It has been shown that clusterin has chaperone-like activity, being able to protect proteins against precipitation under stress conditions. It has also been shown that local acidosis is common at sites of tissue damage or stress. We asked whether acidic pH induces structural changes in clusterin and enhances its ability to bind to other proteins. We found by affinity chromatography and ELISA that the binding of clusterin to glutathione-*S*-transferase, IgG, apolipoprotein A-I, and complement protein C9 was enhanced at mildly acidic compared to physiological pH. Analytical ultracentrifugation and gel filtration studies revealed that clusterin exists in different polymerization states with monomer occurring preferentially at pH 5.5 and multimeric species at pH 7.5. Although circular dichroism showed little difference in the α-helical and *β*-sheet contents of clusterin at pH 5 compared to pH 7.5, evidence for pH-dependent structural changes in clusterin was obtained from fluorescence experiments. pH titrations showed reversible changes in the fluorescence of tryptophan residues in clusterin. There was a reversible 2-fold increase in the fluorescence of the extrinsic probe 4,4′-bis(1-anilinonaphthalene-8-sulfonate) bound to clusterin at pH 5.5 compared to pH 7.5. There was also a 3.5-fold increase in fluorescence resonance energy transfer from tryptophan residues in clusterin to 4,4′-bis(1-anilinonaphthalene-8-sulfonate) at pH 5.5 compared to pH 7.5. These data suggest that pH-induced changes in the structure of clusterin are responsible for its enhanced ability to bind protein ligands at mildly acidic pH.

Clusterin is a highly conserved mammalian glycoprotein, comprising a heterodimer of α- and *β*-polypeptide chains, which are produced by posttranslational cleavage of the primary translation product at the Arg205−Ser206 bond (*1*). Recently, expression of the clusterin gene has been shown to be under the control of a highly conserved 14 bp element which is specifically recognized by HSF-1, a transcription factor important in the expression of heat-shock proteins. This, coupled with the demonstration that there is a parallel accumulation of clusterin and hsp70 mRNA in heat-shocked A431 cells, led to the suggestion that clusterin is an extracellular heat-shock protein (*2*). Clusterin shares functional properties with chaperones; it can protect a number of proteins against stress-induced precipitation. Clusterin was found to bind to the stressed proteins, forming high molecular weight complexes; clusterin thus appears to be an extracellular chaperone (*3*). These findings may collectively provide an explanation for the observation of local overexpression of clusterin following a number of biochemical insults, including ischemia (*4*), neuronal injury (*5*), treatment of cells with cancer chemotherapeutics, pressure-induced kidney damage, and androgen ablation of the prostate gland (*6*). Clusterin may fulfill a cytoprotective function at sites of tissue damage through its ability to prevent inappropriate precipitation of secreted or cell-surface proteins.

It is known that at sites of tissue damage or inflammation the local pH can drop to below 6, a phenomenon known as local acidosis. High hydrogen ion concentrations have been found in vivo not only in inflamed tissue (*7*) but also in cardiac ischemia (*8*), in infarcted brain (*9*), and in the brains of people with Alzheimer's disease (*10*). As a first step in assessing the significance of local acidosis in relation to the cytoprotective function of clusterin, we asked whether mildly acidic conditions promoted the ability of clusterin to bind to other proteins. We have already shown that binding of heparin to clusterin is pH-dependent; minimal binding occurs at pH 7.4, but heparin binds to clusterin with a submicromolar apparent dissociation constant at pH 6 (*11*). We report here that binding of pure clusterin to IgG, GST,[1] and polymerized C9 is highly pH-dependent, and we demonstrate that this is also the case for unfractionated clusterin in human serum. These functional changes do not arise from major changes in the secondary structure of clusterin, monitored by circular dichroism. They are correlated with pH-dependent changes in the polymerization state of clusterin, as probed

* To whom correspondence should be addressed. Phone: (+) 612 9351 3905, FAX: (+) 612 9351 4726, E-mail: sbe@biochem.usyd.edu.au.

[‡] These authors made equal contributions to this work.

[§] Current address: Departments of Chemistry and Biochemistry, University of Western Australia, Nedlands WA 6907, Australia.

[1] Abbreviations: bis-ANS, 4,4′-bis(1-anilinonaphthalene-8-sulfonate); BSA, bovine serum albumin; GST, glutathione-*S*-transferase; b-IgG, biotinylated IgG; MAb, monoclonal antibody; SAM-HRP, horseradish peroxidase-conjugated sheep anti-mouse Ig antibody; SA-HRP, horseradish peroxidase-conjugated streptavidin.

10.1021/bi991581b CCC: $19.00
Published on Web 01/21/2000

by gel filtration and analytical ultracentrifugation, and with increased fluorescence of the hydrophobic probe, bis-ANS, and increased fluorescence resonance energy transfer from tryptophan residues in clusterin to bis-ANS at acidic pH.

MATERIALS AND METHODS

Proteins. Monoclonal antibodies (MAbs) G7 (human clusterin-specific) and DNP9 (dinitrophenol-specific) have been described (*12*, *13*). Hybridoma cells secreting MAb HA-60 (human apoA-I-specific) were from the American Type Culture Collection. Rabbit anti-human C9 IgG was from Serotec, and rabbit anti-human IgG antibody was from Sigma Chemical Co., St. Louis, MO. Horseradish peroxidase-conjugated sheep anti-mouse Ig antibody (SAM-HRP) and horseradish peroxidase-conjugated streptavidin (SA-HRP) were from Silenus Laboratories, Hawthorn, Victoria, Australia, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Sigma. Complement protein C9 was from Sigma, and glutathione-*S*-transferase (GST) was obtained by thrombin cleavage of GST fusion proteins (*14*). Human clusterin and IgG were purified from serum as described (*15*). Contaminating IgG was removed from clusterin preparations by protein G−Sepharose affinity chromatography. This purification procedure resulted in clusterin preparations of about 95% purity, determined by densitometric scanning of SDS−PAGE gels. Clusterin for use in circular dichroism or analytical ultracentrifugation experiments was further purified by FPLC. This was performed by injecting 500 μL of a 2 mg/mL solution of immunoaffinity-purified clusterin onto a Superose 6 column, which was eluted as below. Monomeric clusterin was isolated by pooling the fractions corresponding to the 80 kDa peak and was >98% pure as judged by SDS−PAGE. Biotinylated IgG (b-IgG) was prepared by adding biotin-X-NHS (Calbiochem, San Diego, CA) from a DMSO stock to human IgG in 0.1 M Na_2CO_3, 0.1 M NaCl, pH 8.5, to give a 1:5 w/w ratio. After a 2 h incubation at room temperature, the biotinylated IgG was dialyzed against 10 mM phosphate, 150 mM NaCl, pH 7.4 (PBS).

Immunochemical Methods. (A) Binding of Clusterin to IgG, Polymerized C9, and GST in ELISA. ELISA trays were coated with human IgG at 10 μg/mL in 0.1 M $NaHCO_3$, pH 9.5, or with GST at 5 μg/mL in PBS for 1 h at 37 °C. For polymerization of C9, a solution was prepared containing 100 μg/mL C9 and 100 mM $ZnCl_2$ in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5. The solution was incubated at 37 °C for 2 h, and the resulting polymeric C9 was used the same day. ELISA trays were coated with 2 μg/mL polymeric C9 diluted in the above buffer and incubated overnight at 4 °C. The trays were washed and then blocked with PBS containing 0.04% (w/v) thymol and 1% (w/v) heat-denatured casein (HDC) for 1 h at 37 °C. Clusterin was diluted to between 2 and 20 μg/mL in 10 mM MES, 10 mM phosphate, 150 mM NaCl (PBS/MES) containing 0.1% (w/v) Triton X-100 (TX-100), at either pH 6.0 or pH 7.5. The samples were then transferred onto the coated trays and incubated for 1 h at 37 °C. To ensure that the ELISA signals obtained reflected differences in the binding of clusterin to the target proteins with pH, rather than pH-dependent differences in the binding of subsequent detection molecules (MAbs, SAM-HRP, or SA-HRP), all subsequent steps used buffers at pH 6. The trays were washed 3 times using 10 mg/mL BSA in PBS containing 0.1% TX-100. Bound clusterin was detected by incubating the trays for 1 h at 37 °C with tissue culture supernatant from G7 hybridoma cells. Bound primary antibody was detected using horseradish peroxidase-conjugated sheep anti-mouse IgG applied diluted 1:1000 in HDC for 1 h at 37 °C. Bound horseradish peroxidase was assayed with *o*-phenylenediamine as substrate. When the binding of clusterin in serum to the target proteins was measured, serum diluted 1:200 into MES/PBS adjusted to either pH 6 or pH 7.5 was used. Bound clusterin was then detected as above. Alternatively, ELISA trays were coated with 5 μg/mL clusterin in 0.1 M $NaHCO_3$, pH 9.5, and then washed and blocked as above. Biotinylated IgG (b-IgG), diluted 2.5 μg/mL in MES/PBS, pH 6.0 or 7.5, containing 10 mg/mL BSA was added to the clusterin-coated trays and incubated for 1 h at 37 °C. The trays were then washed 3 times using MES/PBS, pH 6.0, containing 10 mg/mL BSA. b-IgG was detected using SA-HRP peroxidase, diluted 1:2000 in the same buffer, which was assayed as above.

(B) Dot-Blot Assays of Clusterin Preparations. Samples of clusterin which had been affinity-purified using MAb G7-Sepharose were diluted in PBS, and aliquots were applied to nitrocellulose membranes. The nitrocellulose was blocked by incubation in HDC for 1 h at 37 °C and then incubated with primary antibodies for 1 h; MAbs were used as tissue culture supernatant, and rabbit anti-human IgG antibodies were diluted 1:1000 in HDC. Bound primary antibodies were detected with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody or goat anti-rabbit IgG antibody, both applied diluted 1:1000 in HDC and incubated as above. Bound secondary antibodies were detected using 3 mg/mL 4-chloro-1-naphthol in methanol, diluted 1:5 in PBS containing 0.03% hydrogen peroxide. Blots were scanned and analyzed by densitometry using a Fluor-S MultiImager and Multi-Analyst (v 1.0.2) software (BioRad).

FPLC Gel Filtration. A Pharmacia FPLC system including a UV-monitor and fraction collector (Pharmacia Biotechnology, Uppsala, Sweden) was used to operate a Superose 6 column (Pharmacia). The column was eluted at 0.3−0.5 mL/min, and fractions were collected where necessary at 1 fraction/min. All buffers used were degassed by vacuum filtration through a 0.45 μm nitrocellulose filter (Millipore). Samples never exceeded a volume of 500 μL or a total protein amount of 1 mg.

Circular Dichroism Analysis. A Jasco J-720 spectropolarimeter, linked to a Neslab RTE-111 cooling system, was used to obtain circular dichroism data. The spectra of clusterin (200 μg/mL in 10 mM phosphate at pH 5 or 7.5) were acquired using a 1 mm cell, thermostated at 25 °C. The data sets contained 50 accumulated spectra acquired between 180 and 200 nm and 10 accumulated spectra acquired between 200 and 260 nm, with step resolutions of 0.5 nm, speeds of 20 nm/min, 1 nm bandwidths, 1 s response times, and 50 mdeg sensitivity in both cases. Secondary structure predictions from these data were obtained using the variable selection program VARSLC1 (*16*).

Spectrofluorometric Analyses. Fluorometric analysis was performed using an LS 50B luminometer controlled by FL Winlab software (Perkin-Elmer) with the cell holder held at 20 °C. All samples were degassed and filtered through 0.45 μm filters (Millipore) prior to analysis. Intrinsic tryptophan fluorescence was measured using an excitation wavelength

of 295 nm (5 nm bandwidth), and emission scans were recorded from 300 to 400 nm. The fluorescence of bis-ANS was measured using an excitation wavelength of 385 nm (5 nm bandwidth), and emission scans were recorded from 400 to 550 nm. Fluorescence resonance energy transfer from tryptophan residues to bis-ANS was monitored using an excitation wavelength of 295 nm (5 nm bandwidth), and emission scans were recorded from 300 to 550 nm. In all cases, at least 10 scans were collected and the average fluorescence intensities determined.

Analytical Ultracentrifugation Analyses. Sedimentation equilibrium experiments were performed using cells fitted with conventional Yphantis 12 mm 6-channel equilibrium centerpieces (*17*) and quartz windows in a Beckman optima XL-A analytical ultracentrifuge. This system houses an optics system that is able to directly measure the absorbance of the sample at different radial positions of the chamber while the sample is spinning. The absorption profiles can hence be directly fitted to in order to determine molecular weight or association constants.

Samples of the protein (at 0.6−0.25 mg/mL) were dialyzed against PBS/MES, pH 5.5, 6.5, and 7.5. The dialysate was used to prepare sample dilutions which were centrifuged at 15 000 rpm and at temperatures of 5, 10, or 25 °C. Absorption profiles were recorded at 230, 280, and 360 nm with the absorbance profile from the dialysate automatically subtracted. The data were recorded in 0.001 cm steps and 10 scans averaged to produce each profile. The system was deemed in equilibrium when consecutive profiles recorded 4 h apart were identical.

The absorption profiles were trimmed to exclude any data measuring above 1 absorption unit or displaying any sign of light scattering due to a buildup of pelleted protein. The data from three absorption profiles (representing different dilutions of the protein solution run simultaneously) were then analyzed by fitting models of association to the absorbance versus radial position distribution using the program NONLIN and through the calculation of the apparent weight average molecular weight ($M_{w,app}$) (*17*, *18*).

Sedimentation equilibrium data were fitted by the NONLIN program to the following equation:

$$c_{\text{total}} = \delta c + \sum_{i=1}^{n} C_i(r) = \delta c + \sum_{i=1}^{n} K_{1,}iC_1(r)^q(i)$$

where δc is the concentration offset of the first data point, $q(i)$ is the degree of association for the ith associated species, $C_i(r)$ is the concentration of the ith species at radius r, $K_{1,i}$ is the equilibrium constant for the association of monomer to the $q(i)$-mer, $C_1(r)$ is the concentration of the monomer at radius r, and n is the total number of species present in the model being used for fitting the data. Using values of $q(2) = 2$, 3, and 4, it is possible to fit the data to models of monomer−dimer, monomer−trimer, and monomer−tetramer, respectively.

A value of 0.702 mL/g was used for the partial specific volume of clusterin, calculated from its reported amino acid and sugar composition (*20*), and the reported partial specific volumes of these subunits (*21*). An absorbance coefficient (280 nm) of 38 040 M^{-1} cm^{-1} and MW of 66 366 for clusterin were used in all calculations. The density of the buffer solution was determined to be 1.007 g/mL at the salt concentrations used. Meniscus depletion experiments run at 42 000 rpm were used to determine the base line offset value to be used in the curve-fitting procedure.

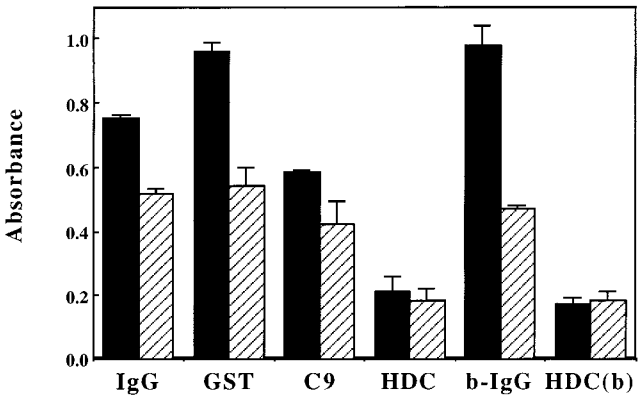

FIGURE 1: pH dependence of the binding of purified clusterin to poly-C9, IgG, and GST in ELISA. ELISA trays were coated with polymerized C9, IgG, GST, clusterin, or HDC blocking solution only as described under Materials and Methods. Clusterin samples (2.5 μg/mL) in MES/PBS containing 0.1% TX-100 at pH 6.0 (solid bars) or pH 7.5 (hatched bars) were then applied to the trays coated with C9, IgG, GST, or HDC blocking solution only (HDC), and bound clusterin was quantified as described under Materials and Methods. The binding of b-IgG to trays coated with clusterin or with HDC blocking solution only [HDC(b)] at either pH 6.0 (solid bars) or pH 7.5 (hatched bars) was quantified as described under Materials and Methods. The data are means ± standard deviation of triplicate measurements.

RESULTS

Preferential Binding of Clusterin to Protein Ligands at Mildly Acidic pH. Clusterin binds to heparin preferentially under mildly acidic conditions (*11*), and conference reports[2] suggest that other ligands also bind to clusterin preferentially under these conditions. We therefore investigated whether the binding of clusterin to its known protein ligands, IgG (*15*, *22*), C9 (*23*), and GST (*3*), occurred preferentially at pH 6 compared to pH 7.5. We found increased ELISA signals for bound clusterin at pH 6 compared to pH 7.5 in all cases. In the case of the clusterin−IgG interaction, increased binding of biotinylated IgG at low pH to clusterin adsorbed to ELISA trays was also observed (Figure 1). These increases presumably arose from increased binding of clusterin to the target proteins at low pH, rather than pH-dependent differences in the binding of subsequent detection molecules (MAbs, SAM-HRP, or SA-HRP) because these detection molecules were used at pH 6 in all cases. Similar data to those shown were obtained for clusterin concentrations in the range 2−20 μg/mL. The finding that the binding of clusterin to trays treated with HDC blocking solution only was pH-independent and was between 2- and 5-fold lower than its binding to the target proteins suggests that the pH-dependent interaction of clusterin with the target proteins is specific. Clusterin in unfractionated serum also bound preferentially to C9 and GST, but not to IgG, at pH 6 compared to pH 7.5 (Figure 2A). A possible explanation for the latter finding is that the IgG in serum (present at about

[2] J. Lakins and M. Tenniswood, data presented at the 3rd International Workshop on Clusterin, Villars sur Ollon, Jan 1997.

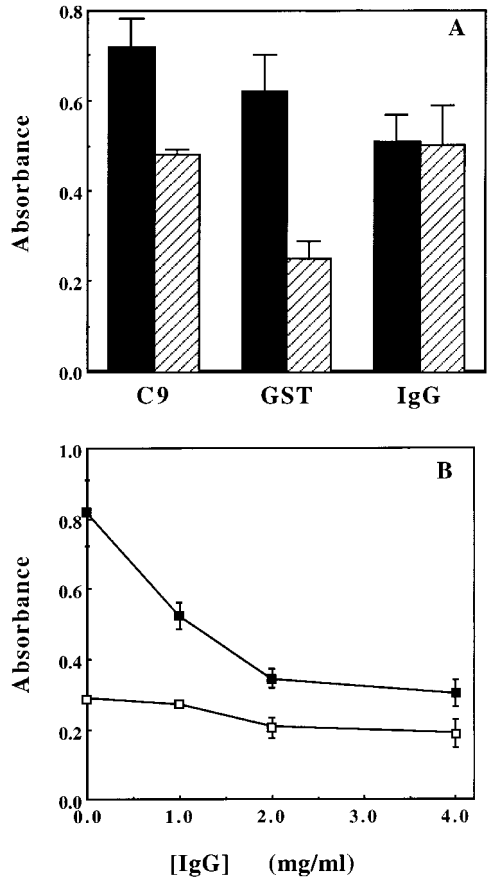

FIGURE 2: pH dependence of the binding of clusterin in serum to poly-C9, IgG, and GST in ELISA. (A) Retrieval of clusterin from serum. ELISA trays were coated with polymerized C9, IgG, or GST as described under Materials and Methods. Serum, diluted 1:200 in MES/PBS containing 0.1% TX-100 at pH 6.0 (solid bars) or pH 7.5 (hatched bars), was then applied to the trays and bound clusterin quantitated as described under Materials and Methods. The data are means ± standard deviation of triplicate measurements. (B) Effects of preincubation of clusterin with IgG on binding to IgG immobilized on ELISA trays. Clusterin at 5 μg/mL was preincubated for 2 h at 37 °C with IgG at the indicated concentrations in MES/PBS containing 0.1% TX-100 at pH 6.0 (■) or pH 7.5 (□). Aliquots were then applied to ELISA trays coated with IgG, and bound clusterin was quantitated as described under Materials and Methods. The data are means ± standard deviation of triplicate measurements.

15 mg/mL) binds preferentially to serum clusterin at acidic pH, thus acting as a competitive inhibitor of the IgG immobilized on the ELISA tray, so preventing increased binding of clusterin to the ELISA tray at acidic pH. The absence of similar inhibition of the binding of serum clusterin to immobilized C9 by serum C9 may reflect the far lower serum concentration of C9 (about 0.2 mg/mL). This interpretation is supported by the finding that preincubation of purified clusterin with purified human IgG at pH 6.0 led to more than 60% inhibition of the subsequent binding of the clusterin to IgG immobilized on an ELISA tray; in contrast, only about 30% inhibition was observed when the experiment was carried out at pH 7.5 (Figure 2B).

Using an alternative approach, we compared the relative levels of IgG and apoA-I which copurified with clusterin in MAb affinity chromatography of dilute human serum at pH 5.5 compared to pH 7.4. Dot-blot analyses of clusterin purified in this way showed that approximately 2.5-fold more clusterin was retrieved from serum by MAb G7 at pH 5.5

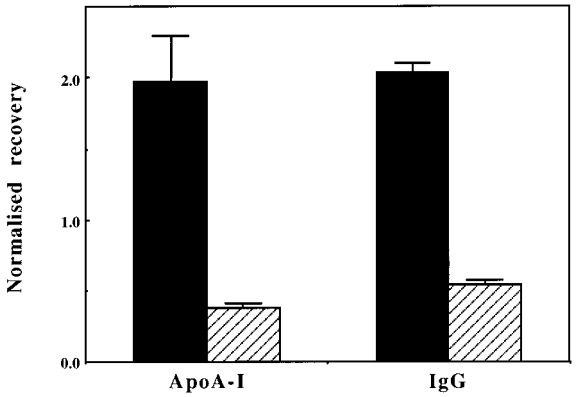

FIGURE 3: Dot-blot analysis of apoA-I and IgG copurified with clusterin in immunoaffinity chromatography. Samples of clusterin which had been purified from serum by MAb G7 affinity chromatography at pH 5.5 (solid bars) or pH 7.4 (hatched bars) were analyzed for apoA-I and IgG by dot-blot as described under Materials and Methods. The data were normalized by dividing the dot-blot signal obtained for the target proteins by the dot-blot signal obtained for clusterin at each pH value. The data are means ± standard deviations of triplicate determinations.

compared to pH 7.4 (data not shown). Dot-blot analyses also showed that increased amounts of apoA-I and IgG copurified with clusterin at pH 5.5 compared to pH 7.4. Normalizing the amounts of these proteins to the yield of clusterin at each pH showed that the relative yields of apoA-I and IgG were respectively 6-fold and 3.8-fold higher at pH 5.5 compared to pH 7.4 (Figure 3).

pH-Dependent Changes in the Aggregation State of Clusterin. Purified clusterin has a propensity to form high molecular weight aggregates (*24*). Hence, an explanation for the pH-dependent binding of clusterin to protein ligands (Figures 1−3) is that it exhibits different aggregation states at different pH values, with the different aggregate forms showing different affinities for these ligands. Therefore, purified clusterin was subjected to Superose 6 gel filtration chromatography in a series of experiments in which the pH of the elution buffer was varied between 6.0 and 7.5 (Figure 4).

With increasing pH, there was a progressive decrease in the species eluting at about 45−50 min, with concomitant increases in species eluting between 35 and 45 min. The elution times of standard proteins (IgG, 150 kDa; bovine serum albumin, 66 kDa; and lysozyme, 14 kDa, applied separately to the column) all decreased slightly at pH 7.5 compared to 6.0 (data not shown). This is presumably a consequence of a general pH-dependent change in the properties of the column used in these experiments. Calibration curves constructed using these proteins showed that the species eluting at 48 min at pH 7.5 and the species eluting at 49 min at pH 6.0 could both be assigned to the clusterin $\alpha\beta$ heterodimer at about 70−80 kDa and also that the species eluting between 35 and 45 min had sizes consistent with dimer−tetramer multimers of the clusterin $\alpha\beta$ heterodimer. Thus, the data in Figure 4 suggest that purified clusterin exists as a complex mixture of monomeric and polymeric forms of the $\alpha\beta$ heterodimer, with the former becoming more dominant at lower pH.

We assessed the reversibility of the pH-dependent changes in the aggregation state of clusterin by first comparing the gel filtration profiles of clusterin samples which had either

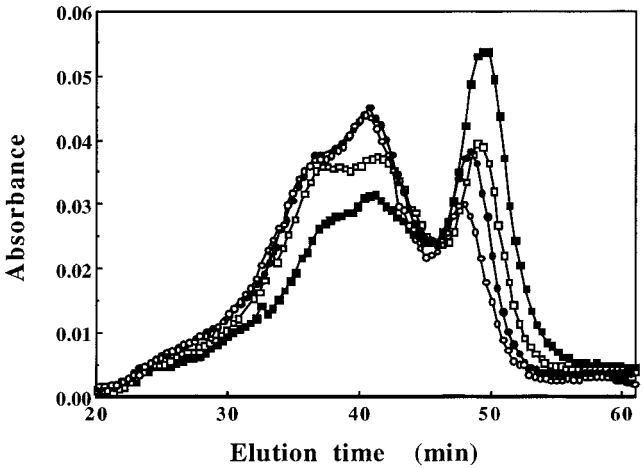

FIGURE 4: FPLC gel filtration of clusterin as a function of pH. Aliquots (0.5 mL) of clusterin at 1 mg/mL in PBS/MES at pH 6.0 (■), 6.5 (□), 7.0 (●), and 7.5 (○) were analyzed by FPLC gel filtration using a Superose 6 column equilibrated with PBS/MES at each pH. The elution profiles were scanned and converted into digital images using the analog-to-digital conversion program DataThief 2.0. The figure shows overlays of individually scanned profiles, and the data are representative of three independent experiments.

been maintained at pH 7.5 buffer or first had been dialyzed against pH 5.5 buffer and then dialyzed back to pH 7.5. There was little difference between the gel filtration profiles of these samples (data not shown), implying that clusterin could reversibly change its aggregation state over this pH range. We then pooled the polymer fraction from clusterin analyzed by gel filtration as in Figure 4 and reapplied it to the column. The reappearance of a $\alpha\beta$ heterodimer peak (Figure 5A) suggests that the aggregation of clusterin is at least in part a reversible process. Finally, we found that the relative size of the polymeric clusterin peak increased with increasing concentrations of clusterin applied to the column (Figure 5B), also suggesting that clusterin can reversibly interconvert between monomer and polymer forms of the $\alpha\beta$ heterodimer.

Sedimentation equilibrium of clusterin at different pH values also showed pH-dependent changes in its polymerization state. The sedimentation equilibrium profiles acquired for clusterin at pH values of 5.5, 6.5, and 7.5 were used to construct $M_{w,app}$ vs concentration plots. These showed a slight increase in $M_{w,app}$ with concentration from approximately 66 kDa at the lowest concentration, which was most apparent for the sample at pH 7.5 (Figure 6). This is consistent with a monomeric species of 66 kDa undergoing association at all three pHs, but particularly pH 7.5. The data were fitted to models of a 66 kDa species in equilibrium with polymers in order to derive association constants at the different pH values using the program NONLIN (see Materials and Methods). The data were approximately consistent with the monomer−dimer model with an association constant of the order of 10^5 M^{-1}. However, no simple model (monomer−dimer, monomer−trimer, monomer−tetramer) could simulate the profiles with high integrity. It is likely that the samples contained a mixture of multimeric species that could not be described by a simple model. These data were, however, consistent with the gel filtration data; the pH 7.5 sedimentation equilibrium data showed a larger amount of polymeric clusterin relative to the lower pH values.

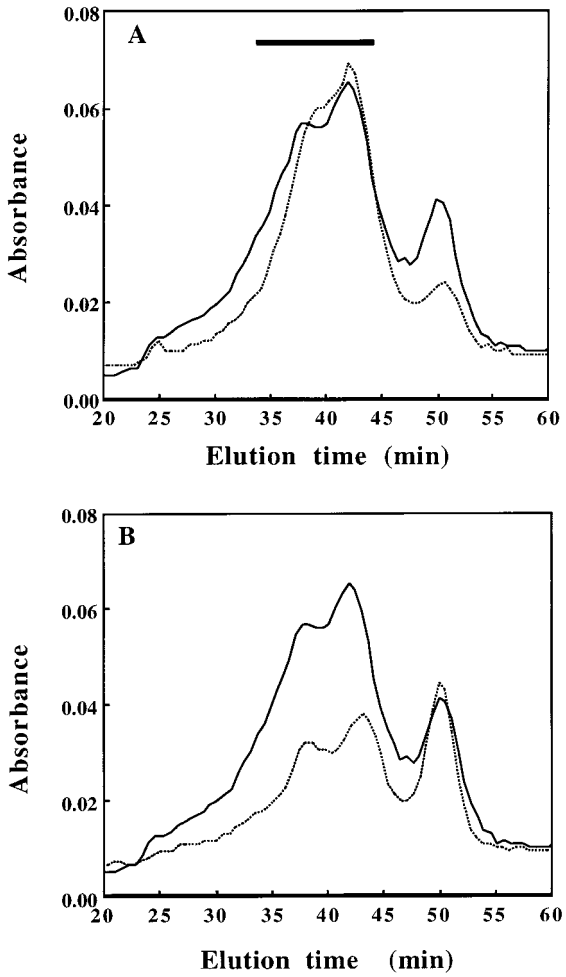

FIGURE 5: FPLC gel filtration of clusterin as a function of polymerization state and protein concentration. (A) Effect of polymerization state. A 100 μL aliquot of clusterin (2 mg/mL in PBS, pH 7.5) was analyzed by FPLC gel filtration using a Superose 6 column (solid line). The high molecular weight peak fraction (indicated by the bar) was pooled, concentrated to 2 mg/mL, and then reapplied to the column (dotted line). The data shown are representative of two independent experiments. (B) Effect of protein concentration. Aliquots (100 μL) of clusterin at 1 mg/mL (dotted line) and at 2 mg/mL (solid line) in PBS, pH 7.5, were analyzed by gel filtration as in panel A. The data shown are representative of two independent experiments.

Spectroscopic Evidence for pH-Dependent Changes in the Structure of Clusterin. To obtain further evidence for pH-dependent changes in the structure of clusterin, we first compared its far-UV circular dichroism spectra at pH 5 and 7.5 (Figure 7). Analysis of these spectra using the variable selection program VARSLC1 (*16*) showed that there were no significant differences in the calculated α-helical, β-sheet, and β-turn contents of clusterin between these pH values (Table 1). These spectra are qualitatively similar to those of Zlokovic et al. (*25*), who showed that iodination of clusterin did not lead to large changes in its secondary structure.

We then examined the effects of changing pH on the fluorescence of the hydrophobic probe bis-ANS bound to clusterin. Bis-ANS is a compound that fluoresces intensely in hydrophobic environments and practically not at all in water and has been used as a probe to measure changes in exposed hydrophobic regions on proteins (e.g., see ref *26*). Titration of bis-ANS into clusterin solutions at pH 5.5 and 7.5 gave dose-dependent increases in probe fluorescence in

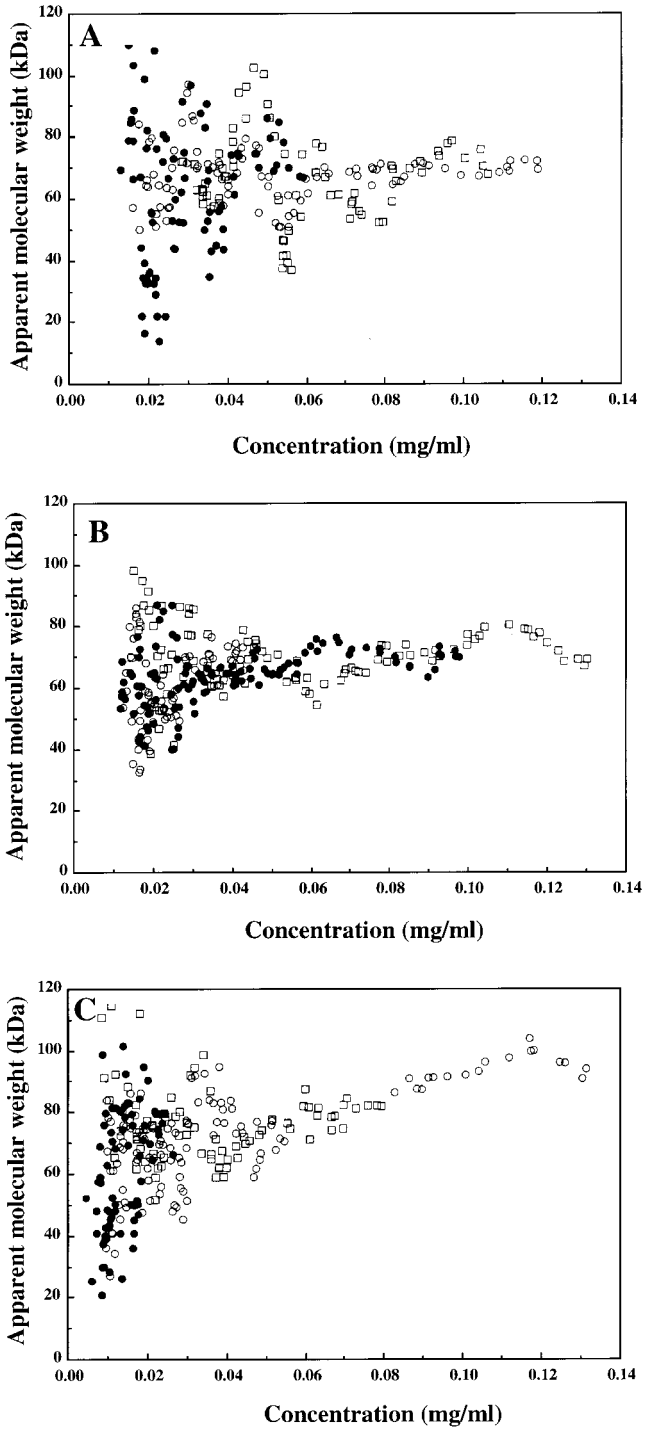

FIGURE 6: Sedimentation equilibrium analysis of clusterin. Samples of clusterin in PBS/MES at pH 5.5 (panel A), pH 6.5 (panel B), and pH 7.5 (panel C) were analyzed by analytical ultracentrifugation as described under Materials and Methods. The initial loading concentrations were 0.25 mg/ml (●), 0.12 mg/mL (□), and 0.06 mg/mL (○).

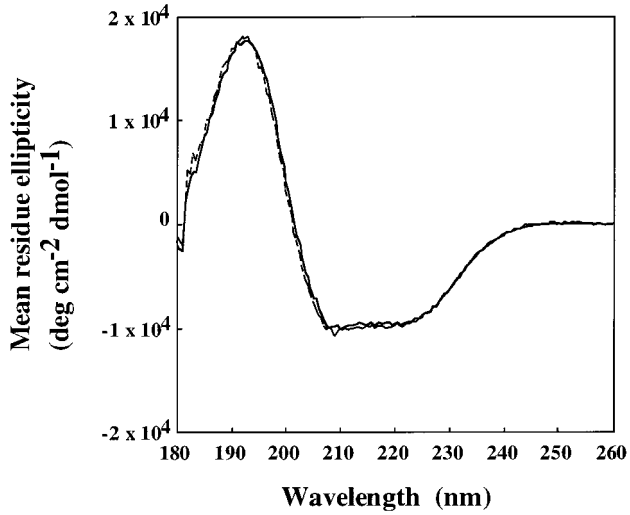

FIGURE 7: Circular dichroism spectra of clusterin at pH 5.0 and 7.5. Clusterin which had been purified by immunoaffinity chromatography and then by FPLC gel filtration was dialyzed at 200 μg/mL into 10 mM sodium phosphate at either pH 5.0 (solid line) or pH 7.5 (dashed line) and analyzed using a Jasco J-720 CD spectropolarimeter as described under Materials and Methods.

Table 1: Predicted Secondary Structure Contents of Clusterin at pH 7.5 and 5.0[a]

pH	α-helix (%)	antiparallel β-sheet (%)	parallel β-sheet (%)	β-turn (%)	others (%)	total (%)
5.0	25 ± 3	18 ± 2	5 ± 1	18 ± 1	31 ± 1	97
7.5	23 ± 2	19 ± 2	5 ± 1	17 ± 1	33 ± 1	98

[a] The circular dichroism spectra shown in Figure 7 were analyzed with the variable selection program VARSLC1 (*16*).

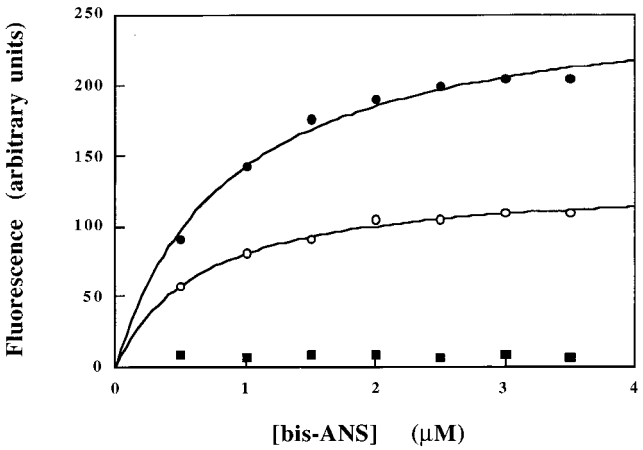

FIGURE 8: Fluorescence of clusterin-bound bis-ANS at pH 5.5 and 7.5. Bis-ANS was titrated into solutions of clusterin at 40 μg/mL clusterin in PBS/MES at pH 5.5 (●) or pH 7.5 (○), and its fluorescence was measured as described under Materials and Methods. In the control, bis-ANS was titrated into buffer only (■). The data shown are representative of three independent experiments.

both cases (Figure 8). Nonlinear regression of these data, assuming a single class of bis-ANS binding sites on clusterin, showed that there was little difference in the apparent dissociation constants for bis-ANS−clusterin complexes (0.85 ± 0.1 μM at pH 5.5 and 0.64 ± 0.06 μM at pH 7.5) but that the maximum probe fluorescence at pH 5.5 was about twice that at pH 7.5 (264 ± 10 and 132 ± 3 arbitrary units, respectively). This difference implies that there is an increase in the exposed hydrophobic surface on clusterin at acidic pH. To assess the extent to which this change was reversible, samples of clusterin were subjected to pH changes by dialysis against buffers of appropriate pH: pH 5.5−7.5; pH 7.5−5.5; pH 5.5−7.5 and then back to 5.5; pH 7.5−5.5 and then back to 7.5. They were then analyzed by spectrofluorometry, measuring both the intrinsic fluorescence of tryptophan residues in the protein and the probe fluorescence of bis-ANS (Table 2). Two conclusions can be drawn from these data. First, the finding that the intrinsic fluorescence

Table 2: Changes in Bis-ANS-Associated and Tryptophan Fluorescence of Clusterin at Different pH Values[a]

initial pH	dialysis protocol	bis-ANS fluorescence	tryptophan fluorescence
7.5	pH 7.5 to pH 5.5	134 ± 1	130 ± 1
5.5	pH 5.5 to pH 7.5	81 ± 1	148 ± 0.5
7.5	pH 7.5 to pH 5.5 to pH 7.5	88 ± 0.5	152 ± 0.5

[a] Clusterin at 40 μg/mL in MES/PBS at either pH 5.5 or pH 7.5 was subjected to dialysis for 24 h against MES/PBS at pH 5.5 or 7.5 as indicated. The tryptophan fluorescence of the samples was measured, and then bis-ANS was added to 1 μM. The fluorescence of bis-ANS was susequently measured. The data are means ± standard deviations of 10 scans in each case.

of tryptophan residues in clusterin was lower at pH 5.5 than at pH 7.5 supports the conclusion drawn from Figure 9 that there are pH-induced changes in the structure of clusterin over this range. Second, the finding that both the tryptophan and bis-ANS fluorescence of the samples at either pH was independent of their previous different pH exposures suggests that the pH-induced changes in clusterin are largely reversible.

As a further method to analyze potential structural changes in clusterin with changing pH, fluorescence resonance energy transfer (FRET) from tryptophan residues to associated bis-ANS was compared at pH 5.5 and 7.5. The excitation wavelength was 295 nm, and evidence for FRET, manifest as parallel bis-ANS dose-dependent increases in fluorescence at 480 nm and decreases at 350 nm, was obtained at both pH values (Figure 9A,B). However, FRET was significantly enhanced at pH 5.5 compared to pH 7.5; this is shown in Figure 9C, in which the fluorescence at 480 nm (normalized to the fluorescence at 350 nm in the absence of bis-ANS at each pH) is plotted as a function of bis-ANS concentration. Nonlinear regression of these data showed that the apparent dissociation constants for bis-ANS−clusterin complexes (0.44 ± 0.04 μM at pH 5.5 and 0.40 ± 0.08 μM at pH 7.5) were comparable to those obtained from similar analyses of the data in Figure 8. The analysis also showed that, at saturating levels of bis-ANS, there was about 3-fold more FRET at pH 5.5 than at pH 7.5 (normalized fluorescence values of 1.81 ± 0.07 and 0.51 ± 0.04, respectively).

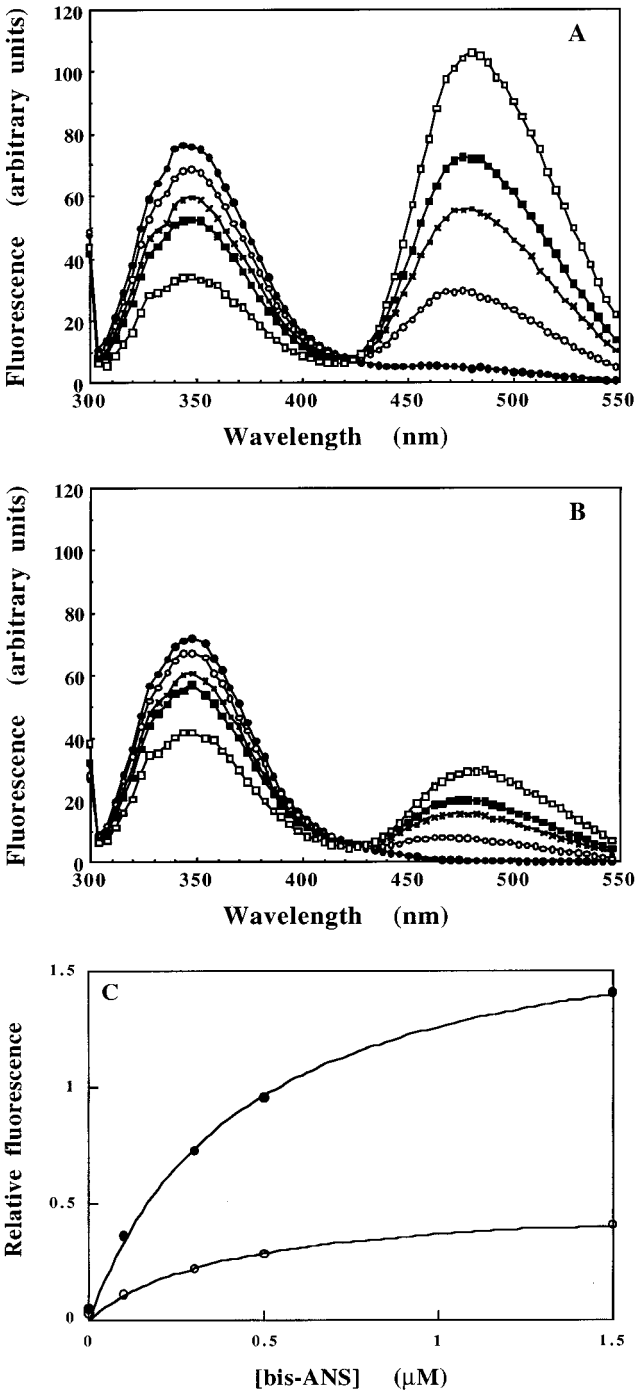

FIGURE 9: FRET of clusterin−bis-ANS complexes at pH 5.5 and 7.5. Bis-ANS was titrated into solutions of clusterin at 80 μg/mL clusterin in PBS/MES at pH 5.5 (panel A) or pH 7.5 (panel B), and FRET was measured as described under Materials and Methods. The final bis-ANS concentrations were as follows: 0 (●), 0.1 μM (○), 0.3 μM (×), 0.5 μM (■), and 1.5 μM (□). In panel C, the fluorescence at 480 nm, relative to the fluorescence at 350 nm in the absence of bis-ANS, is plotted against bis-ANS concentration at pH 5.5 (●) and pH 7.5 (○).

DISCUSSION

We have shown above that binding of clusterin to four macromolecules, IgG, complement protein C9, apoA-I, and GST, is enhanced at mildly acidic pH (Figures 1−3) and have previously reported similar observations for the binding of clusterin to heparin (*11*). Given that these five macromolecules are structurally and functionally disparate, it seems unlikely that each of them undergoes pH-dependent structural changes, leading to an enhancement in their ability to bind clusterin. An alternative possibility is that there may be pH-induced changes in the structure of clusterin, resulting in a conformation that has enhanced binding capacities for various molecules.

We obtained evidence for such changes, over the same pH range that clusterin exhibits enhanced binding to macromolecules, using FPLC gel filtration, analytical ultracentrifugation, and spectrofluorometry. The results from the first two of these indicated that clusterin exists in an array of multimer forms; low pH favored the formation of clusterin monomer whereas higher order polymers formed preferentially at normal physiological pH (Figures 4−6). Therefore, one explanation for the enhanced binding of macromolecules to clusterin at acidic pH is that the binding site(s) on clusterin for these is (are) preferentially exposed when clusterin is monomeric. However, the finding that biotinylated IgG bound preferentially at acidic pH to clusterin immobilized on ELISA trays (Figure 1) and our previous finding that the

same is true for biotinylated heparin (*11*) suggest that factors other than pH-dependent changes in the polymerization state of clusterin are involved. This conclusion arises from the assumption that once clusterin has adsorbed to an ELISA tray it would be unlikely to be able to undergo pH-dependent changes in its polymerization state.

The finding from circular dichroism analysis that the α-helical and β-sheet contents of clusterin were (within experimental error) the same at pH 5 and 7.5 (Figure 7, Table 1) implies that the changes in its ligand-binding properties over this pH range do not reflect gross structural changes in the clusterin monomer. However, the observation of reversible changes in the fluorescence of tryptophan residues in clusterin between pH 7.5 and 5.5 (Table 2) implies that there are local pH-dependent changes in the protein. This conclusion was supported by the findings of increased fluorescence of clusterin-bound bis-ANS at acidic pH (Figure 8) and the observation of increased FRET from tryptophan residues to bis-ANS under these conditions (Figure 9). The first of these findings suggests that there is increased exposure of hydrophobic regions on clusterin at acidic pH. The finding of increased FRET implies that there are pH-dependent changes in the distances between the bis-ANS binding site(s) on clusterin and its tryptophan residues. Although FRET is commonly used to calculate the distances between donor and acceptor fluorophors, this is not possible from our data because clusterin contains four tryptophan residues and we have yet to establish if clusterin contains a unique bis-ANS binding site.

How then can the findings of increased binding of proteins to clusterin, a shift in favor of its monomer form, little change in its α-helical and β-sheet contents, and increased exposure of hydrophobic regions under mildly acidic conditions be integrated? The COILS algorithm (*27*) strongly predicts that clusterin contains two coiled-coil α-helices, residues 15−80 in the α chain and residues 295−345 in the β chain. Coiled-coil α-helices are motifs which are commonly associated with formation of protein polymers, and it is known that changes in pH can modulate the propensity of proteins to polymerize through these motifs. For example, Seidel et al. (*28*) found that the thermal stability of a coiled-coil dimer of a peptide derived from delta sleep-inducing immunoreactive peptide decreased at acidic pH with little change in circular dichroism, and Beck et al. (*29*) observed that formation of homopolymers of a coiled-coil peptide derived from cartilage matrix protein was strongly pH-dependent.

Therefore, a speculative model for the pH-dependent changes in the structure and ligand-binding properties postulates that clusterin can form homopolymers through its coiled-coil α-helices. Preferential formation of clusterin monomer under mildly acidic conditions would lead to an increase in the proportion of clusterin molecules in which the hydrophobic residues normally buried in the coiled-coil interface are exposed to the solvent. These residues could provide binding sites for bis-ANS, thus accounting for the enhanced fluorescence of this probe when bound to clusterin under acidic conditions (Figures 8 and 9). These residues may also provide recognition sites for hydrophobic regions of other proteins, thus explaining the enhanced binding of these to clusterin at relatively low pH (Figures 1−3).

The preferential binding of clusterin to other macromolecules at mildly acidic pH occurred at physiological temperature (*11*, Figures 1 and 2 above) and at clusterin concentrations less than but comparable to those in vivo (*30*), suggesting that this effect may be physiologically relevant. What, therefore, could be the physiological significance of the finding that the binding of clusterin to other macromolecules is enhanced at low pH? Clusterin has a chaperone-like function, in that it can prevent precipitation of GST, catalase, α-lactalbumin, and bovine serum albumin under denaturing conditions (*3*). Clusterin has a similar effect on precipitation of denatured IgG, ovotransferrin, and lysozyme.[3] This ability presumably arises from the presence of one or more binding sites on clusterin with a broad specificity for other proteins. It is known that clusterin is produced locally at sites of tissue damage or inflammation (*4*−*6*). This may be augmented by increases in the local concentration of clusterin derived from platelets, which are recruited to sites of tissue damage (*31*, *32*). The clusterin consequently present at sites of tissue damage or inflammation would experience low pH; the local acidosis at these sites. The resulting enhancement of its ability to bind to other macromolecules could therefore lead to an enhancement of its chaperone-like function.

[3] T. Adamkiewicz, D. Humphreys, S. Poon, and M. R. Wilson, unpublished results.

ACKNOWLEDGMENT

We thank the NSW Red Cross Blood Bank for gifts of human serum and Dr. Mark R. Wilson for constructive comments.

REFERENCES

1. Burkey, B. F., deSilva, H. V., and Harmony, J. A. (1991) *J. Lipid Res. 32*, 1039−1048.
2. Michel, D., Chatelain, G., North, S., and Brun, G. (1997) *Biochem. J. 328*, 45−50.
3. Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B., and Wilson, M. R. (1999) *J. Biol. Chem. 274*, 6875−6881.
4. Kida, E., Pluta, R., Lossinsky, A. S., Golabek, A. A., Choi-Miura, N. H., Wisniewski, H. M., and Mossakowski, M. J. (1995) *Brain Res. 674*, 341−346.
5. May, P. C., and Finch, C. E. (1995) *Trends Neurosci. 15*, 391−396.
6. Buttyan, R., Olsson, C. A., Pintar, J., Chang, C., Bandyk, M., Ng, P. Y., and Sawczuk, I. S. (1989) *Mol. Cell. Biol. 9*, 3473−3481.
7. Punnia-Moorthy, A. (1987) *J. Oral Pathol. 16*, 36−44.
8. Jacobus, W. E., Taylor, G. J., Hollins, D. P., and Nunnally, R. L. (1977) *Nature 265*, 756−758.
9. Back, T., Hoehn-Berlage, M., Kohno, K., and Hossmann, K.-A. (1994) *Stroke 25*, 494−500.
10. Yates, C. M., Butterworth, J., Tennant, M. C., and Gordon, A. (1990) *J. Neurochem. 55*, 1624−1630.
11. Pankhurst, G. J., Bennett, C. A., and Easterbrook-Smith S. B. (1998) *Biochemistry 37*, 4823−4830.
12. Murphy, B. F., Kirszbaum, L., Walker, I. D., and d'Apice, A. J. (1988) *J. Clin. Invest. 81*, 1858−1864.
13. Wilson, M. R., and Easterbrook-Smith, S. B. (1993) *Anal. Biochem. 209*, 183−187.
14. Heuer, K. H., Mackay, J. P., Podzebenko, P., Bains, N. P. S., Weiss, A. S., King, G. F., and Easterbrook-Smith, S B. (1996) *Biochemistry 35*, 9069−9075.
15. Wilson, M. R., and Easterbrook-Smith, S. B. (1992) *Biochim. Biophys. Acta 1159*, 319−326.
16. Johnson, W. C., Jr. (1990) *Proteins: Struct., Funct., Genet. 7*, 205−214.

17. Yphantis, D. A. (1964) *Biochemistry 3*, 297−317.
18. Johnson, M. L., Correia, J. A., Yphantis, D. A., and Halvorson, H. R. (1981) *Biophys. J. 36*, 575−588.
19. Teller, D. C. (1973) *Methods Enzymol. 27*, 346−441.
20. Kapron, J. T., Hilliard, G. M., Lakins, J. N., Tenniswood, M. P. R., West, K. A., Carr, S. A., and Crabb, J. W. (1997) *Protein Sci. 6*, 2120−2133.
21. Durschlag, H. (1986) in *Thermodynamic data for biochemistry and biotechnology* (Hinz, H.-J., Ed.) pp 45−128, Springer-Verlag, Berlin.
22. Wilson, M. R., Roeth, P. J., and Easterbrook-Smith, S. B. (1991) *Biochem. Biophys. Res. Commun. 177*, 985−990.
23. Tschopp, J., Chonn, A., Hertig, S., and French, L. E. (1993) *J. Immunol. 151*, 2159−2165.
24. Blaschuk, O., Burdzy, K., and Fritz, I. B. (1983) *J. Biol. Chem. 258*, 7714−7720.
25. Zlokovic, B. V., Martel, C. L., Matsubara, E., McComb, J. G., Zheng, G., McCluskey, R. T., Frangione, B., and Ghiso, J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4229−4234.
26. Sharma, K. K., Kumar, G. S., Murphy A. S., and Kester, K. (1998) *J. Biol. Chem. 273*, 15474−15478.
27. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science 252*, 1162−1164.
28. Seidel, G., Adermann, K., Schindler, T., Ejchart, A., Jaenicke, R., Forssmann, W.-G., and Rösch, P. (1997) *J. Biol. Chem. 272*, 30918−30927.
29. Beck, K., Gambee, J. E., Kamawal, A., and Bächinger, H. P. (1997) *EMBO J. 13*, 3767−3777.
30. Jenkins, S. H., Stuart, W. D., Bottoms, L. A., and Harmony, J. A. (1996) *Methods Enzymol. 263*, 309−316.
31. Witte, D. P., Aronow, B. J., Stauderman, M. L., Stuart, W. D., Clay, M. A., Gruppo, R. A., Jenkins, S. H., and Harmony, J. A. (1993) *Am. J. Pathol. 143*, 763−773.
32. Tschopp, J., Jenne, D. E., Hertig, S., Preissner, K. T., Morgenstern, H., Sapino, A. P., and French, L. (1993) *Blood 82*, 118−125.

BI991581B