Clusterin (CLU) is a potent extracellular chaperone that inhibits protein aggregation and precipitation otherwise caused by physical or chemical stresses (e.g. heat, reduction). This action involves CLU forming soluble high molecular weight (HMW) complexes with the client protein. Other than their unquantified large size, the physical characteristics of these complexes were previously unknown. In this study, HMW CLU-citrate synthase (CS), HMW CLU-fibrinogen (FGN), and HMW CLU-glutathione S-transferase (GST) complexes were generated in vitro, and their structures studied using size exclusion chromatography (SEC), ELISA, SDS-PAGE, dynamic light scattering (DLS), bisANS fluorescence, and circular dichroism spectrophotometry (CD). Densitometry of Coomassie Blue-stained SDS-PAGE gels indicated that all three HMW CLU-client protein complexes had an approximate mass ratio of 1:2 (CLU:client protein). SEC indicated that all three clients formed complexes with CLU ≥ 4 × 10^7 Da; however, DLS estimated HMW CLU-FGN to have a diameter of 108.57 ± 18.09 nm, while HMW CLU-CS and HMW CLU-GST were smaller with estimated diameters of 51.06 ± 6.87 nm and 52.61 ± 7.71 nm, respectively. Measurements of bisANS fluorescence suggest that the chaperone action of CLU involves preventing the exposure to aqueous solvent of hydrophobic regions that are normally exposed by the client protein during heat-induced unfolding. CD analysis indicated that, depending on the individual client protein, CLU may interact with a variety of intermediates on protein unfolding pathways with different amounts of native secondary structure. In vitro, soluble complexes like those studied here are likely to serve as vehicles to dispose of otherwise dangerous aggregation-prone misfolded extracellular proteins.

Controlled unfolding is important in many biological processes including protein translocation, degradation by proteases, and regulation of enzyme activity. Uncontrolled unfolding and the consequent accumulation of insoluble protein aggregates is implicated in the pathology of many diseases including Alzheimer disease and type II diabetes and is promoted by various stresses such as oxidative stress (1), shear stress (2), and thermal stress (3). Cells have extensive quality control mechanisms to ensure that intracellular proteins are maintained predominantly in their native conformations. Molecular chaperones are known to play a central role in these systems by targeting unfolded proteins for refolding or degradation (4–7). However, little is known about the existence of corresponding systems for protein folding quality control in the extracellular environment (8).

A large number of alternative functions have been proposed for clusterin (CLU), nevertheless, the potent chaperone activity of this protein (9–13) and its constitutive presence in many biological fluids suggests that it is likely to be important in extracellular protein folding quality control. Recently haptoglobin (14) and α2-macroglobulin (15, 16) have also been identified as extracellular chaperones. All three proteins exhibit small heat shock protein (sHsp)-like activity, preferentially binding to stressed client proteins to prevent their precipitation in an ATP-independent manner (9, 11, 14, 16). When acting alone, extracellular chaperones lack refolding activity; however it has been shown that CLU can hold partially unfolded proteins in a state competent for refolding by Hsc70 (11).

CLU is found associated with extracellular protein deposits in numerous diseases including drusen in age-related macular degeneration (17), renal immunoglobulin deposits in kidney disease (18), Lewy bodies in Parkinson disease (19), prion deposits in Creutzfeldt-Jakob disease (20), and amyloid plaques in Alzheimer disease (21). Knock-out studies have shown that CLU-deficient mice accumulate insoluble protein deposits in the kidneys and develop progressive glomerulopathy (22). These findings suggest a role for CLU in the clearance of extracellular misfolded proteins; however, the mechanism by which this may occur has yet to be determined.

Currently, little is known about the physical characteristics of the soluble complexes formed during the interaction of CLU with chaperone client proteins (9–12). This is the first study to investigate the physical properties of CLU-client protein complexes. The present study provides new insights into the properties of complexes formed in vitro between CLU and citrate synthase (CS), fibrinogen (FGN), and glutathione S-transferase (GST).

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4 The abbreviations used are: CLU, clusterin; HMW, high molecular weight; CS, citrate synthase; FGN, fibrinogen; GST, glutathione S-transferase; sHSP, small heat shock protein; Hsp, heat shock protein; LDL, low density lipoprotein; IgG, immunoglobulin; SEC, size exclusion chromatography; G7, anti-human clusterin monoclonal antibody; FPLC, fast protein liquid chromatography; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; bisANS, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid; AFU, arbitrary fluorescence units; DLS, dynamic light scattering.
EXPERIMENTAL PROCEDURES

Materials—4,4′-Bis(1-anilino-8-naphthalene sulphonate; bisANS), bovine serum albumin (BSA), CS, and FGN were all obtained from Sigma-Aldrich. All buffer salts and H₂O₂ were obtained from Ajax Chemical Co. Human blood was obtained as a kind gift from Wollongong Hospital (Wollongong, NSW, Australia) and processed to yield plasma, which was stored frozen at −20 °C until used. CLU was purified from human plasma by immunoaffinity chromatography as previously described (23). GST was expressed in Escherichia coli using the vector pGEX-2T (without an insert; Invitrogen) as previously described (24) and purified using a glutathione-Sepharose™ High Performance column according to the manufacturer’s directions (GE Healthcare).

Precipitation Assays—CS (6.0 μm), FGN (6.0 μm), or GST (20 μm) were incubated at 41, 45, or 60 °C, respectively, in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄, pH 7.4) containing 0.1% (w/v) Na₃P₄ (PBS/Az) in the presence or absence of CLU (6.6, 6.5, and 3.2 μm) respectively or the control protein, BSA (at the same respective molar concentrations). The solutions were added in triplicate-μl aliquots to a 384-well plate (Greiner Bio-One). Protein precipitation was monitored by measuring the absorbance at 360 nm (A₃60 nm, an indication of turbidity) with a FLUOStar Optima incubator microplate reader (BMG Labtechnologies).

Preparation of Preheated Protein Controls—Preheated control proteins were prepared by incubating client proteins or CLU (alone) at the same temperature and for the same duration used to form the HMW complexes. Preheated controls were filtered (0.45 μm) and the clarified solutions assayed for residual protein content using the bichinchoninic microprotein assay (25), or the respective extinction coefficients at 280 nm.

Size Exclusion Chromatography (SEC)—SEC was carried out using a Superose™ 6 10/300 column (GE Healthcare) at the recommended flow rate of 0.5 ml/min and the absorbance at 280 nm continuously monitored using an ÄKTA FPLC system (GE Healthcare). Mass standards were from a commercial high molecular weight calibration kit (GE Healthcare). All buffers and samples were filtered (0.45 μm) before use. SEC-purified HMW complexes were collected from fractions between 7–8 ml in the peak corresponding to the size exclusion limit of ≈4 × 10⁷ Da. The integrity of the complexes was checked periodically by reanalysis using the same column.

Sandwich ELISA—The wells of an ELISA plate (Greiner Bio-One) were coated with purified G7 anti-CLU antibody (23), then blocked with 1% (w/v) BSA in PBS (BSA/PBS). Mixtures of CLU and client protein were heated as described under “Precipitation Assays,” and fractionated by SEC (as described above). After washing with PBS, proteins eluting at the exclusion limit (≥4 × 10⁷ Da), preheated CLU or client proteins, or a mixture of CLU and individual client proteins were incubated in the wells of the ELISA plate (diluted to 50 μg/ml in BSA/PBS). Then a primary antibody (or antiserum) reactive with the client protein, diluted in BSA/PBS, was added following the manufacturer’s instructions. Finally an appropriate horseradish peroxidase-conjugated secondary antibody diluted in BSA/PBS was added following the manufacturer’s instructions. All incubations were carried out for 1 h at 37 °C with shaking and washing was performed with PBS. After a final wash, ortho-phenylenediamine at 2.5 mg/ml in 50 mM citric acid, 100 mM Na₂HPO₄, pH 5, was added. The absorbance at 490 nm (A₄90 nm) was measured using a SpectraMax Plus 384 microplate reader (Molecular Devices). Nonspecific binding was assessed using a species-matched polyclonal antibody or serum of irrelevant specificity and the appropriate secondary antibody. Primary antibodies used were sheep polyclonal anti-CS (IgG fraction, Abcam), goat anti-FGN antiserum, and rabbit polyclonal anti-GST (IgG fraction, Chemicon). The respective controls were polyclonal anti-apolipoprotein A1 (IgG fraction, Abcam), normal goat serum (Sigma-Aldrich), and purified normal rabbit IgG fraction (Sigma-Aldrich).

Dynamic Light Scattering (DLS)—Solutions of SEC-purified HMW CLU-CS, HMW CLU-FGN, and HMW CLU-GST complexes, CLU, FGN, CS, or GST (controls were preheated or untreated), or mixtures of these proteins, were made between 0.1 and 1.0 mg/ml in PBS and filtered (0.45 μm). Triplicate samples were examined in low volume plastic cuvettes using a Zetasizer Nano ZS (Malvern). Particle diameters were recorded as a frequency distribution curve, and the average diameter and range (average peak and width of 9 normally distributed curves) reported.

Densitometry—Several milligrams of CS, FGN, GST, or CLU were extensively dialyzed against distilled water and then freeze-dried. The recovered protein was weighed using a XS205 Dual Range analytical balance (Mettler Toledo). The proteins were redissolved in an appropriate volume of filtered (0.45 μm) PBS and the absorbance at 280 nm of several aliquots measured using a SpectraMax Plus 384 microplate reader (Molecular Devices). The average absorbance at 280 nm and the known protein concentration were used to calculate the extinction coefficient using Beer’s law. These proteins, as well as SEC-purified HMW CLU-CS, HMW CLU-FGN, and HMW CLU-GST complexes were reduced by boiling in SDS-PAGE loading buffer containing 100 mM dithiothreitol and 1% (v/v) β-mercaptoethanol and separated on a 12% SDS gel. Six sample wells containing unknown amounts of reduced HMW CLU-CS, HMW CLU-FGN, or HMW CLU-GST complexes, and triplicate wells containing 0.5–6 μg of reduced CS, FGN, GST, or CLU were also loaded onto the gel. Following Coomassie Blue staining and destaining, the major bands corresponding to reduced CS, FGN, GST, and CLU were analyzed using a GS 800 calibrated densitometer (Bio-Rad) and Quantity One software (Bio-Rad). The average optical density/mm² of the major bands was used to construct a standard curve for each protein. Using these standard curves, the relative amounts of CLU and FGN, CS or GST present in the SEC-purified HMW complexes were calculated.

4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic Acid (bisANS)—For bisANS analyses, CLU client proteins (0.5 mg/ml CS, 2 mg/ml FGN, or 0.5 mg/ml GST), or mixtures of client proteins (at the same concentrations) and CLU (at 0.4 mg/ml for experiments using CS or FGN, or at 0.2 mg/ml for experiments using GST) were incubated under the same conditions used to form HMW CLU-client protein complexes (see above). At specified
time points, samples were taken from the solutions and snap-frozen in liquid nitrogen. Following completion of the time courses, all samples were thawed and immediately diluted in PBS containing bisANS to give final concentrations of 50 μg/ml client protein and 10 μM bisANS. Samples containing CLU alone were diluted to give equivalent concentrations to that present in the (CLU + client protein) samples (i.e. 15 μg/ml or 30 μg/ml). Fluorescence was measured on a FLUOstar Optima fluorescence plate reader using excitation and emission windows of 360 ± 10 and 490 ± 10 nm, respectively.

Circular Dichroism (CD)—For CD analyses, samples were analyzed as previously described (26); all samples were in 10 mM Na₂HPO₄, pH 7.4. Individual proteins were analyzed before and after heating (the latter were residual soluble protein) at the following concentrations: CLU at 164 μg/ml, CS at 119 μg/ml, FGN at 164 μg/ml, and GST at 110 μg/ml. In separate experiments, spectra were acquired for HMW complexes of CLU-CS (119 μg/ml), CLU-FGN (164 μg/ml), CLU-GST (110 μg/ml), and solutions of CLU or the individual client proteins at concentrations corresponding to those present in the complexes analyzed (calculated on the basis of the mass ratios of CLU: client protein in the complexes). Estimates of secondary structure were obtained using the program CDSSTR (27).

Thioflavin T Analyses—For thioflavin T fluorescence assays, all samples were prepared at 50 μg/ml in PBS and contained 62.5 μM thioflavin T; fluorescence was measured using a FLUOstar Optima microplate reader (BMG Labtechnologies), with an excitation wavelength of 440 nm and an emission wavelength of 490 nm (slit-width 10 nm). Lysozyme amyloid was formed as described in Ref. 28.

RESULTS

In Vitro Formation of CLU-Client Protein Complexes—When incubated alone at 41 °C, 6 μM CS showed a progressive increase in turbidity (increasing A₃60 nm, Fig. 1A) from 0 to about 500 min. No further increase in turbidity was observed after ~500 min. Under the same conditions, when CLU was present with CS at a near equimolar concentration, no increases in turbidity were detected, indicating that CS was stabilized in solution. At the same concentration, BSA had little effect on the precipitation of CS. Incubation of FGN alone at 45 °C resulted in progressive precipitation of the protein after an initial lag phase of ~200 min, no further increases in turbidity were measured after 800 min (Fig. 1B). Under the same conditions, co-incubation of 6.5 μM CLU with 6.0 μM FGN completely abolished any increase in turbidity; in contrast, 6.5 μM BSA had little effect on the precipitation of FGN. Incubation of 20 μM GST at 60 °C resulted in rapid precipitation of the protein after a lag phase of ~25 min (Fig. 1C). After 40 min, the protein solution reached maximum turbidity and prolonged heating had no further effect. Co-incubation of 20 μM GST with 3.2 μM CLU almost completely inhibited the precipitation of GST under the same conditions. This was in contrast to co-incubation with 3.2 μM BSA, where the protein solution exhibited a similar precipitation profile to that observed when GST was incubated alone. However, the maximum turbidity was marginally less in the presence of BSA. Both CLU and BSA were stable when incubated alone at 41–60 °C (data only shown for 60 °C; Fig. 1C). As first reported by Humphreys et al. (9) the effect of CLU on protein precipitation was dose-dependent for all client proteins (data not shown).

Production of Preheated Protein Controls—To produce preheated protein controls, solutions of individual proteins were heated as described above and insoluble protein removed by filtration through a 0.45-μm filter. There was no difference in the SEC profiles of unheated CS, FGN, GST, or CLU and the residual corresponding proteins remaining in solution after the respective heat treatments (Fig. 2). Also, when the residual, soluble heat-treated proteins were mixed, very little interaction was detected by SEC between CLU and CS (Fig. 2A), CLU and FGN (Fig. 2B), or CLU and GST (Fig. 2C).

Detection of HMW Complexes by SEC—SEC fractionation of the heat-stressed mixtures of CLU and CS (41 °C), CLU and FGN (45 °C), or CLU and GST (60 °C) showed that they con-
tained HMW species eluting at the exclusion limit of the column (≥4 × 10^7 Da) that were absent from the same mixtures left unheated, and from solutions of the individual proteins (Fig. 2). Compared with FGN and GST, co-incubation of CS with CLU at 41 °C produced proportionally less HMW species; however, it was evident that complexes comparable in mass to those formed at higher temperatures by CLU and FGN or GST were also formed by CLU and CS. The exclusion limit peak was collected in each case and represented putative HMW CLU-client protein complexes. The identity of these complexes was confirmed by sandwich ELISA (see below). These complexes were stored in PBS/Az at 4 °C, and their integrity checked at intervals by SEC; under these conditions they remained stable for months.

Sandwich ELISA to Confirm the Identity of CLU-Client Protein Complexes

—Sandwich ELISA designed to capture CLU and subsequently detect CS, FGN, or GST was used to confirm the identity of the putative complexes purified by SEC. Relative to the samples containing the HMW complexes, little absorbance was obtained for control samples (Fig. 3). The traces shown are representative of more than three independent experiments.

Characterization of Clusterin-Client Protein Complexes

formed at higher temperatures by CLU and FGN or GST were also formed by CLU and CS. The exclusion limit peak was collected in each case and represented putative HMW CLU-client protein complexes. The identity of these complexes was confirmed by sandwich ELISA (see below). These complexes were stored in PBS/Az at 4 °C, and their integrity checked at intervals by SEC; under these conditions they remained stable for months.

Sandwich ELISA to Confirm the Identity of CLU-Client Protein Complexes—Sandwich ELISA designed to capture CLU and subsequently detect CS, FGN, or GST was used to confirm the identity of the putative complexes purified by SEC. Relative to the samples containing the HMW complexes, little absorbance was obtained for control samples (Fig. 3).

Estimates of Stoichiometry within Complexes—To estimate the stoichiometry of individual proteins within the HMW complexes, SEC-purified complexes were separated by SDS-PAGE
under reducing conditions, the gels stained with Coomassie Blue, and the intensity of individual protein bands quantified by densitometry. After standard curves were generated for each protein, the approximate mass ratios and molar ratios for each complex were calculated. The mass ratio of CLU to client protein was similar for CS, FGN, and GST complexes (about 1:2 in each case). However, the molar ratios were very different; HMW CLU-CS complexes contained approximately the same number of molecules of CS and CLU, HMW CLU-FGN complexes contained about 3 molecules of CLU for every FGN molecule, while CLU-GST complexes contained 5 molecules of GST for each molecule of CLU (Table 1). These estimated CLU:client protein ratios were employed when selecting controls for structural studies of the HMW complexes.

Size Estimation by Dynamic Light Scattering—The results of DLS were consistent between 0.1 and 1 mg/ml; representative results obtained at 0.5 mg/ml are shown (Fig. 4). In contrast to SEC, DLS was unable to resolve the differently sized oligomers of CLU in solution; DLS analysis of CLU samples indicated a normally distributed particle size. There was no apparent difference in size between native and preheated control proteins. The limitations of DLS in determining the respective diameters of particles of similar size, resulted in a peak corresponding to an intermediate size (compared with the individual proteins) when mixtures of residual preheated CLU and CS FGN or GST were analyzed. DLS indicated that SEC purified HMW CLU-CS and HMW CLU-GST were approximately six times larger than either of their respective components, while HMW CLU-FGN was more than 8 times larger than CLU or FGN (Fig. 4).

**TABLE 1**

| HMW complex | Conditions | Mass ratio (CLU:Client) | Standard error | Standard error |
|-------------|------------|-------------------------|----------------|----------------|
|             |            | **z, n = 3**             |                |                |
| CLU-CS      | 9 h, 41 °C | 1: 1.81                 | 0.027          | 1: 1.17        |
| CLU-FGN     | 12 h, 45 °C| 1: 1.88                 | 0.146          | 1: 0.34        |
| CLU-GST     | 50 min, 60 °C | 1: 2.11               | 0.168          | 1: 5.14        |

**FIGURE 4.** Dynamic light scattering estimates of the mean diameters of HMW CLU-client protein complexes and other proteins. Samples analyzed were SEC-purified HMW complexes of CLU-CS, CLU-FGN, and CLU-GST, unheated CS, FGN, GST, and CLU, preheated CS, FGN, GST, and CLU (*CS, *FGN, *GST, and *CLU, respectively), and mixtures of the preheated client proteins and *CLU. The conditions used to generate the complexes and the preheated proteins were as described under “Experimental Procedures”. There was no significant difference in the mean diameters of *CLU preheated at 41, 45, or 60 °C (only data for *CLU preheated at 60 °C is shown). Histograms represent mean diameter ± range of 9 normally distributed curves.

**FIGURE 5.** Plots showing time-dependent changes in bisANS fluorescence during heating of CS, FGN, GST ± CLU, and CLU alone, in arbitrary fluorescence units (AFU). Samples analyzed were (A) CS or CLU alone or CS co-incubated with CLU, (B) FGN or CLU alone or FGN co-incubated with CLU, (C) GST or CLU alone or GST co-incubated with CLU. Data points shown represent the mean fluorescence of three replicates ± S.E. For each plot the 0 h time value has been normalized to a value of 10,000 AFU. The conditions used to generate the complexes and the preheated proteins were as described under “Experimental Procedures.” + denotes increased bisANS fluorescence compared with the respective 0 h time value (Tukey HSD, \( p < 0.01 \)).
Characterization of Clusterin-Client Protein Complexes

![Graphs showing CD spectra for various samples](image)

FIGURE 6. Far-UV CD spectra. Samples analyzed were (A) CLU and *CLU preheated as indicated, unheated and preheated (B) CS, (C) FGN, and (D) GST; (E) SEC-purified HMW CLU-CS and mixtures of unheated or preheated CS and CLU, (F) SEC-purified HMW CLU-FGN and mixtures of unheated or preheated FGN and CLU, and (G) SEC-purified HMW CLU-GST and mixtures of unheated or preheated GST and CLU. The conditions used to generate the complexes and the preheated proteins were as described under “Experimental Procedures.” The data shown are means of six scans.

Table 2

| Sample          | Helix (%) | Sheet (%) | Turn (%) | Unordered (%) |
|-----------------|-----------|-----------|----------|---------------|
| CS              | 69.45 ± 1.39 | 7.25 ± 1.36 | 7.70 ± 1.01 | 15.93 ± 1.16 |
| *CS             | 63.15 ± 0.98 | 11.43 ± 1.10 | 11.62 ± 0.84 | 12.92 ± 1.05 |
| FGN             | 11.34 ± 0.32 | 41.36 ± 0.77 | 20.21 ± 0.69 | 26.72 ± 1.06 |
| *FGN            | 7.09 ± 0.15 | 32.90 ± 0.12 | 25.25 ± 0.25 | 33.58 ± 0.31 |
| GST             | 13.88 ± 0.24 | 31.88 ± 0.52 | 21.81 ± 0.54 | 29.96 ± 0.66 |
| *GST            | 7.36 ± 0.22 | 34.32 ± 0.39 | 24.58 ± 0.50 | 32.72 ± 0.56 |
| CLU             | 34.19 ± 0.10 | 18.70 ± 0.17 | 19.89 ± 0.19 | 26.69 ± 0.25 |
| *CLU 41 °C      | 39.26 ± 0.23 | 17.18 ± 0.39 | 16.81 ± 0.34 | 26.57 ± 0.52 |
| *CLU 45 °C      | 15.75 ± 0.22 | 30.55 ± 0.47 | 23.83 ± 0.49 | 29.39 ± 0.61 |
| *CLU 60 °C      | 14.69 ± 0.21 | 31.52 ± 0.43 | 23.00 ± 0.45 | 30.38 ± 0.63 |
| HMW CLU-CS      | 30.59 ± 0.21 | 21.26 ± 0.37 | 18.84 ± 0.28 | 29.33 ± 0.44 |
| *CLU-CS         | 63.59 ± 0.67 | 10.15 ± 0.80 | 10.45 ± 0.78 | 15.35 ± 1.08 |
| *CLU-FGN        | 46.98 ± 0.41 | 15.69 ± 0.64 | 15.27 ± 0.51 | 22.45 ± 0.79 |
| HMW CLU-FGN     | 26.25 ± 0.10 | 22.67 ± 0.18 | 21.21 ± 0.19 | 28.82 ± 0.24 |
| *CLU-FGN        | 26.99 ± 0.09 | 22.52 ± 0.19 | 21.15 ± 0.18 | 28.57 ± 0.27 |
| *CLU-GST        | 9.38 ± 0.17 | 32.49 ± 0.22 | 23.85 ± 0.28 | 33.64 ± 0.27 |
| HMW CLU-GST     | 11.55 ± 0.24 | 36.83 ± 0.39 | 21.29 ± 0.38 | 29.32 ± 0.47 |
| *CLU-GST        | 33.80 ± 0.18 | 21.66 ± 0.35 | 18.54 ± 0.40 | 25.75 ± 0.54 |
| *CLU-GST        | 11.78 ± 0.25 | 29.48 ± 0.38 | 25.93 ± 0.49 | 32.70 ± 0.46 |

Table 2. However, heating at 41 °C only produced small increases in the predicted content of β-sheet and β-turn structure (Fig. 6A and Table 2). Preheated CS had a CD spectrum shown). In all cases, at times when preheated soluble client protein showed increased bisANS fluorescence, the corresponding fluorescence of the respective preheated mixtures of CLU and client protein showed a lesser change. This suggests that the interaction between CLU and partially unfolded client proteins in these mixed solutions reduces the extent to which hydrophobic regions on the client proteins are exposed to solvent.

Thioflavin T analyses were carried out to determine if SEC-purified complexes possessed any amyloid-like characteristics (i.e. contained β-sheet rich structures). SEC-purified HMW CLU-FGN and CLU-GST complexes and the relevant native and preheated protein controls all produced less than 8% of the fluorescence arising from a sample of lysozyme amyloid present at the same mass concentration (data not shown).

Circular Dichroism Spectrophotometry—The CD spectrum for unheated CLU indicated high α-helical content with minima at ~208 nm and ~222 nm. The molar ellipticity at these wavelengths slightly increased when CLU was preheated at 43 or 60 °C. Under these conditions, CDSSTR analysis predicted a large decrease in α-helical content, a similarly large increase in predicted β-sheet content and smaller increases in the predicted contents of β-turn and unordered structure (Fig. 6A and
Characterization of Clusterin-Client Protein Complexes

Measurements of bisANS fluorescence indicated that heat treatment induced CS, FGN, and GST to expose more hydrophobicity to solution (Fig. 5). However, at least for FGN and GST, after 4 h of heating the level of solvent-exposed hydrophobicity had returned to that of the respective time 0 samples (or less). This may be because at these later time points a significant fraction of the client protein had precipitated from solution and was no longer available to bind bisANS. The bisANS fluorescence of solutions of CLU alone heated at 41 and 60 °C did not show any significant changes over the time courses measured (Fig. 5, A and C). Furthermore, although there were some statistically significant differences measured, there was no large or consistent change in the bisANS fluorescence of CLU heated alone at 45 °C (Fig. 5B). A clear trend in all three cases is that the bisANS fluorescence of mixtures of CLU and client protein increased significantly less during heating than was seen for corresponding heated solutions of client protein alone (Fig. 5). Thus, the data shown strongly suggest that co-incubation with CLU reduced the extent to which client proteins exposed hydrophobicity to solution when heated. Therefore it appears likely that the molecular interactions involved in the formation of CLU-client protein complexes either shield hydrophobic regions on the client protein from exposure to solution and/or prevent structural changes that would otherwise occur to result in their exposure. Previous work has implicated the binding of CLU to regions of exposed hydrophobicity on client proteins as an integral part of its chaperone action (10).

Remarkably, the CD spectra acquired indicated that HMW CLU-FGN complexes had a predicted overall content of the various secondary structures indistinguishable from that of a mixture of native CLU and FGN at the same concentrations (Fig. 6F). The most likely interpretation of this result is that, under the conditions tested, the interaction between CLU and FGN resulted in a mutual stabilization of secondary structures. Interestingly, the situation was quite different when examining complexes formed between CLU and CS or GST. In these cases, the predicted loss of overall α-helical structure in the complexes was the same or greater than that in the corresponding mixture of preheated CLU and soluble preheated client protein. However, the complexes had more predicted β-sheet content and slightly less β-turn and unordered structure than the corresponding mixture of previously heated proteins (Table 2). The differences observed may be due to the differing inherent stabilities of the client proteins and the nature of secondary structure of unfolded intermediate states they display.

Especially under conditions of pathological stress, but also under normal physiological conditions, it is likely that in extra-cellular fluids CLU-client protein complexes will form as a mechanism to combat the development of insoluble protein aggregates, which can give rise to a variety of disease conditions (8). Results presented here suggest that CLU may interact with unfolding proteins at different points along their respective unfolding pathways. Depending on the point at which this interaction occurs, CLU may maintain the native secondary structures of the client protein or stabilize the client in some other non-native but stable conformation. The interactions between CLU and the client protein are likely to involve CLU shielding regions of exposed hydrophobicity.

Discussion

Although CS (normally intracellular), FGN (normally extra-cellular and highly glycosylated), and GST (recombinant) are very different proteins with a large discrepancy in mass (~52, 340, and 23 kDa, respectively), and the complexes with CLU were formed using very different conditions (41, 45, or 60 °C, respectively), in all cases the estimated mass stoichiometry of CLU-client protein in the complexes was about 1:2 (Table 1). In other words, in each case, CLU formed soluble complexes in which it “carried” about twice its own mass in the form of client protein. The molar ratio of CLU-client protein was quite different for each type of complex (Table 1), suggesting that the relative total masses of CLU and client protein limit the structure of the complexes and not their respective molar ratios. However, investigation of a larger number of client proteins is needed to confirm this interpretation. SEC indicated that all types of client protein generated complexes of $\approx 4 \times 10^7$ Da in vitro (Fig. 2). DLS suggested that CLU-FGN complexes had a diameter approximately twice that of CLU-CS or CLU-GST complexes (~100 versus 50 nm; Fig. 4). At this scale, the soluble CLU-client protein complexes are very large indeed, being a similar size to virus particles.

Characterization of Clusterin-Client Protein Complexes

superimposable on that of unheated CS (Fig. 6B). In contrast, following heating at 45 °C, FGN showed a change in minima from ~222 nm to ~200 nm, consistent with the following predicted changes: a small decrease in α-helical content, a moderate decrease in β-sheet, and small increases in β-turn and unordered structure content (Fig. 6C and Table 2). After heating at 60 °C, GST also showed a change in minima from that typical of high α-helical to a more disordered structure. CDSSTR predicted a substantial decrease in α-helical content and much smaller increases in β-sheet, β-turn, and unordered structure content (Fig. 6D and Table 2). Comparing the CD spectra of SEC-purified HMW CLU-CS complexes with those of mixtures of the native or soluble preheated client proteins suggested that secondary structure was significantly altered in HMW CLU-CS complexes (Fig. 6E). This was largely attributed to a predicted loss of more than half the native α-helical content, compared with the unheated proteins (Table 2). Analysis of the spectrum of a mixture of soluble preheated CS and preheated CLU predicted that, relative to the CLU-CS complex, the proteins had a smaller decrease in α-helical content and an increase in unordered structure. In contrast the CD spectrum for HMW CLU-FGN complexes was virtually superimposable on that of a mixture of native CLU and FGN (Fig. 6F and Table 2). However, a corresponding mixture of preheated CLU and preheated FGN had a very different CD spectrum, consistent with the loss of predicted structure for the individual proteins following heating. It was evident that the formation of HMW CLU-GST complexes was accompanied by changes in secondary structure (Fig. 6G). For these complexes, relative to a mixture of the native proteins, there were predicted significant losses of α-helical content, a large increase in β-sheet content, and small increases in β-turn and unordered structure (Table 2). The CD spectrum and predicted losses of secondary structure were similar for HMW CLU-GST complexes and a mixture of soluble preheated GST and CLU.
Physiological factors such as macromolecular crowding and shear stress are likely to favor protein aggregation in vitro compared with low concentrations of purified proteins in simple buffers (2, 29, 30). Thus, like many other studies of chaperone action, we used elevated temperature to induce client proteins to unfold and interact with CLU in vitro. CLU is very heat stable and heating up to at least 60 °C does not inhibit its chaperone action (9, 12). Mammals experience sporadic increases in body temperature as a result of physical activity (31), environmental exposure (32), and infection (33); fevers of up to 42 °C have been reported (34). Thus, the mild heat stress (41 °C) used to induce the precipitation of CS is encountered physiologically. Despite the differing conditions required for their formation in vivo, all three types of complexes shared the following features: a CLU: client protein mass ratio of 1:2, very large size (≈4 × 10^7 Da) by SEC, and diameters of 50–100 nm by DLS, and reduced exposed hydrophobicity on the client protein (compared with client protein heated alone). It appears reasonable to expect that these shared characteristics give us important insights into the biophysical properties of CLU-client protein complexes in general and add to our mechanistic understanding of the chaperone action of CLU.

In unpublished work, we have shown that when human plasma is “stressed” by gentle rotation for 10 days at 37 °C, the plasma contains CLU-FGN complexes (detected by sandwich ELISA) and when fractionated by SEC both CLU and FGN are present in fractions corresponding to molecules of ≈4 × 10^7 Da.5 Thus, it appears likely that CLU-client protein complexes generated in situ in plasma are likely to include species of the sizes reported here for complexes formed in vitro from purified proteins. It is not possible to purify intact CLU-client protein complexes from plasma by immunoaffinity chromatography because the harsh elution conditions (2 M GdHCl) will at least partly disrupt intermolecular interactions. Inappropriately aggregating proteins can be cytotoxic and also give rise to large pathological deposits that can interfere with organ/tissue function (35–39). In vivo, the sequestration of misfolded proteins into large, soluble complexes with extracellular chaperones like CLU is likely to be the first step in preventing them from forming toxic or otherwise pathological aggregates. On the basis of evidence reviewed elsewhere, we have proposed that these soluble complexes are probably rapidly cleared from the body by receptor-mediated endocytosis and subsequent lysosomal degradation (8, 40). It will be important to demonstrate that this process operates in a whole animal model, this work is currently under way.

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