The Acute Phase Protein Haptoglobin Is a Mammalian Extracellular Chaperone with an Action Similar to Clusterin †

ARTICLE *in* BIOCHEMISTRY · SEPTEMBER 2005

Impact Factor: 3.02 · DOI: 10.1021/bi050764x · Source: PubMed

5 AUTHORS, INCLUDING:

Justin J Yerbury
University of Wollongong
39 PUBLICATIONS **971** CITATIONS

SEE PROFILE

Mark R Wilson
University of Wollongong
98 PUBLICATIONS **3,650** CITATIONS

SEE PROFILE

The Acute Phase Protein Haptoglobin Is a Mammalian Extracellular Chaperone with an Action Similar to Clusterin†

Justin J. Yerbury,‡ Mark S. Rybchyn,§ Simon B. Easterbrook-Smith,§ Cindy Henriques,|| and Mark R. Wilson*,‡

School of Biological Sciences and the Institute for Biomolecular Science, University of Wollongong, Northfields Avenue, Wollongong, New South Wales 2522, Australia, School of Molecular and Microbial Biosciences, University of Sydney, Sydney, New South Wales 2006, Australia, and Centenary Institute of Cancer Medicine and Cell Biology, Locked Bag number 6, Newtown, New South Wales 2042, Australia

Received April 27, 2005; Revised Manuscript Received June 9, 2005

ABSTRACT: Haptoglobin (Hp) is an acidic glycoprotein present in most body fluids of humans and other mammals. Although the functions of Hp are not yet fully understood, the available evidence indicates that it is likely to play an important role in suppressing inflammatory responses. Some earlier work suggested that Hp might be a newly identified member of a small group of extracellular chaperones found at significant levels in human body fluids. Previously, the only well-characterized member of this group was clusterin, which shares functional similarities with the small heat-shock proteins. We report here that Hp specifically inhibited the precipitation of a variety of proteins induced by either heat or oxidation, including proteins in unfractionated human serum. We also show that, like clusterin, Hp (i) inhibits the precipitation of stressed proteins by forming solubilized high molecular weight complexes with them, (ii) cannot protect enzymes from heat-induced loss of function, and (iii) lacks ATPase activity and the ability to independently refold proteins following stresses. Furthermore, we show that Hp has maximum chaperone activity at mildly alkaline pH and, unlike clusterin, does not undergo significant changes in oligomerization state coincident with pH-induced changes in chaperone activity. Our results raise the possibility that Hp may exert an anti-inflammatory action *in vivo* by inhibiting the inappropriate self-association of "damaged" (misfolded) extracellular proteins.

Haptoglobin (Hp)[1] is an acidic glycoprotein present in most body fluids of humans and other mammals. Individual humans (but not other mammals) express one of three major phenotypic forms of Hp, designated Hp1-1, Hp2-1, and Hp2-2. These phenotypes occur as a consequence of two alleles (HP 1 and HP 2); a crossing-over event is thought to have generated the variant alleles by transposing a large part of the sequence encoding the α chain (*1*). In its simplest form, Hp1-1 is a tetramer comprised of two (light) α^1 and two (heavy) β chains linked by disulfide bonds; Hp2-1 and Hp2-2 are disulfide-linked polymerized forms of higher molecular mass. Hp2-2 lacks α^1 chains but contains higher mass α^2 chains instead; the most common phenotype, Hp2-1, contains both α^1 and α^2 chains and is thought to be comprised of a series of polymeric forms of the formula $(\alpha^1{\cdot}\beta)_2{\cdot}(\alpha^2{\cdot}\beta)_n$, where $n = 0, 1, 2, 3, \ldots$ (*2*). The levels of Hp in human plasma are increased to 8-fold during inflammation, various infections, trauma, tissue damage, and in association with neoplasia, leading to Hp being designated as an "acute phase protein" (*1*, *2*). It has been reported that some animals (e.g., cattle) do not constitutively synthesize Hp but only do so in response to stresses such as inflammation (*3*).

The best known ligand of Hp is hemoglobin (Hb), which it binds to with extremely high affinity [$K_D \sim 10^{-15}$ M (*1*)]. Previously, it was widely believed that a primary biological function of Hp was to clear the body of vascular Hb released from damaged red blood cells. However, recent work has shown in a mouse model that the absence of Hp expression had no significant effect on the clearance of Hb following experimentally induced severe hemolysis; the Hp knock-out mice showed substantially higher mortality under these conditions, which appeared to result from greater oxidative stress (*4*). Formation of the Hp−Hb complex inhibits Hb-mediated generation of lipid peroxides and hydroxyl radical, which is thought to occur in areas of inflammation (*2*). Thus, the available evidence indicates that Hp is likely to exert an important anti-inflammatory action *in vivo* by inhibiting oxidative damage mediated by free Hb (*5*). A variety of other putative biological functions have also been ascribed to Hp. It has been implicated in immune regulation (*6*), shown to inhibit cathepsin B activity (*7*), and to have pro-angiogenic effects (*8*). Binding of Hp to human neutrophils has been reported to inhibit respiratory burst activity (*9*). In addition, neutrophils have been shown to take up exogenous Hp and store it within cytoplasmic granules; they subsequently secrete it into the local extracellular environment in response to a variety of pro-inflammatory stimuli [e.g., yeast, TNFα,

† Parts of this work were supported by project grants from the Australian Research Council to S.B.E. (DP0208572) and M.R.W. (DP0211310).

* To whom correspondence should be addressed. Telephone: 61-242-214534. Fax: 61-242-214135. E-mail: mrw@uow.edu.au.

‡ University of Wollongong.

§ University of Sydney.

|| Centenary Institute of Cancer Medicine and Cell Biology.

[1] Abbreviations: CS, citrate synthase; GST, glutathione-*S*-transferase; Hp, haptoglobin; lys, lysozyme; ovo, ovotransferrin; PBS, phosphate-buffered saline.

10.1021/bi050764x CCC: $30.25
Published on Web 07/21/2005

or the chemotactic peptide fMLP (*10*, *11*)]. Thus, although the functions of Hp are not yet fully understood, the available evidence indicates that it is likely to play an important role in suppressing inflammatory responses.

We have described and characterized the chaperone action of clusterin, a widely distributed and highly conserved glycoprotein found at high levels in human blood (*12*−*15*). Some recent work suggested that Hp might also have chaperone activity, which, if true, would make it one of a very few known mammalian chaperones present in human body fluids at substantial levels. Both Hp and clusterin are (i) comprised of disulfide-linked α and *β* chains, (ii) heavily glycosylated [Hp and clusterin are 20 and 30% sugar by mass, respectively (*1*, *16*)], (iii) constitutively present at high levels in human plasma [Hp at 0.3−1.9 mg/mL (*1*) and clusterin at 60−140 *μ*g/mL (*17*)], and (iv) expressed at higher levels during a variety of stresses and disease states (*2*, *18*). Given these similarities, the recent suggestion that Hp might be a chaperone appeared worthy of a closer examination. Previous studies of the putative chaperone action of Hp were restricted to only two stressed proteins and did not demonstrate that, under the conditions tested, the effects measured were specific to Hp (*19*, *20*). The primary aims of this study were to establish whether Hp is a genuine extracellular chaperone and, if so, to compare its mechanisms of action with those of clusterin. To determine whether Hp had a genuine chaperone activity, we assayed the aggregation of a variety of proteins subjected to stresses, including unfractionated proteins in human serum, and tested the effects of Hp and control proteins in these systems. We also investigated whether Hp, like clusterin and the small heat-shock proteins (sHSPs), binds preferentially to stressed proteins to form solubilized high molecular weight complexes. In addition, we tested whether Hp had any ability to inhibit the heat-induced loss of enzyme activity or to refold denatured enzymes and whether ATP might be required for either of these processes. Last, because it is known that clusterin is activated by mildly acidic pH (*15*), we tested the effects of pH on the structure and chaperone action of Hp. This report establishes that Hp exerts a genuine chaperone action, provides insights into the mechanism of this action, and identifies similarities and differences between it and that of clusterin and the sHSPs.

MATERIALS AND METHODS

Materials. Human serum was obtained from Wollongong Hospital (Wollongong, NSW, Australia) and stored frozen at −20 °C until use. CNBr-activated Sepharose, human hemoglobin, ovotransferrin (ovo), lysozyme (lys, from chicken egg white), catalase (from bovine liver), citrate synthase (CS, from porcine heart), alcohol dehydrogenase (from bakers' yeast), and superoxide dismutase (SOD, from bovine erythrocytes) were purchased from Sigma (MO). Complete protease inhibitor cocktail was obtained from Roche (Sydney, Australia).

Glutathione-*S*-transferase (GST) from *Schistosoma japonicum* was prepared by thrombin cleavage of recombinant Jun leucine zipper-GST fusion protein and purified by GSH-agarose-affinity chromatography as described in ref *21*. ovo and GST were biotinylated using NHS-LC-biotin (Pierce, Sydney, Australia) following the instructions of the manufacturer. Streptavidin-agarose was purchased from Oncogene (Merck, Sydney, Australia). Rabbit polyclonal anti-hemoglobin antibody (IgG fraction) was obtained from Dako (Sydney, Australia). The 3A8 murine hybridoma, which secretes a monoclonal antibody reactive with human haptoglobin, was obtained as a kind gift from Dr. Ludwig Wagner (University of Vienna, Austria). A plasmid-encoding mutant (chaperone inactive) A30P α synuclein was a gift from Dr. Robert Cappai (Department of Pathology, University of Melbourne, Melbourne, Australia). A30P α-synuclein protein was expressed in *Escherichia coli* and purified by acid precipitation as described in ref *22*. 4,4′-Bis(1-anilinonaphthalene-8-sulfonate) (bisANS) was obtained from Molecular Probes (Eugene, OR). Human clusterin was purified by immunoaffinity chromatography from human serum as previously described (*23*).

Purification of Hp. Hp was purified from human serum using a well-established hemoglobin-affinity chromatography method; this method yields native Hp, which retains the ability to bind to hemoglobin with high affinity (*24*). Briefly, human serum (80 mL) was diluted 3:1 with 10 mM Tris-HCl at pH 7 (TB) containing 5 M NaCl and then mixed with Hb-Sepharose (5 mL, containing about 50 mg of bound Hb) on an end-over-end stirrer for 1 h at 4 °C. Human Hb was bound to CNBr-activated Sepharose using standard methods. The Hb-Sepharose was then washed with TB and unwanted Hb-binding proteins eluted with TB containing 1.6 M guanidine-HCl. Hp was eluted using 10 mM sodium acetate at pH 5 containing 3.5 M guanidine-HCl. The eluate was dialyzed extensively against TB and any Hp−Hb complexes subsequently removed by immunoaffinity chromatography using anti-Hb antibody-Sepharose. The Hp concentration was determined by absorbance at 280 nm using the molar extinction coefficient 5.1×10^4 (corresponding to a 50-kDa $\alpha\beta$ dimer) (*25*).

Protein Precipitation Assays: Heat Stress. CS (3 *μ*M) with or without Hp2-1 in CS buffer (14 mM Na_2HPO_4, 3.2 mM Tris, 26 mM NaCl, and 0.6 mM EDTA at pH 7.4) was heated in the wells of a 96-well microplate (100 *μ*L/well) at 43 °C, and protein precipitation was measured as turbidity (A^{360}), quantified using a Spectramax 250 plate reader (Molecular Devices, CA). GST (7.8 *μ*M), with or without Hp2-1, in phosphate buffer (50 mM Na_2HPO_4 at pH 7.4), was heated at 55 °C for 20 min in an automated seven-chambered diode array spectrophotometer (Hewlett−Packard GMBH, Germany) and A^{360}-measured as a function of time. Similarly, ovo (13 *μ*M), with or without Hp2-1, in phosphate buffer, was heated to 60 °C for 35 min, and protein precipitation was measured as above. In other experiments, ovo (3.3 *μ*M, 0.25 mg/mL) with or without Hp1-1, Hp2-1, or Hp2-2 (all at 40 *μ*g/mL), also in phosphate buffer, was heated to 60 °C for 30 min and A^{360} measured as a function of time; in all subsequent experiments, the Hp2-1 phenotype was used.

Oxidative Stress. lys (70 *μ*M), with or without Hp2-1, was incubated in the wells of a 96-well microplate (100 *μ*L/well) for 18 h at room temperature in oxidative stress buffer (1 mM H_2O_2, 2 mM EDTA, 2 mM Na ascorbate, and 2 mM $FeCl_3$), which produces hydroxyl radicals (*26*); changes in A^{360} were measured in a Spectramax 250 microplate reader. To confirm that the effects of Hp were specific, in some experiments, SOD or the chaperone-inactive A30P mutant of α synuclein (*22*) were used as control proteins. These

were added to the following final concentrations for each of the indicated substrates: 6.7 μM (CS), 17 μM (GST), 8.3 μM (ovo), and 33 μM (lys), with other conditions as specified above.

The Effects of pH on the Ability of Hp To Inhibit Heat-Induced Precipitation of Proteins. CS (0.3 mg/mL) with or without Hp2-1 (0.15 mg/mL) in CS buffer (adjusted to pH 6.0−7.5) was heated at 43 °C, and precipitation was measured using a Spectramax 250 microplate reader. In other experiments, ovo (1 mg/mL) with or without Hp2-1 (0.25 mg/mL) in phosphate buffer (adjusted to pH 6.0−8.0) was heated to 60 °C in either quartz cuvettes or a 384-well microplate, and precipitation was measured using a diode array spectrophotometer (as described above) and a FluoStar microplate reader (BMG Labtech, Melbourne, Australia), respectively.

Precipitation of Proteins in Whole Human Serum. To selectively deplete human serum of Hp, an immunoaffinity column bearing the monoclonal anti-Hp antibody 3A8 was used. 3A8 was purified from tissue culture supernatant using protein G chromatography and then coupled to CNBr-activated Sepharose using standard methods. Unfractionated normal human serum (NHS; Hp2-1 phenotype, 5 mL, supplemented with Complete protease inhibitor cocktail) was passed repeatedly over a 3 mL packed volume column of Sepharose CL 4B (control) or 3A8-Sepharose (to remove Hp). To minimize dilution, on the first pass, in each case, the first 1.5 mL eluted was discarded; this procedure resulted in negligible dilution of the total protein concentration in the serum fractions collected. The yield of Hp from this procedure was about 2.0 mg/mL of serum processed. The 3A8 column was subsequently washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4 at pH 7.4) and bound Hp eluted with 2 M guanidine hydrochloride in PBS. Eluted Hp was dialyzed extensively against PBS. There was no difference in chaperone activity between Hp purified by Hb-Sepharose versus 3A8-Sepharose chromatography (data not shown). To confirm depletion of Hp from serum, 10 μL aliquots of sera were separated under nonreducing conditions on a 7.5% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Sartorius, Melbourne, Australia), which was subsequently blocked using 1% (w/v) heat-denatured casein in PBS (HDC/PBS). The blocked membrane was probed using tissue-culture supernatant containing 3A8 antibody followed by a 1:2000 dilution of sheep-anti-mouse Ig-horseradish peroxidase conjugate (Silenus, Melbourne, Australia) in HDC/PBS. Bound secondary antibody was detected using an enhanced chemiluminescence kit (Pierce, IL). Aliquots (50 μL) of control NHS or haptoglobin-depleted serum (HDS), prepared from the same batch of serum, were diluted 1:2 with PBS, supplemented with a final concentration of 7.5 mM sodium azide and incubated in Eppendorf tubes at 43 °C for 48 h. The precipitate was recovered from the samples by filtering them using 0.45 μm ULTRAFREE centrifugal filtration units (Millipore, Sydney, Australia). Each filter was washed with 3 × 500 μL aliquots of PBS before solubilizing the filtered and washed precipitate with 4 M guanidine hydrochloride in PBS (heated at 60 °C for 2 h). Protein content was determined using a bicinchoninic acid microprotein assay (*27*).

Detection of High Molecular Weight Complexes Formed Between Hp and Stressed Proteins: HPLC. Size-exclusion chromatography (SEC) was used as a first step to investigate the formation of high molecular weight complexes between Hp and stressed proteins. ovo (0.5 mg/mL), Hp2-1 (0.25 mg/mL), and mixtures of both Hp2-1 and ovo (at the same final concentrations) in phosphate buffer, were incubated for 1 h at either 37 or 60 °C. Using the same buffer, GST (0.2 mg/mL), Hp2-1 (0.5 mg/mL), and mixtures of GST and Hp2-1 (at the same final concentrations) were incubated at 37 or 57 °C for 40 min. All samples were centrifuged at 10000*g* for 5 min immediately before analysis. A Biosep-SEC-S4000 column (Phenomenex, CA) was equilibrated with 50 mM Na_2HPO_4 at pH 7.4 and a 100 μL sample volume loaded onto the column using a Shimadzu VP series HPLC system (Shimadzu, Kyoto, Japan). Chromatography was performed at room temperature with a flow rate of 0.5 mL/min.

Affinity Adsorption. Purified Hp2-1 (0.5 mg/mL) or mixtures of Hp2-1 (0.5 mg/mL) and either biotinylated ovo (1 mg/mL) or GST (0.2 mg/mL) (in a total volume of 60 μL of PBS) were heated at 60 °C for 1 h (Hp, Hp + GST) or 2 h (Hp + ovo), or incubated at room temperature for the same periods of time (controls). All samples were then centrifuged (5 min at 10000*g*) to remove insoluble material and shaken for 1 h at room temperature with streptavidin-agarose (50 μL packed volume). The streptavidin-agarose was washed by centrifugation 3 times with PBS and then boiled in SDS−PAGE sample buffer for several minutes to elute bound protein, which was subsequently analyzed by SDS−PAGE under nonreducing conditions.

Testing the Effects of Hp on Heat-Induced Loss and Subsequent Recovery of Enzyme Activity. Catalase and GST were heated in the presence or absence of Hp2-1, and the remaining enzyme activity was assayed. Catalase (150 μg/mL in 44 mM Na_2HPO_4, 21 mM NaCl, 50 mM KCl, and 5 mM $MgCl_2$ at pH 7.4) was incubated for 15 min at either 37 °C (unstressed control) or 55 °C (heat stressed), in the presence or absence of 300 μg/mL Hp2-1 (which is sufficient to inhibit most precipitation of catalase under these conditions). Immediately after incubation, samples were diluted 1:1 in refolding buffer (50 mM Na_2HPO_4, 50 mM KCl, and 5 mM $MgCl_2$ at pH 7.4). To assay catalase activity, 50 μL samples of the above solutions were taken at intervals and added to 0.12% (v/v) H_2O_2 in 50 mM Na_2HPO_4 buffer at pH 7.4; the consumption of H_2O_2 was measured as a decrease in absorbance at 210 nm. Similarly, GST (200 μg/mL in 40 mM Na_2HPO_4, 35 mM NaCl, 50 mM KCl, and 5 mM $MgCl_2$ at pH 7.4) was incubated for 5 min at either 37 or 57 °C in the presence or absence of 500 μg/mL Hp2-1 (which is sufficient to inhibit most GST precipitation under these conditions). The samples were then immediately diluted 1:1 in refolding buffer. To assay GST activity, 200 μL samples were taken at intervals and added to 800 μL of 2 mM glutathione, 2 mM 1-chloro-2,4-dinitrobenzene, and 50 mM Na_2HPO_4 at pH 7.4 and the production of 1-*S*-glutathionyl-2,4-dinitrobenzene was measured as an increase in absorbance at 340 nm. For both catalase and GST, in some experiments, 2 mM ATP was included in the buffer during the initial incubation and also in the refolding buffer.

ATPase Assays. Production of ADP from ATP was measured using an enzyme-coupled assay in which ADP production is linked to oxidation of NADH. The ATPase

reaction buffer contained 2 mM Hepes at pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 10 μM EDTA, 170 μM ATP, 840 μM phosphoenol pyruvate, 105 μM NADH, 37.4 units/mL of lactate dehydrogenase, and 31.5 units/mL of pyruvate kinase (*28*). The reaction buffer (0.8 mL) was held in a quartz cuvette, maintained at 37 °C, and NADH oxidation was monitored as a decrease in absorbance at 340 nm, measured as a function of time after (i) no additions or (ii) the addition of a final concentration of 20 μg/mL Hp2-1. The validity of this assay was confirmed by showing that (i) addition of exogenous ADP (21 nmol) or (ii) generation of ADP from ATP through phosphorylation of creatine (1.1 μmol added) catalyzed by creatine phosphokinase (0.87 units added) both led to NADH oxidation (data not shown). To test the possibility that Hp might exhibit ATPase activity only when complexing with stressed proteins, a 20 μL aliquot of a mixture of Hp2-1 (0.8 mg/mL) and ovo (1.0 mg/mL) that had been heated at 60 °C for 30 min was added to the ATPase reaction buffer above and the assay was performed as described.

Dynamic Light Scattering (DLS). Samples of Hp2-1 in phosphate buffer (adjusted to pH 6.0−7.5) were analyzed by DLS using a Zetasizer Nano ZS and Dispersion Technology software version 3.00 (Malvern Instruments Ltd., U.K.). Solutions were passed through a 0.2 μm pore-size filter immediately before analysis. For the purpose of these analyses, Hp was assigned a typical protein refractive index of 1.45 (*29*).

Circular Dichroism (CD) Spectroscopy. A Jasco J-720 spectropolarimeter, linked to a Neslab RTE-111 cooling system, was used to acquire CD data. Far-UV (190−260 nm) CD studies were performed using a 1 mm cell with Hp2-1 at 200 μg/mL in 5 mM sodium phosphate at pH 7.5 or 5 mM MES at pH 6.0 and 20 °C. Spectra were acquired at 20 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 CD spectral data using CDSSTR (*30*) and CONTIN-LL (*31*).

Fluorescence Measurements. The fluorescence of bisANS bound to Hp was measured using an Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Victoria, Australia). Samples of Hp2-1 (100 μg/mL in either 50 mM MES and 50 mM NaCl at pH 6.0 or 50 mM phosphate and 50 mM NaCl at pH 7.5) were maintained at 20 °C. bisANS was added to give various concentrations up to 16 μM, and after each addition, the fluorescence of the samples at 500 nm was measured with an excitation wavelength of 385 nm. The data shown have been corrected for the fluorescence of control samples containing bisANS only. Equation 1, which describes the binding of bisANS to a single class of binding sites on Hp, was fitted to the data by nonlinear regression analysis using SigmaPlot version 8.02 (SPSS, Chicago, IL)

$$F = F_{max}[\text{bisANS}]/(K_d + [\text{bisANS}]) \quad (1)$$

In this equation, F, F_{max}, and K_d are the observed fluorescence, the fluorescence at saturating concentrations of bisANS, and the apparent dissociation constant, respectively.

RESULTS

Hp Protects Proteins from Stress-Induced Precipitation. Supraphysiological temperatures (and other stresses) will induce proteins to unfold and subsequently aggregate. For a given heat stress, different proteins will unfold at different rates, depending on their individual structural stability. The available evidence indicates that, although different proteins may require a greater or lesser heat stress to unfold at comparable (experimentally convenient) rates, the pathways of unfolding remain the same regardless of the temperature (*32*). We tested the ability of Hp to inhibit the aggregation of several different proteins induced by heating to 43−60 °C and also one protein induced to aggregate by oxidative stress. Like clusterin (*15*), Hp is very structurally stable; it does not precipitate when heated for extended periods at 60 °C (data not shown). Furthermore, even after prolonged heating at 70 °C, there is only a small change in its CD spectrum and it actually has a slightly enhanced ability to inhibit protein precipitation (*33*).

Heating CS at 43 °C resulted in a gradual precipitation of protein, shown by an increase in absorbance at 360 nm (A^{360}), reaching a maximum after about 120 min [Figure 1A (i)]. GST at 55 °C produced extensive protein precipitation within 5−10 min [Figure 1A (ii)]. In the absence of Hp (trace represented by ◆), the reduction in A^{360} after about 5 min corresponded to the formation of large macroscopically visible aggregates, with a concomitant "clearing" of finer microaggregates from suspension. Heating ovo at 60 °C resulted in extensive protein precipitation within 30 min [Figure 1A (iii)]. Oxidative stress produced a slow precipitation of lys, which approached a maximum over a period of 10−20 h [Figure 1A (iv)]; when Hp2-1 was incubated alone, a low level of turbidity (less than 15% of that detected for lys alone) was detected after 20 h of oxidative stress (data not shown). Hp2-1 gave dose-dependent inhibition of heat-induced precipitation of GST, ovo, and CS [Figure 1A (i−iii)] and oxidative stress-induced precipitation of lys [Figure 1A (iv)]. In each case, further increases in the ratio of Hp to substrate protein further reduced the extent of substrate protein precipitation (data not shown). These effects were specific to Hp2-1 because SOD and the A30P mutant of α synuclein did not prevent precipitation of any of the target proteins under these conditions (data for SOD represented in Figure 1A (i, iii, and iv) as ◇; data for SOD/GST and for the effects of A30P on all proteins tested are not shown).

Because many chaperones exist in solution as aggregates of an ill-defined number of monomers, a convention that has been adopted when dealing with the interactions between chaperones and other proteins is to define stoichiometry in relation to the individual subunits of the chaperone and the protein with which it interacts [the subunit molar ratio (SMR) (*12*)]. In the case of human Hp, this is complicated because it occurs in three different phenotypes comprised of different and variable assemblies of α and β chains (*1*, *2*). Most experiments were done with Hp2-1; in these cases, for simplicity, when molar ratios were required for calculations of SMR, we based our calculations on the assumption that each Hp "subunit" is comprised of $\alpha^2\beta$ [with a mass of about 50 kDa, inclusive of glycosylation (*1*)]. The potency of the inhibition of target protein precipitation by Hp2-1 varied, depending on the target protein. This is shown in Figure 1B, in which the percent inhibition of turbidity (A^{360}) by Hp2-1 at the end of each of the time courses shown in Figure 1A is plotted as a function of the SMR of the target protein to

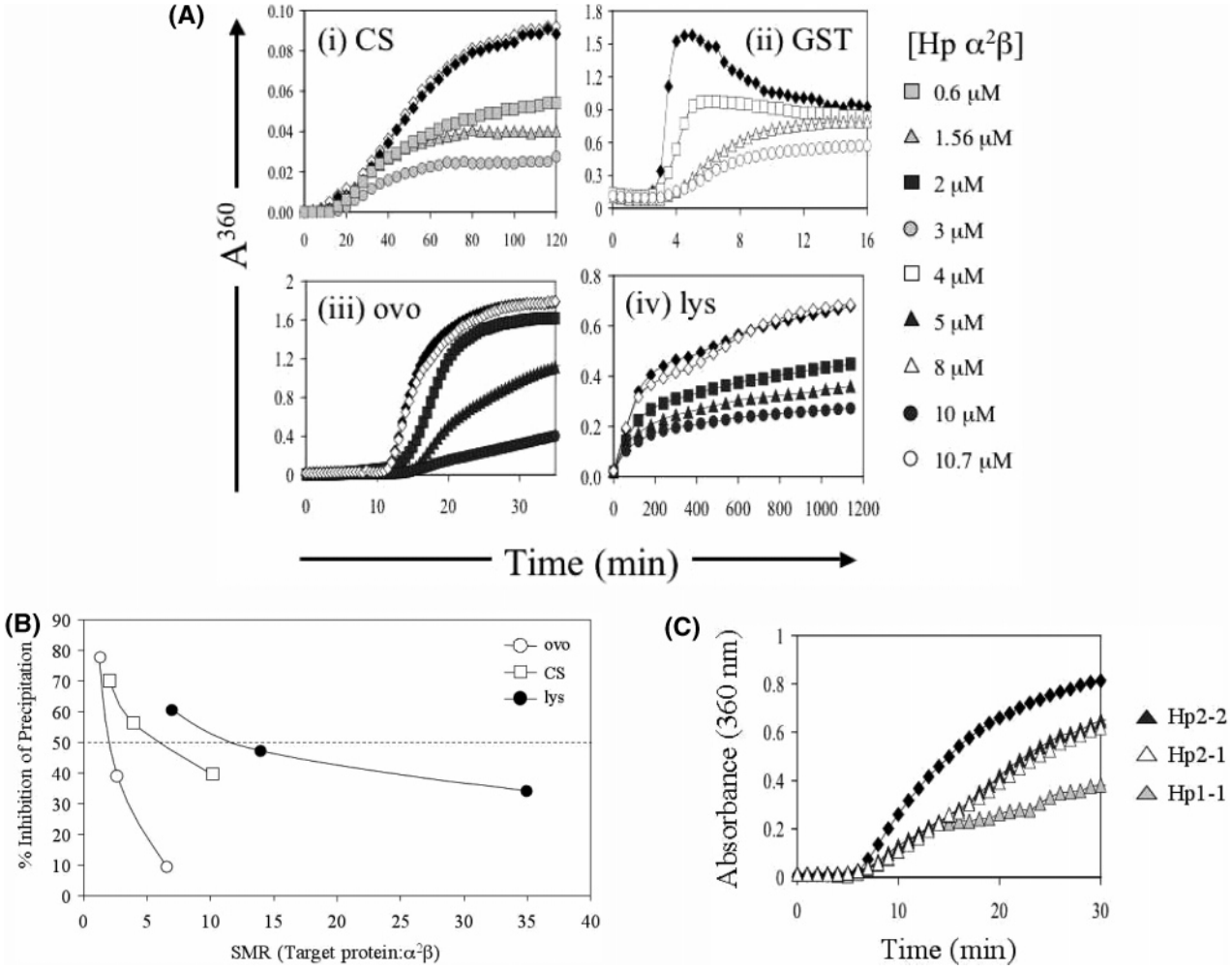

FIGURE 1: Inhibition of stress-induced protein precipitation by Hp. (A) Turbidity (measured as A^{360}) as a function of time for (i) CS (3 μM) and Hp2-1 heated at 43 °C, (ii) GST (7.8 μM) and Hp2-1 heated at 55 °C, (iii) ovo (13 μM) and Hp2-1 heated at 60 °C, and (iv) lys (70 μM) and Hp2-1 exposed to oxidative stress as described in the Materials and Methods. The key indicates the molar concentrations of the Hp2-1 "subunit" (assumed to be comprised of $\alpha^2\beta$ with a mass of 50 kDa) used. In all panels, ◆ represents the result in the absence of Hp or other additions. On panels i, iii, and iv, ◇ represents the result in the presence of SOD added to the following final concentrations for each of the indicated substrates: 6.7 μM (CS), 8.3 μM (ovo), and 33 μM (lys). (B) Percentage inhibition by Hp2-1 of the turbidity of solutions of (i) CS and ovo induced by heat and (ii) lys induced by oxidative stress, plotted as a function of the SMR of the target protein/Hp $\alpha^2\beta$ "subunit". The lines drawn simply indicate the trends in the data and have no theoretical significance. (C) Turbidity (measured as A^{360}) as a function of time for ovo (0.25 mg/mL, 3.3 μM) and 40 μg/mL of either Hp1-1, Hp2-1, or Hp2-2 (see the legend), heated at 60 °C. Owing to the variable structures of Hp2-1 and Hp2-2 (*2*), it is not possible to meaningfully compare their respective molar concentrations. The data points shown are individual measurements and in each case are representative of at least three independent experiments.

Hp $\alpha^2\beta$ dimers; data for GST are not plotted because the formation of macroscopic aggregates in the control makes the calculation of percent inhibition problematic. It is apparent that the inhibition was most potent for lys, with 50% inhibition being achieved at a lys/$\alpha^2\beta$ ratio of ~12:1. To achieve comparable inhibition of heat-induced turbidity for CS and ovo, the data suggest that target protein/$\alpha^2\beta$ ratios of ~6:1 and ~2:1, respectively, are required. Although all of the phenotypic forms of Hp were capable of inhibiting stress-induced protein precipitation, they differed in their potency. When tested at equal mass ratios (0.25 mg/mL ovo and 40 μg/mL of Hp1-1, Hp2-1, or Hp2-2), Hp1-1 inhibited heat-induced turbidity for ovo at 30 min by about 50%, while the corresponding inhibition by both Hp2-1 and Hp2-2 was about 20% (Figure 1C).

Immunoaffinity chromatography successfully depleted nearly all Hp from human serum (Figure 2A). If Hp acts as a chaperone *in vivo*, then its selective depletion from whole human serum should enhance stress-induced precipitation of proteins in serum. This was found to be the case. Under the conditions tested, from 50 μL aliquots of sera, about 0.1 mg of protein precipitated from NHS but about 0.25 mg of protein precipitated from HDS under the same conditions (Figure 2B). Thus, the selective depletion of Hp more than doubled the amount of protein precipitated in human serum incubated at 43 °C for 48 h. This difference was reproducible and statistically significant ($p = 0.02$, Student's t test). Collectively, the results indicate that the chaperone action of Hp can protect both purified proteins and unfractionated proteins in whole human serum from stress-induced precipitation.

Hp Interacts with Stressed Proteins to Form High Molecular Weight (HMW) Complexes. Chaperones such as clusterin and the sHSPs are known to form HMW complexes with partly unfolded proteins under stress conditions (*12*, *34*). We used SEC as a first step to investigate whether Hp forms complexes with stressed proteins; ovo and GST were used as "target" proteins in these assays. The variably sized

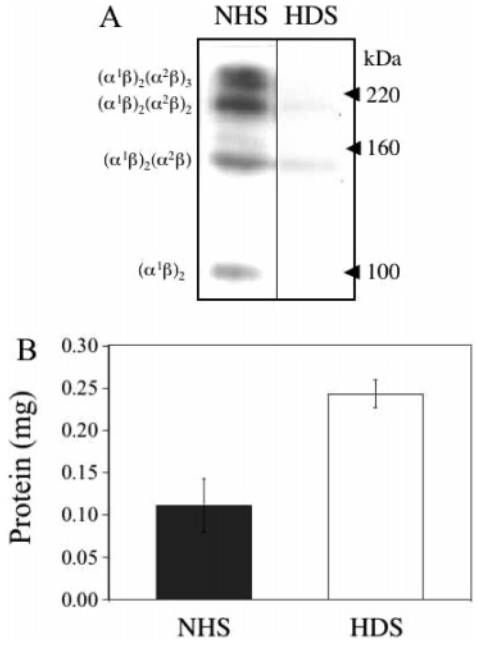

FIGURE 2: Endogeneous Hp inhibits heat-induced precipitation of proteins in human serum. (A) Immunoblot showing the depletion of Hp2-1 from human serum by immunoaffinity chromatography. Aliquots (10 μL) of NHS and HDS were analyzed using 3A8 anti-Hp antibody (see the Materials and Methods for more details). The position of molecular mass standards are indicated by arrowheads at the right of the image. (B) Histogram showing the amount of protein precipitated from 50 μL aliquots of NHS and HDS heated at 43 °C for 48 h. Data points shown are means of triplicate measurements, and error bars represent standard errors of the means. The results shown are representative of two independent experiments.

absorbance peaks migrating at a position corresponding to a species of molecular weight less than 15 kDa (indicated by the dashed arrows in Figure 3A) represent azide present in some of the samples analyzed or minor "buffer fronts" in other samples. The range of the molecular mass standards available limit what can be deduced from the SEC profiles for Hp alone; however, it is clear that Hp migrated under these conditions as a broad, asymmetric peak maximal at an elution time of about 380 s, with more than half the material eluting before this time and a small fraction eluting at or near the exclusion limit of the column (indicated by the arrow labeled 2000 kDa). Thus, most of the Hp2-1 species migrated at a rate consistent with a mass in the range of 220−2000 kDa, with a small fraction migrating as species with a mass of 2000 kDa or greater; this is consistent with it being comprised of a series of variably glycosylated disulfide-linked polymers spanning a wide range of masses. The elution profiles of untreated versus heat-treated Hp were very similar (Figure 3A), indicating that there was no major change in the aggregation state of Hp in solution in response to heat. In the case of ovo, when the protein was heated in the absence of Hp, most but not all of it precipitated and was removed by centrifugation before loading onto the column; the remaining ovo migrated with the same mobility as unheated ovo [Figure 3A (i)].

When GST was heated in the absence of Hp, essentially all of it precipitated and it was therefore not detected by SEC [trace 4 in Figure 3A (ii)]. When unheated mixtures of Hp and either ovo or GST were analyzed by SEC, the resulting absorbance profiles were consistent with the proteins migrating independently of one another (Figure 3A). However, when stressed mixtures were analyzed, in each case, a HMW fraction eluted either at or near the exclusion limit of the column (Figure 3A, indicated by the horizontal bars). When analyzed by SEC, in comparison to heated mixtures of Hp and GST, there appeared to be more species migrating at 300−350 s in heated mixtures of Hp and ovo, suggesting that the average size of complexes formed between Hp and ovo was less than those formed between Hp and GST. SDS−PAGE analysis of HMW fractions obtained by SEC of mixtures of Hp and ovo or GST indicated that they only contained both Hp and the respective target protein following heat stress (data not shown). Similar results were obtained from analyses of heat-stressed CS and oxidatively stressed lys (data not shown). This suggested that Hp was forming soluble, HMW complexes with stressed proteins. This interpretation was confirmed by using streptavidin-agarose to affinity adsorb proteins from unheated and heated solutions of Hp and mixtures of Hp and biotinylated ovo/GST. The adsorbed material was subsequently analyzed by SDS−PAGE. In fractions prepared from unstressed mixtures, only the biotinylated target protein was detected (Figure 3B). Furthermore, after being heated when in solution alone, Hp did not bind to the streptavidin-agarose beads (Figure 3B). However, fractions prepared from stressed mixtures of Hp and target protein contained Hp (Figure 3B). This demonstrates that Hp formed complexes with the target proteins only after heating. Similar results were obtained for affinity adsorption analyses of mixtures of Hp and CS (exposed to heat stress) or lys (exposed to oxidative stress) (data not shown). Collectively, the above results establish that, during experimental stresses but not otherwise, Hp bound to stressed proteins to form soluble HMW complexes.

Hp Does Not Protect Enzymes from Heat-Induced Loss of Activity or Independently Promote Protein Refolding and Lacks ATPase Activity. To investigate whether Hp is capable of protecting enzymes from stress-induced loss of function and whether ATP has an effect on any such ability, we tested the enzyme activity of catalase and GST before and after exposure to heat stress in the presence or absence of Hp and ATP. The presence of Hp2-1 (at sufficient concentrations to provide protection against precipitation) had no effect on the loss of catalase or GST activity in either case, regardless of the presence of ATP (parts A and B of Figure 4, time 0). Furthermore, when acting alone and regardless of the presence of ATP, Hp2-1 was unable to promote the recovery of catalase or GST activity following heat stress (parts A and B of Figure 4, times 2 and 5 h). Last, purified Hp2-1 had no detectable ATPase activity either when tested alone or following heat-induced association with ovo (data not shown).

Effects of pH on the Chaperone Action and Structure of Hp. We previously showed that mildly acidic pH (i) increased the efficiency with which clusterin inhibited the precipitation of stressed proteins (*15*), (ii) induced dissociation of clusterin oligomers in solution into the heterodimeric form (*15*), and (iii) increased the exposure of regions of hydrophobicity on the molecule (*35*). Given the functional parallels between clusterin and Hp, we investigated the effects of pH on the chaperone action and structure of Hp. In assays of heat-induced protein precipitation, in the absence of Hp, changes in pH between 6.0 and 7.5 had little effect on the time course

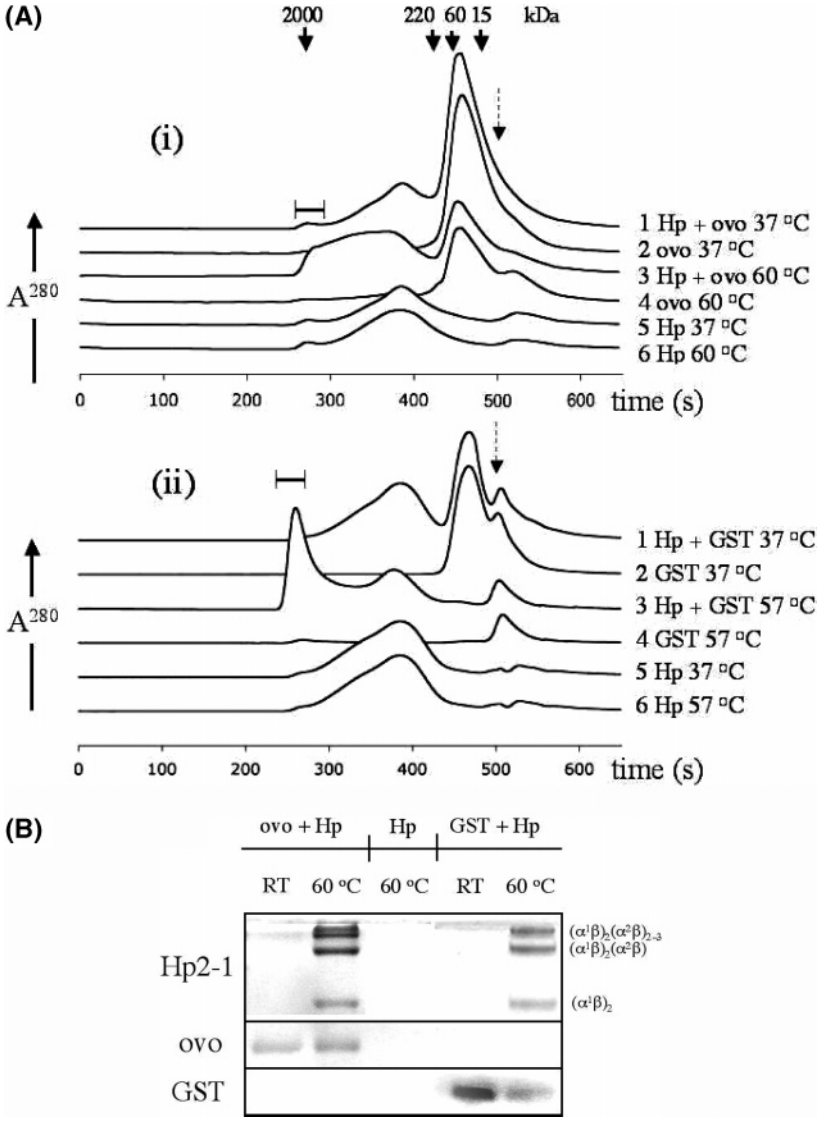

FIGURE 3: Hp forms HMW complexes with stressed proteins. (A) Results of SEC analyses of (i) Hp2-1 and ovo or (ii) Hp2-1 and GST following incubation alone or as mixtures at either 37 or 57/60 °C (indicated in the legend to the right of the traces; see the Materials and Methods for other details). The positions of molecular mass standards are indicated by arrows at the top of the figure. (B) Image of sections of a Coomassie blue stained SDS−PAGE gel (electrophoresed under nonreducing conditions), showing proteins affinity adsorbed by streptavidin-agarose from mixtures that had been heated at 60 °C or incubated at room temperature (RT), containing Hp2-1 alone or Hp2-1 and either biotinylated ovo or biotinylated GST. In each case, the results shown are representative of at least two independent experiments. The identity of the bands labeled at the right of the figure was established by comparison with molecular mass standards (not shown) and the known masses of Hp2-1 molecular species (*46*).

or extent of precipitation of CS [Figure 5A (i)]. In the case of ovo, the extent of precipitation was similar at all pH values but proceeded at a slightly faster rate at pH 6.0−6.5 than at pH 7.0−7.5 [Figure 5A (ii)]; this may indicate that changes in the ionization of ovo side chains induced by mildly acidic pH promote more rapid intermolecular interactions when the protein partly unfolds in response to heat. In contrast, in the presence of Hp, the extent of protein precipitation was significantly greater at lower pH. Under the conditions tested, the ability of Hp to inhibit heat-induced turbidity in solutions of CS and ovo was greatest at pH 7.5 (approximately 70 and 64% inhibition, respectively) and was significantly less at pH 6.0 (approximately 40 and 26% inhibition, respectively) (Figure 5A). In the case of ovo, the rate of precipitation in the presence of Hp (like those in its absence) was faster at pH 6.0−6.5 than at pH 7.0−7.5. Collectively, the results show that, in comparison to pH 7.5, at mildly acidic pH, Hp is less efficient at inhibiting the precipitation of stressed proteins from solution. Using ovo as a substrate we also examined the relative efficiency of the chaperone actions of Hp and clusterin over the pH range of 6.0−8.0. The ability of Hp to inhibit heat-induced turbidity in solutions of ovo increased nearly linearly over the pH range of 6.0−7.5 and remained at a similar level over the pH range of 7.5−8.0 (Figure 5B). In contrast, the ability of clusterin to inhibit heat-induced turbidity of solutions of ovo was greatest at pH 6.0 and decreased with increasing pH, particularly between pH 7.0−7.5. Surprisingly, under these conditions, the chaperone efficiency of clusterin increased again at pH 8.0 to approach near maximum levels (Figure 5B).

The results indicated that the efficiency of the chaperone action of Hp was strongly affected by changes in pH over the range of 6.0−7.5. We then examined the effects of pH over this range on the solution size of Hp (probed by DLS), its secondary structure (probed by far-UV CD), and the extent to which it exposed hydrophobicity to solution (probed by

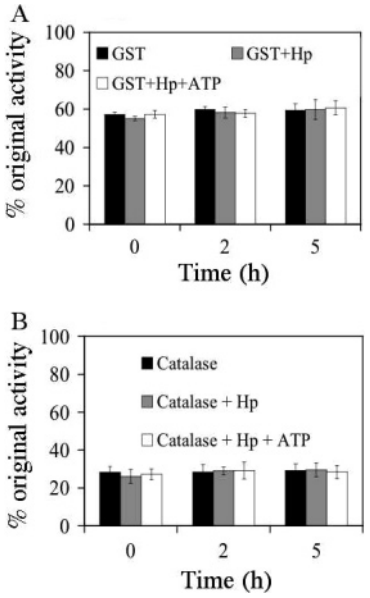

FIGURE 4: Hp does not protect enzymes from heat-induced loss of activity or enhance their subsequent recovery of activity. Bar graphs showing the level of (A) GST and (B) catalase activity as a function of time after heat stress. Additions present during and after heat stress are indicated in the respective keys. The data shown are means of triplicate measurements, and the error bars represent standard errors of the means. The data shown are representative of three independent experiments.

bisANS fluorescence). Dynamic light-scattering measurements indicated that there was no significant change in the solution size of Hp2-1 at pH 6.0 versus 7.5. At both pH values, about 98% of Hp2-1 species in solution had a diameter in the range of 9−25 nm with a mean of 15.1 nm (Figure 6A). At pH 7.5 (but not at pH 6.0), a minor additional population of much larger particles (representing about 1% of all particles in solution) was present; these particles had a diameter in the range of 250−400 nm with a mean diameter of about 317 nm (indicated by the arrow in Figure 6A). The far-UV CD spectra of Hp2-1 at pH 6.0 and 7.5 were virtually superimposable (Figure 6B); deconvolution of the spectra into their α-helical, β-sheet, β-turn, and unordered components showed that there was no significant differences between these at pH 6 compared to pH 7.5 (data not shown). In contrast, there were pH-dependent differences in the binding of the fluorescent hydrophobic probe bisANS to Hp; dose-dependent increases in the fluorescence of bisANS bound to Hp2-1 were observed at both pH 6 and 7.5, but the fluorescence at saturating levels of bisANS was about 2-fold higher at pH 6 than at 7.5 (Figure 6C). It was assumed that there is a single class of noncooperative bindings sites for bisANS on Hp, and the data were fitted to eq 1 (see the Materials and Methods) by nonlinear regression; these analyses gave values for F_{max} of 527 ± 5 and 275 ± 8 at pH 6.0 and 7.5, respectively, with corresponding values for K_d of 7.3 ± 1.6 and 3.5 ± 0.3 μM.

DISCUSSION

Previous work showed that human Hp inhibited the aggregation of two proteins induced by *in vitro* stresses: γ

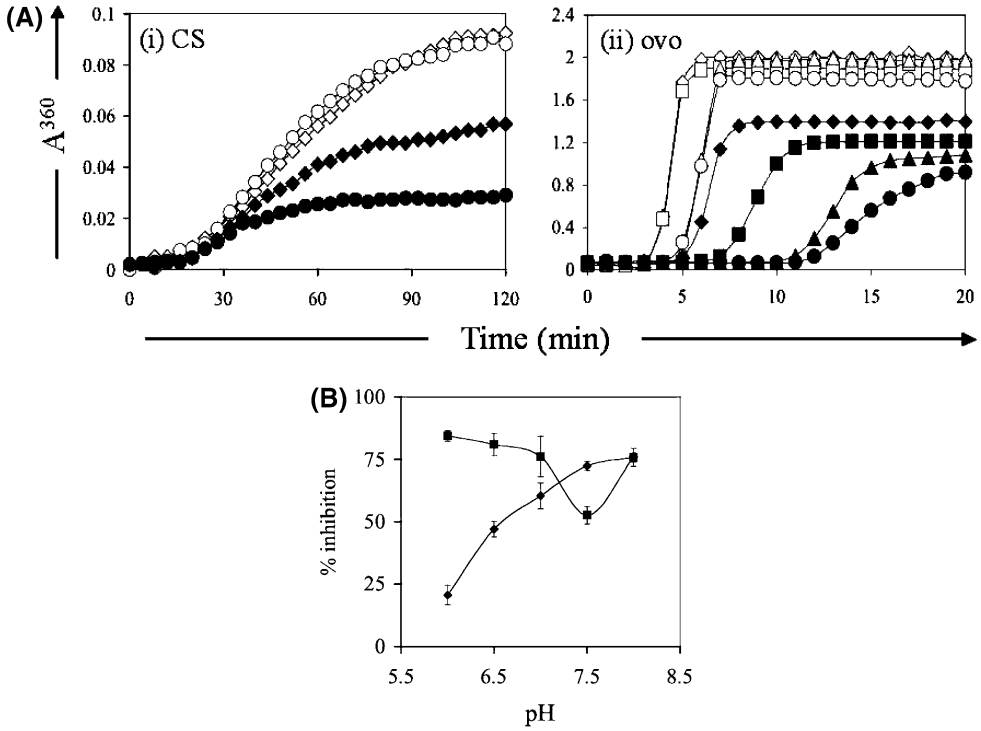

FIGURE 5: Effects of pH on the chaperone action of Hp. (A) (i) Turbidity of solutions of CS alone (0.3 mg/mL) (◇ and ○) or CS (0.3 mg/mL) and Hp2-1 (0.15 mg/mL) (◆ and ●) at pH 6.0 (◇ and ◆) and 7.5 (○ and ●), as a function of time of heating at 43 °C. (ii) Turbidity of solutions of ovo alone (1 mg/mL) (◇, □, △, and ○) or ovo (1 mg/mL) and Hp2-1 (0.25 mg/mL) (◆, ■, ▲, and ●) at pH 6.0 (◇ and ◆), 6.5 (□ and ■), 7.0 (△ and ▲), or 7.5 (○ and ●), as a function of time of heating (in water-jacketed quartz cuvettes) at 60 °C. Data points shown are individual measurements and in each case are representative of at least three independent experiments. (B) Plot showing the percent inhibition of the heat-induced turbidity of solutions of ovo (1 mg/mL) by Hp2-1 (0.25 mg/mL; ◆) and clusterin (40 μg/mL; ■), as a function of pH over the range 6.0−8.0 (these experiments were done using 384-well microplates; see the Materials and Methods). The percentages are calculated from the differences in A^{360} at the end of a 2 h time course. Data points are means of three replicates, and the error bars represent standard errors of the means. The results shown are representative of three independent experiments.

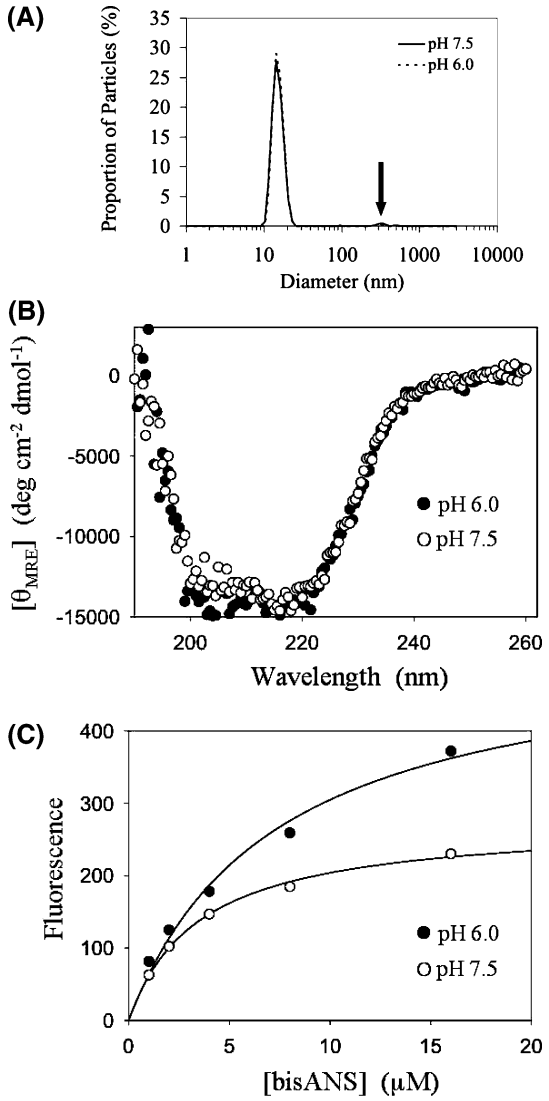

FIGURE 6: Effects of pH on the structure of Hp. (A) Histogram plot showing the results of dynamic light-scattering analyses of Hp2-1 at pH 6.0 and 7.5. The proportions of particles with different estimated diameters is plotted (see the Materials and Methods for further details). (B) CD spectra of Hp2-1 at pH 6.0 (●) and 7.5 (○). The data shown are means of three scans; mean residue ellipticity is plotted, calculated for Hp2-1, assuming a molar extinction coefficient of 5.1×10^4 M^{-1} cm^{-1} (*25*). (C) Dependence of the fluorescence (in arbitrary units) of Hp2-1/bisANS complexes on the bisANS concentration at pH 6.0 (●) and 7.5 (○) (measured as described in the Materials and Methods). Data shown are representative of two independent experiments.

crystallin exposed to heat or oxidative stress (*19*) and catalase exposed to heat (*20*). It was also shown that, in comparison to its effects on aggregation induced by heat or oxidative stress, Hp less efficiently inhibited the aggregation of γ crystallin following its rapid dilution from 8 M urea (*20*). These earlier studies (i) were restricted to two substrate proteins, (ii) did not demonstrate that the effects of Hp were specific, and (iii) did not establish a mechanism for the putative chaperone action of Hp. Results presented here show that Hp inhibited the stress-induced precipitation of a variety of purified proteins. This included the heat-induced precipitation of CS, GST, and ovo and oxidative stress-induced precipitation of lys; the use of control proteins demonstrated that the protective effects of Hp were specific (Figure 1A and data not shown). All three Hp phenotypes inhibited the heat-induced precipitation of ovo, although at equivalent mass concentrations, Hp1-1 was the most efficient (Figure 1C). Furthermore, although we have not identified which specific protein(s) in human serum precipitate in response to mild heating, we showed that immunoaffinity depletion of Hp from serum more than doubled the amount of protein that precipitated in response to the treatment (Figure 2B). Collectively, the results indicate that Hp has the ability to protect many different proteins from stress-induced precipitation, and its effects in whole human serum suggest that this activity may be relevant *in vivo*.

The SMRs of the interaction between the four proteins tested and Hp2-1 (calculated on the basis of a 50-kDa $\alpha^2\beta$ Hp "subunit") sufficient to inhibit most stress-induced precipitation were 2.0:1 (CS), 0.73:1 (GST), 1.3:1 (ovo), and 7.0:1 (lys). This indicates that Hp is a more efficient chaperone than the intracellular sHSPs; the available data suggest that sHSPs bind stressed proteins at a SMR of one or more subunits of sHSP to one partially folded protein subunit [i.e., at best, SMR = 1:1 (*36*, *37*)]. However, SMR-based comparisons indicate that Hp is a less efficient chaperone than clusterin, which, under similar conditions to those used in the current study, only required SMRs of target protein/clusterin of 12.5:1 (*12*) and 21:1 (*13*) to inhibit most of the heat-induced precipitation of GST and ovo, respectively (these ratios are calculated assuming a clusterin "subunit" is comprised of the 80-kDa $\alpha\beta$ heterodimer). The different SMRs required for Hp to give equivalent levels of inhibition of aggregation for the various proteins tested may reflect individual differences in the extent of exposure of regions of hydrophobicity by the proteins. Those requiring greater amounts of Hp to inhibit aggregation may, under the conditions tested, expose a greater area of hydrophobicity to solution.

Size-exclusion and affinity adsorption analyses indicated that Hp2-1 formed soluble, HMW complexes with stressed proteins (Figure 3 and data not shown). Clusterin and the sHSPs form similar HMW complexes with stressed proteins (*12*, *34*). Many other chaperones but not clusterin (*13*) have an ATP-dependent ability to refold unfolded proteins following stresses. A BLAST analysis of the NCBI nonredundant protein sequence database showed that there is no sequence similarity between Hp and any known ATPases or other proteins with nucleotide-binding domains. However, to eliminate the possibility that Hp might exert refolding activity by utilizing a novel ATP-binding domain, we tested whether Hp could protect enzymes from heat-induced loss of activity or promote poststress recovery of enzyme activity, in either the presence or absence of ATP. Using GST and catalase as targets, our results demonstrate that, when acting alone, Hp has no effect on the heat-induced loss of enzyme activity or on their ability to recover activity after heat stress, regardless of the presence or absence of ATP (Figure 4). We also demonstrated that Hp lacks detectable ATPase activity (data not shown). These results indicate that Hp lacks the ability to independently refold heat-stressed enzymes and that ATP does not play a direct role in its chaperone action. We have not excluded the possibility that, like the sHSPs, Hp may hold stressed proteins in a refolding-competent state, from which the native conformations may be recovered by interaction with ATP-dependent chaperones *in vivo*. However, the only known chaperones with established refolding

activity present in human body fluids (e.g., Hsp70) occur at very low (ng/mL) levels (*38*). Thus, even if Hp could stabilize stressed proteins in a refolding-competent state, the physiological relevance of this in extracellular body fluids would be questionable.

Clusterin is the first identified chaperone activated by mildly acidic pH; it exists in solution as heterogeneous aggregates and is induced to deoligomerize at mildly acidic pH, which may expose more chaperone-binding sites to solution (*15*). Results presented here indicate that, compared to pH 7.5, clusterin also has increased chaperone activity under mildly alkaline conditions (Figure 5B). The reasons for this are unknown but may reflect pH-induced changes in the tertiary or quaternary structure of clusterin. In solution, both Hp2-1 and Hp2-2 are known to consist of variably sized oligomers made up of different numbers of disulfide-linked $\alpha\beta$ units (*2*); the quaternary structure of Hp in aqueous solution is unknown. In contrast with clusterin but like the sHSP α crystallin (*15*), the efficiency of the chaperone action of Hp2-1 rapidly decreases as the pH is reduced from 7.5 to 6.0 (Figure 5). Furthermore, at least when acting on heat-stressed ovo, the chaperone action of Hp2-1 is near maximum efficiency at pH 7.5 and remains high at pH 8.0 (Figure 5B); even at pH 9.0, Hp2-1 maintains this high level of chaperone efficiency (data not shown).

The difference between clusterin and Hp with respect to the effects of pH on their chaperone actions suggests that their mechanisms may be quite distinct. We examined the mass of Hp2-1 in solution using SEC and DLS. HPLC SEC analyses of Hp2-1 at pH 7.4 indicated that it migrated as a broad asymmetrical peak, consistent with species in the range of 220−2000 kDa (Figure 3A). DLS measurements indicated that over the pH range of 6.0−7.5, pH had little effect on the solution size of Hp2-1; at either pH, the vast majority of Hp species was distributed normally about a mean diameter of approximately 15 nm (Figure 6A). At pH 7.5 only, about 1% of particles had an estimated diameter of more than 300 nm (which would correspond to oligomers containing up to hundreds of $\alpha\beta$ units). These very large oligomers were not detected at pH 6.0, suggesting that they were not stabilized by disulfide bonds and resulted from pH-sensitive non-covalent interactions. However, given that they comprise such a small proportion of the Hp2-1 species in solution, it appears very unlikely that the effects of pH on their formation is an important mechanism affecting the chaperone action of Hp. Thus, although changes in the oligomerization state have been implicated in the effects of pH on the chaperone activity of clusterin (*15*), there is no evidence that a similar mechanism operates in the case of Hp. Therefore, it follows that pH primarily affects the chaperone action of Hp by altering some aspect(s) of its secondary and/or tertiary structure.

Far-UV CD analyses of Hp2-1 indicated that varying pH over the range 6.0−7.5 had no significant effect on the predicted contents of α-helical, β-sheet, and β-turn regions (Figure 6B), suggesting that the secondary structure of Hp does not undergo any gross changes over this pH range. However, an inherent limitation of CD measurements is that they do not identify specific locations of structural changes in a molecule; they only provide overall structural information. The results of experiments measuring the fluorescence of bisANS bound to Hp2-1 indicate that the protein undergoes a pH-dependent structural change (not detected by far-UV CD analyses). As a consequence of these changes, bisANS fluoresced more intensely when bound to Hp at pH 6 compared to pH 7.5; however, Hp had a higher affinity for bisANS at pH 7.5 than at pH 6 (Figure 6C). This latter observation may provide an explanation for our finding that Hp is more efficient at inhibiting protein precipitation at pH 7.5 compared to pH 6.0; the higher affinity hydrophobic binding sites exposed at pH 7.5 may be important in the interactions of Hp with stressed proteins (which presumably involves its binding to exposed hydrophobic surfaces on these proteins). The pH-induced changes in bisANS binding detected appear unrelated to the isoelectric point of the protein, which is 3.9−4.2 (*1*).

Although we have not measured the dissociation constants, the demonstration of stable complexes formed between Hp and stressed proteins (Figure 3) suggests that Hp binds to stressed proteins with high affinity. Therefore, collectively, the available data suggests that, *in vitro*, Hp potently inhibits stress-induced protein aggregation by binding (via an ATP-independent mechanism) to partly unfolded proteins to form stable HMW-solubilized complexes but does not itself effect protein refolding. In these respects, the chaperone action of Hp is like that of clusterin and the sHSPs. However, these three types of chaperones clearly differ with respect to the molar efficiences with which they inhibit protein aggregation (see above) and the effects of pH and temperature on their actions. Current understanding indicates that increased temperatures induce deoligomerization of sHSPs (which like clusterin aggregate in solution) to yield 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 (*39*−*41*). An activation mechanism analogous to that proposed for the sHSPs has been suggested for clusterin, in which reduced pH (rather than increased temperature) induces enhanced chaperone action (*15*). Mildly acidic pH has no effect on the size of most Hp species in solution and decreases the chaperone efficiency of both the sHSP α crystallin (*15*) and Hp (Figure 4B). Therefore, at least over the pH range of 6.0−7.5, the chaperone action of Hp shows a similar pattern of dependence on pH as the sHSP α crystallin. The effects of temperature on the solution size and chaperone efficiency of Hp have yet to be examined in detail.

In healthy individuals, the proportion of Hp in circulation complexed with Hb is very low because Hp−Hb complexes are rapidly cleared and degraded by liver-resident macrophages (*42*). Hp probably has discrete chaperone and Hb-binding sites because complexation with Hb has only a limited effect on the ability of Hp to inhibit the heat-induced aggregation of catalase [when compared with uncomplexed Hp, the Hp−Hb complex is about 25% less effective (*33*)]. Hp inhibits the pro-inflammatory oxidative effects of free Hb by binding to it with very high affinity and mediates its uptake via the CD163 macrophage cell-surface receptor (*43*). Our results raise the possibility that another, complementary, mechanism by which Hp may exert an anti-inflammatory action *in vivo* is via it inhibiting the inappropriate self-association of "damaged" (misfolded) extracellular proteins. Misfolded proteins have been implicated as underlying inflammatory events in atherosclerosis (*44*) and Alzheimer's disease (*45*). It is interesting to speculate that, like its role

in directing the uptake of Hb via CD163, Hp may also facilitate the cellular uptake and degradation of misfolded proteins to which it complexes.

Last, we propose that Hp and clusterin are the first clearly identified members of a small family of known extracellular mammalian chaperones, present in human plasma at levels of about 100 μg/mL or greater, which comprise a critical defense mechanism against the clinically dangerous consequences of inappropriate protein aggregation in extracellular body spaces. Other chaperones normally found inside cells, such as Hsp70, are also found in human plasma, but their low concentrations in this fluid (in the ng/mL range) mean that they are unlikely to substantively inhibit precipitation of plasma proteins; their release from damaged cells is likely to at least partly explain their occurrence in extracellular fluids (*38*).

ACKNOWLEDGMENT

M. R. W. and S. B. E. gratefully acknowledge the support provided by Discovery-Project Grants from the Australian Research Council. J. J. Y. and M. S. R. are supported by Australian Postgraduate Award scholarships.

REFERENCES

1. Bowman, B. H., and Kurosky, A. (1982) Haptoglobin: The evolutionary product of duplication, unequal crossing over, and point mutation, *Adv. Hum. Genet. 12*, 189−261.
2. Dobryszycka, W. (1997) Biological functions of haptoglobin−New pieces to an old puzzle, *Eur. J. Clin. Chem. Clin. Biochem. 35*, 647−654.
3. Heegaard, P. M., Godson, D. L., Toussaint, M. J., Tjornehoj, K., Larsen, L. E., Viuff, B., and Ronsholt, L. (2000) The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus, *Vet. Immunol. Immunopath. 77*, 151−159.
4. Lim, S. K., Kim, H., bin Ali, A., Lim, Y. K., Wang, Y., Chong, S. M., Costantini, F., and Baumman, H. (1998) Increased susceptibility in Hp knockout mice during acute hemolysis, *Blood 92*, 1870−1877.
5. Lim, S.-K. (2001) Consequences of haemolysis without haptoglobin, *Redox Rep. 6*, 375−378.
6. Louagie, H., Delanghe, J., Desombere, I., De Buyzere, M., Hauser, P., and Leroux-Roels, G. (1993) Haptoglobin polymorphism and the immune response after hepatitis B vaccination, *Vaccine 11*, 1188−1190.
7. Snellman, O., and Sylven, B. (1967) Haptoglobin acting as a natural inhibitor of cathepsin B activity, *Nature 216*, 1033.
8. Cid, M. C., Grant, D. S., Hoffman, G. S., Auerbach, R., Fauci, A. S., and Kleinman, H. K. (1993) Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis, *J. Clin. Invest. 91*, 977−985.
9. Oh, S. K., Pavlotsky, N., and Tauber, A. I. (1990) Specific binding of haptoglobin to human neutrophils and its functional consequences, *J. Leukocyte Biol. 47*, 142−148.
10. Wagner, L., Gessl, A., Parzer, S. B., Base, W., Waldhausl, W., and Pasternack, M. S. (1996) Haptoglobin phenotyping by newly developed monoclonal antibodies. Demonstration of haptoglobin uptake into peripheral blood neutrophils and monocytes, *J. Immunol. 156*, 1989−1996.
11. Berkova, N., Gilbert, C., Goupil, S., Yan, J., Korobko, V., and Naccache, P. H. (1999) TNF-induced haptoglobin release from human neutrophils: Pivotal role of the TNF p55 receptor, *J. Immunol. 162*, 6226−6232.
12. Humphreys, D., Carver, J. A., Easterbrook-Smith, S. B., and Wilson, M. R. (1999) Clusterin has chaperone-like activity similar to that of small heat-shock proteins, *J. Biol. Chem. 274*, 6875−6881.
13. Poon, S., Rybchyn, M. S., Easterbrook-Smith, S. B., Carver, J. A., and Wilson, M. R. (2000) Clusterin is an ATP-independent chaperone with very broad substrate specificity that stabilizes stressed proteins in a folding-competent state, *Biochemistry 39*, 15953−15960.
14. Poon, S., Treweek, T. M., Wilson, M. R., Easterbrook-Smith, S. B., and Carver, J. A. (2002) Clusterin, a unique extracellular chaperone, specifically interacts with slowly aggregating proteins on their off-folding pathway, *FEBS Lett. 513*, 259−266.
15. Poon, S., Rybchyn, M. S., Easterbrook-Smith, S. B., Carver, J. A., Pankhurst, G. J., and Wilson, M. R. (2002) Mildly acidic pH activates the extracellular molecular chaperone clusterin, *J. Biol. Chem. 277*, 39532−39540.
16. Kapron, J. T., Hilliard, G. M., Lakins, J. N., Tenniswood, M. P. R., West, K. A., Carr, S. A., and Crabb, J. W. (1997) Identification and characterization of glycosylation sites in human serum clusterin, *Protein Sci. 6*, 2120−2133.
17. Morrissey, C., Lakins, J., Moquin, A., Hussain, M., and Tenniswood, M. (2001) An antigen capture assay for the measurement of serum clusterin concentrations, *J. Biochem. Biophys. Methods 48*, 13−21.
18. Rosenberg, M. E., and Silkensen, J. (1995) Clusterin: Physiologic and pathophysiologic considerations, *Int. J. Biochem. Cell Biol. 27*, 633−645.
19. Pavlicek, Z., and Ettrich, R. (1998) Chaperone-like activity of human haptoglobin, *Chem. Papers−Chemicke Zvesti 52*, 309−310.
20. Pavlicek, Z., and Ettrich, R. (1999) Chaperone-like activity of human haptoglobin: Similarity with α-crystallin, *Collect. Czech. Chem. Commun. 64*, 717−725.
21. Heuer, K. H., Mackay, J. P., Podzebenko, P., Bains, N. P., Weiss, A. S., King, G. F., and Easterbrook-Smith, S. B. (1996) Development of a sensitive peptide-based immunoassay: Application to detection of the Jun and Fos oncoproteins, *Biochemistry 35*, 9069−9075.
22. Souza, J. M., Giasson, B. I., Lee, V. M., and Ischiropoulos, H. (2000) Chaperone-like activity of synucleins, *FEBS Lett. 474*, 116−119.
23. Wilson, M. R., and Easterbrook-Smith, S. B. (1992) Clusterin binds by a multivalent mechanism to the Fc and Fab regions of IgG, *Biochim. Biophys. Acta 1159*, 319−326.
24. Javid, J., and Liang, J. C. (1973) The hemoglobin−haptoglobin bond. I. Dissociation of the complex and recovery of the native haptoglobin in an affinity chromatography system, *J. Clin. Lab. Med. 82*, 991−1002.
25. El-Ghmati, S. M., Arredouani, M., van Hoeyveld, E. M., Ceuppens, J. L., and Stevens, E. A. M. (2002) Haptoglobin interacts with the human mast cell line HMC-1 and inhibits its spontaneous proliferation, *Scand. J. Immunol. 55*, 352−358.
26. Wang, K., and Spector, A. (1994) The chaperone activity of bovine α crystallin. Interaction with other lens crystallins in native and denatured states, *J. Biol. Chem. 269*, 13601−13608.
27. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid, *Anal. Biochem. 150*, 76−85.
28. Lilley, R., and Portis, R. (1997) ATP hydrolysis activity and polymerization state of ribulose-1,5-bisphosphate carboxylase oxygenase activase−Do the effects of Mg^{2+}, K^+, and activase concentrations indicate a functional similarity with actin, *Plant Physiol. 114*, 605−613.
29. Kohl, M., Cope, M., Essenpreis, M., and Bocker, D. (1994) Influence of glucose concentration on light scattering in tissue-simulating phantoms, *Optics Lett. 19*, 2170−2172.
30. Johnson, W. C. (1999) Analyzing protein circular dichroism spectra for accurate secondary structures, *Proteins: Struct., Funct., Genet. 35*, 307−312.
31. Provencher, S. W., and Glochner, J. (1981) Estimation of protein secondary structure from circular dichroism, *Biochemistry 20*, 33−37.
32. Day, R., Bennion, B. J., Ham, S., and Daggett, V. (2002) Increasing temperature accelerates protein unfolding without changing the pathway of unfolding, *J. Mol. Biol. 322*, 189−203.
33. Ettrich, R., Brandt, W., Kopecky, V., Baumruk, V., Hofbauerova, K., and Pavlicek, Z. (2002) Study of chaperone-like activity of human haptoglobin: Conformational changes under heat shock conditions and localization of interaction sites, *Biol. Chem. 383*, 1667−1676.
34. Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. W. (1995) On the interaction of α-crystallin with unfolded proteins, *Biochim. Biophys. Acta 1252*, 251−260.

35. Hochgrebe, T., Pankhurst, G. J., Wilce, J., and Easterbrook-Smith, S. B. (2000) pH-dependent changes in the *in vitro* ligand-binding properties and structure of human clusterin, *Biochemistry 39*, 1411−1419.
36. Farahbakhsh, Z. T., Huang, Q. L., Ding, L. L., Altenbach, C., Steinhoff, H. J., Horwitz, J., and Hubbell, W. L. (1995) Interaction of α-crystallin with spin-labelled peptides, *Biochemistry 34*, 509−516.
37. Lindner, R. A., Kapur, A., Mariani, M., Titmuss, S. J., and Carver, J. A. (1998) Structural alterations of α-crystallin during its chaperone action, *Eur. J. Biochem. 258*, 170−183.
38. Wright, B. H., Corton, J. M., El-Nahas, A. M., Wood, R. F., and Pockley, A. G. (2000) Elevated levels of circulating heat shock protein 70 (Hsp70) in peripheral and renal vascular disease, *Heart Vessels 15*, 18−22.
39. Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H. E., Chen, S. X., Saibil, H. R., and Buchner, J. (1999) Hsp26: A temperature-regulated chaperone, *EMBO J. 18*, 6744−6751.
40. van Montfort, R. L. M., Basha, E., Friedrich, K. L., Slingsby, C., and Vierling, E. (2001) Crystal structure and assembly of a eukaryotic small heat shock protein, *Nat. Struct. Biol. 8*, 1025−1030.
41. Gu, L. X., Abulimiti, A., Li, W., and Chang, Z. Y. (2002) Monodisperse Hsp16.3 nonamer exhibits dynamic dissociation and reassociation, with the nonamer dissociation prerequisite for chaperone-like activity, *J. Mol. Biol. 319*, 517−526.
42. Higa, Y., Oshiro, S., Kino, K., Tsunoo, H., and Nakajima, H. (1981) Catabolism of globin−haptoglobin in liver cells after intravenous administration of hemoglobin−haptoglobin to rats, *J. Biol. Chem. 256*, 12322−12328.
43. Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O., Hoffman, H. J., Law, S. K. A., and Moestrup, S. K. (2001) Identification of the haemoglobin scavenger receptor, *Nature 409*, 198−201.
44. Ursini, F., Davies, K. J., Maiorino, M., Parasassi, T., and Sevanian, A. (2002) Atherosclerosis: Another protein misfolding disease? *Trends Mol. Med. 8*, 370−374.
45. Casserly, I., and Topol, E. (2004) Convergence of atherosclerosis and Alzheimer's disease: Inflammation, cholesterol, and misfolded proteins, *Lancet 363*, 1139−1146.
46. Pastewka, J. V., Ness, A. T., and Peacock, A. C. (1975) Hemoglobin binding by isolated polymeric proteins from human haptoglobin types 2−1 and 2−2. Some suggested polymer subunit compositions, *Biochim. Biophys. Acta 386*, 530−537.

BI050764X