α₂-Macroglobulin and Haptoglobin Suppress Amyloid Formation by Interacting with Prefibrillar Protein Species*‡

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α₂-Macroglobulin (α₂M) and haptoglobin (Hp) are both abundant secreted glycoproteins that are best known for their protease trapping and hemoglobin binding activities, respectively. Like the small heat shock proteins, both these glycoproteins have in common the ability to protect a range of proteins from stress-induced amorphous aggregation and have been described as extracellular chaperones. Using an array of biochemical techniques, this study establishes that in vitro at substoichiometric levels and under physiological conditions α₂M and Hp both inhibit the formation of amyloid fibrils from a range of proteins. We also provide evidence that both α₂M and Hp interact with prefibrillar species to maintain the solubility of amyloidogenic proteins. These findings suggest that both α₂M and Hp are likely to play an important role in controlling the inappropriate aggregation of proteins in the extracellular environment.

The pathology of more than 40 human degenerative diseases is associated with the deposition of proteinaceous fibrils or plaques commonly known as amyloid (1). These protein deposition diseases affect many of the tissues and organs of the human body and are comprised of various sporadic and occasionally familial amyloidogenic disorders, e.g. Alzheimer and Parkinson diseases, a number of transmissible prion-based disorders (e.g. Creutzfeldt-Jakob disease), and non-neuropathic disorders such as Type II diabetes and systemic lysozyme amyloidosis. Amyloid formation in vitro is now recognized as a common phenomenon and follows a kinetic pathway characteristic of crystallization; there is an initial “lag” or nucleation phase followed by a rapid exponential “growth” or polymerization phase (2) and finally a plateau phase in which no further fibril growth occurs. The lag phase is usually agreed to represent the time required for the formation of soluble (prefibrillar) oligomers or nuclei that are required to seed fibril growth.

It has been proposed that proteins are able to aggregate and form amyloid deposits in vivo when the normally efficient protein quality control machinery is overwhelmed (3). As a consequence, a great deal of current research is focused on the protein quality control machinery and the role it plays in these disorders (4). However, in many cases, the disease-associated protein deposits are located in the extracellular environment outside the reach of the well studied intracellular protein quality control machinery. Molecular chaperones are central components of the intracellular protein control system, and it is only recently that the existence of extracellular counterparts to these species has been proposed (5). One such extracellular chaperone, clusterin, has been shown to potently inhibit amyloid formation when it is present during the early stages of the fibril-forming process (6–9). Clusterin appears not to bind to the native form of the substrates tested nor does it detectably bind to mature fibrils. Interestingly binding to some species important for the nuclease event was observed to result in inhibition of fibril formation (8, 9). Recently two other secreted glycoproteins, haptoglobin (Hp) and α₂-macroglobulin (α₂M), have been shown to have chaperone activity similar to that of clusterin and to suppress the amorphous aggregation of a range of unrelated proteins (10, 11). The role of these abundant extracellular chaperones in the formation of amyloid deposits in vivo is currently unknown but is of great interest in the context of understanding the triggers of amyloid diseases.

α₂M is an abundant human blood glycoprotein comprised of ~10% carbohydrate by mass (12). It is best known for its ability to inhibit a broad range of proteases. Upon interaction with a protease, α₂M undergoes limited proteolysis in a region containing a variety of protease cleavage sites (known as the bait region) leading to a major conformational change that results in the physical trapping of the protease within a steric “cage” (13). The trapped protease forms a covalent linkage with α₂M by reacting with an intramolecular thiol ester bond to yield a conformationally altered form known as “activated” or “fast” α₂M (α₂M*). Activated α₂M exposes a receptor recognition site for low density lipoprotein receptor-related protein (13). Small nucleophiles such as methylamine can also activate α₂M by directly interacting with the thiol ester bond (14).

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4 The abbreviations used are: Hp, haptoglobin; α₂M, α₂-macroglobulin; Aβ, amyloid β peptide; BSA, bovine serum albumin; Calc, calcitonin; I59T, I59T lysozyme; Hb, hemoglobin; Thio T, thioflavin T; ccBz peptide coiled coil β; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay.
Aside from its interactions with proteases, α2M binds to a wide range of ligands, including those associated with protein deposition disorders. Examples of ligands include Aβ (15), prion protein (16) and β2-microglobulin (17), which are strongly linked with Alzheimer disease, the spongiform encephalopathies, and dialysis-related amyloidosis, respectively. In addition, α2M binds to cytokines and growth factors (18) and to a range of hydrophobic structures including endotoxin, phenyl-Sepharose, and liposomes (19). Binding to hydrophobic molecules does not inhibit the trapping of proteases and is not known to be associated with any conformational changes (19). In accord with that discussed above, α2M is found associated with amyloid deposits in Alzheimer disease and spongiform encephalopathies (16, 20). Previous work has indicated that α2M can inhibit the formation of amyloid fibrils by Aβ (6) and protect cells from Aβ toxicity in a low density lipoprotein receptor-related protein-dependent fashion (21). Recently it was shown that α2M has a promiscuous ATP-independent chaperone action (11). It forms stable complexes with misfolded proteins and maintains their solubility but is unable to affect their refolding independently (11). Whether α2M can inhibit amyloid formation by peptides and proteins other than Aβ has not been tested.

Hp is a secreted acidic glycoprotein produced mainly in the liver and found in most bodily fluids of humans and other mammals. The levels of Hp in human plasma are increased up to 8-fold during various physiological stresses (e.g. inflammation), leading to it being described as an “acute phase protein” (12, 22). In humans, a crossover event is thought to have produced Hp1/2 and Hp2/2, resulting in individuals expressing one of three major Hp phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2). In its simplest form (Hp 1-1), Hp exists in a disulfide-linked (αβ)2 structure with a molecular mass of ~100 kDa. However, in Hp 2-1 and Hp 2-2, an additional cysteine residue in the α2 chain allows the formation of a series of disulfide-linked α2 polymers (~100 to ~500 kDa). Hp is best known for its high affinity binding to hemoglobin (Hb) (Kd ~ 10−15 M) (12). Formation of the Hp-Hb complex inhibits Hb-mediated generation of lipid peroxides and hydroxyl radicals, which are thought to occur in areas of inflammation (22). When complexed to Hb, Hp is known to be recognized by the cell surface receptors CD163 and Mac-1 and to be taken up by receptor-mediated endocytosis for degradation (23). Hp has also been implicated in immune regulation (24) and shown to inhibit cathepsin B activity (25). Taken together, the available evidence indicates that Hp is likely to play an important role in suppressing the inflammatory response under a variety of different conditions.

Human Hp specifically inhibits the precipitation of a wide variety of proteins induced by a range of stresses (10, 26). Like clusterin, Hp forms stable and soluble high molecular weight complexes with misfolded proteins but has no independent ability to refold misfolded proteins. Immunoaffinity depletion of Hp from human serum significantly increases the amount of protein that is precipitated in response to stresses (10). Thus, Hp has the ability to protect many different proteins from stress-induced amorphous precipitation, and its effects in whole human serum suggest that this activity is likely to be relevant in vivo. Currently there are no published studies of the effects of Hp on amyloid formation, although Hp is found associated with Aβ amyloid deposits in vivo (27).

The aims of this study were to determine whether α2M and Hp could affect the in vitro formation of amyloid aggregates by a range of unrelated proteins and, if so, to characterize the mechanism(s) involved. We selected three amyloid-forming proteins that are linked to disease (Aβ-(1–42), calcitonin, and lysozyme, which are associated with Alzheimer disease, a localized amyloidosis, and a familial systemic amyloidosis, respectively) (1) and one other system, a designed peptide with no connections with disease (peptide coiled coil β (ccβn)).

**EXPERIMENTAL PROCEDURES**

**Materials**—α2M and Hp (phenotype 2-1) were purified from human plasma obtained from Wollongong Hospital (Wollongong, New South Wales, Australia) as described previously (10, 11). The concentration of Hp was determined by absorbance at 280 nm using the molar extinction coefficient of 5.1 × 104 (corresponding to a 50-kDa α2 dimer) (28). α2M concentrations were determined using an extinction coefficient of 8.93 for a 1% solution (corresponding to a 720-kDa tetramer) (19). Thioflavin T (Thio T), biocinchoninic acid (BCA) micro protein assay reagent, hexafluorooisopropanol, and bovine serum albumin (BSA) were purchased from Sigma. Calcitonin (Calc) and the short ccβn peptide, which transforms from a helical conformation at 20 °C into amyloid fibrils at 37 °C (9), were purchased from Auspep (Melbourne, Australia). Aβ-(1–42) was purchased from Biopetide (San Diego, CA), resuspended in hexafluorooisopropanol, and divided into aliquots in which the solvent was left to evaporate, resulting in a peptide “film” that was frozen at −80 °C. The non-natural variant of human lysozyme, IS9T, was expressed and purified as described previously (29). The monoclonal anti-α2B antibody WO2 (hybridoma culture supernatant) was a kind gift from Dr. Kevin Barnham (Department of Pathology, University of Melbourne, Melbourne, Australia).

**Fibril Formation in Vitro**—Immediately before use, Aβ-(1–42) was resuspended in buffer (2 parts 20 mM NaOH diluted in 7 parts Milli Q water and 1 part 10× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM Na2HPO4, pH 7.5)) and was centrifuged at 13,000 × g for 10 min to remove any aggregated material. Aβ-(1–42) (10 μM) was shaken at 600 rpm for 8 h at 37 °C in PBS supplemented with 0.9 mM CaCl2, 0.5 mM MgCl2, 1 mM CuCl2, and 600 mM glycine (PBScu), ccβW (60 μM) and Calc (150 μM) were shaken at 600 rpm for 300 min at 37 °C in 0.1 mM Na2HPO4, pH 7.8, and 50 mM Na2HPO4, pH 7.4, respectively. Fibril formation by Aβ, ccβW, and Calc, in the presence or absence of either Hp or α2M, was measured in situ by Thio T fluorescence (see below) using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Victoria, Australia). Variant human lysozyme (159T) (6.8 μM in 0.1 mM sodium citrate buffer, pH 5.0) was incubated at 60 °C in a stirred 1-ml cuvette and light-scattering monitored at 500 nm (5-nm slit width) using a Cary Eclipse spectrophotometer (Varian Ltd., Oxford, UK); the stirred cuvette was found to give more reproducible IS9T aggregation than incubation in multi-well plates. Furthermore co-incubation of Thio T with protein
mixture containing I59T was found to affect the rate of protein aggregation; thus Thio T analyses were performed only at the end point of each assay (see below). Molar concentrations were calculated using the monomeric molecular masses of the amyloid-forming peptides and masses of 720 kDa for the α2M tetramer and 50 kDa for Hp (based on the αβ dimer). To confirm that Hp and α2M do not themselves form Thio T-reactive aggregates under the conditions used, each protein (at 1.0–12.5 μM) was treated as described above, and the Thio T fluorescence was then measured.

**Effects of Hemoglobin Binding on the Ability of Hp to Inhibit Amyloid Formation**—To generate Hp-Hb complexes, 15 μM Hp was incubated with Hb in PBS in an equimolar ratio for 2 h at room temperature. To form amyloid, 10 μM Aβ-(1–42) was shaken at 600 rpm for 8 h at 37 °C in PBSCu; 60 μM ccβw was shaken at 600 rpm for 300 min at 37 °C in PBS. Fibril formation in the presence or absence of either Hp or Hp-Hb complexes was measured as in situ Thio T fluorescence (see below) using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech). Molar ratios of 1:20 Hp:Aβ and 1:30 Hp:ccβw were used as these ratios did not completely prevent aggregation, and any effects, positive or negative, on chaperone action could be determined. To confirm that Hb alone did not affect the formation of Thio T-reactive aggregates under the conditions used, Hb alone at the same final concentration was added to reactions as described above, and the Thio T fluorescence was then measured.

**Thioflavin T Fluorescence Assays**—Thio T (50 μM) was added to aggregation mixtures of Aβ-(1–42), ccβw, or Calc, and the fluorescence was measured at intervals using a FLUOstar OPTIMA fluorescence plate reader with 440-nm excitation (10-nm slit width) and 490-nm emission (10-nm slit width). In the case of I59T, only the end point Thio T fluorescence was measured using a Cary Eclipse spectrofluorometer with 440-nm excitation (10-nm slit width) and an emission scan from 450 to 600 nm (10-nm slit width).

**Effects of α2M and Hp on Mature Amyloid Fibrils**—Amyloid fibrils were formed using conditions outlined above. At the conclusion of in vitro fibril formation time courses, α2M or Hp was added to give molar ratios of α2M/Hp:Aβ = 1:10, α2M/Hp:Calc = 1:15, and α2M/Hp:ccβw = 1:15 and incubated for a period of 500 min at 37 °C. The samples were subsequently analyzed by 10% SDS-PAGE and subsequent immunoblotting with WO2 (an anti-αβ monoclonal antibody). Electrophoretic slicing of Calc was not performed because its low molecular mass (3418 kDa) results in a very low efficiency of transfer to the membrane. Instead Calc samples were analyzed by spotting them directly onto a nitrocellulose membrane (Pall Corp.) before blocking with heat-denatured casein/PBS and probing with a rabbit anti-Calc antibody (Abcam, Sydney, Australia). I59T samples were analyzed by 10% SDS-PAGE and subsequent immunoblotting using a polyclonal rabbit anti-lysozyme antibody (Washington Biotechnology, Simpsonville, MD). Bound antibodies were detected with either horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG or sheep anti-rabbit IgG antibodies (Silenus, Melbourne, Australia) followed by enhanced chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Immunodot Blots**—Samples of Aβ (1 μg), taken at various time points during fibril formation and stored frozen at −20 °C, were spotted onto nitrocellulose membranes (Pall Corp.) and allowed to air dry. The membranes were then blocked with 1% (w/v) heat-denatured casein in PBS and incubated for 2 h at 37 °C in PBS containing 10 μg/ml α2M or Hp or control protein glutathione S-transferase before being washed with PBS. Bound α2M and Hp were detected using specific rabbit polyclonal antibodies (Sigma and Dako (Glostrup, Denmark), respectively). A rabbit anti-glutathione S-transferase antibody (Chemicon, Melbourne, Australia) was used to detect any bound control protein. Bound primary antibodies were detected as described above.

**Sandwich ELISA**—To determine whether stable complexes are formed between α2M or Hp and amyloid-forming peptides, we sampled aggregation reactions containing sufficient α2M or Hp to inhibit most of the protein aggregation that is observed in their absence (i.e. molar ratios of α2M/Hp:Aβ/I59T = 1:10 and α2M/Hp:Calc = 1:15). These samples contained almost all the substrate protein in the soluble fraction (see immunoblot analyses of corresponding samples in Fig. 4, A–C) and were centrifuged at 13,000 × g for 10 min before analysis to remove any traces of insoluble materials. Samples were taken at a time corresponding to the midpoint of the aggregation process in the absence of the chaperones and were subsequently analyzed by a sandwich ELISA. Aggregation reactions were initiated as above using molar ratios of α2M/Hp to substrate of 1:10 (Aβ and I59T) or 1:15 (Calc and ccβw). In the cases of Aβ and ccβw, the peptides were biotinylated using standard methods prior to the aggregation reaction. This modification did not affect the ability of the peptides to form Thio T-positive aggregates (data not shown). 96-well plates (Greiner Bio-one, Sydney, Australia) were coated with a 10 μg/ml concentration of either anti-α2M (Dako) or anti-Hp (Sigma) antibodies raised in rabbit and then analyzed using the SIS Megaview II Image Capture system (Olympus, Hamburg, Germany).

**Effects of α2M and Hp on the Sedimentation Properties of Substrate Proteins**—At the end points of the aggregation reactions, samples of Aβ, Calc, and I59T, with or without α2M or Hp, were centrifuged for 30 min at 10,000 × g. The supernatant was removed, and the pellet was resuspended and subsequently washed repeatedly in PBS. Aβ samples were analyzed by 15% SDS-PAGE and subsequent immunoblotting using WO2 (an anti-αβ monoclonal antibody).
**RESULTS**

\( \alpha_2 \text{-Macroglobulin and Haptoglobin Inhibit Fibril Formation in Vitro} \) — In the absence of \( \alpha_2 \text{M} \) and Hp, aggregation of all the substrates tested showed a lag phase followed by a rapid growth phase, which eventually approached a plateau. The lag phases were somewhat variable between individual experiments but ranged from ~25 to 50, 20 to 30, and 75 to 150 min for A\( \beta \), cc\( \beta_\omega \), Calc, and I59T, respectively (see Figs. 1, A–D, and 2, A–D). The addition of \( \alpha_2 \text{M} \) to all fibril-forming reactions produced a dose-dependent decrease in Thio T-positive aggregation in each case (Fig. 1). In the case of I59T, for which aggregation was followed by measuring absorbance at 500 nm, analysis of end point samples confirmed that Thio T fluorescence was reproducibly suppressed by \( \alpha_2 \text{M} \) (Fig. 1E; \( \alpha_2 \text{M}: \text{I59T} = 1:10 \)). \( \alpha_2 \text{M} \) extended the lag phase in all reactions, although the final level of aggregation was not always greatly reduced (Fig. 2, A–D); this observation suggests that \( \alpha_2 \text{M} \) affects the nucleation step in the aggregation reaction. A substoichiometric ratio of \( \alpha_2 \text{M} \):substrate was sufficient to inhibit aggregation almost completely in each case (1:10 for A\( \beta \) and I59T and 1:15 for cc\( \beta_\omega \) and Calc). Hp also inhibited the aggregation of all substrates tested in a dose-dependent manner (Fig. 2). Like \( \alpha_2 \text{M} \), at concentrations where aggregation was not completely suppressed, Hp extended the lag phase significantly (Fig. 2, A–D). As with \( \alpha_2 \text{M} \), a substoichiometric ratio of Hp:substrate was sufficient to inhibit almost completely all the Thio T-positive aggregation (1:10 for A\( \beta \) and I59T and 1:15 for cc\( \beta_\omega \) and Calc). In the case of I59T, end point Thio T analysis confirmed that Hp substantially suppressed Thio T fluorescence (Fig. 2E; Hp: \( \text{I59T} = 1:10 \)). The presence of the control protein BSA in the amyloid-forming reactions had no effect on aggregation for any of the substrates tested (supplemental Fig. S1). In addition, when incubated alone, neither Hp nor \( \alpha_2 \text{M} \) generated Thio T-reactive material under any of the conditions tested (data not shown). When complexed with hemoglobin,
Hp retained the ability to inhibit amyloid formation by Aβ and ccβw; however, this ability was less than that of uncomplexed Hp (supplemental Fig. S2).

To verify that both α,2M and Hp can inhibit the formation of amyloid fibrils we examined protein aggregation mixtures with and without α,2M and Hp using TEM. In the absence of either α,2M or Hp, samples from all substrate proteins tested contained fibrillar aggregates (Fig. 3). Addition of α,2M to the Calc and I59T aggregation reactions substantially inhibited the formation of fibrils, and only a small number of short fibril-like structures were observed in 1:10 α,2M:Aβ and 1:15 α,2M:ccβw samples (Fig. 3). The addition of Hp to Aβ, ccβw, and I59T aggregation reactions (at ratios of Hpsubstrate of 1:10, 1:15, and 1:10, respectively) significantly inhibited the formation of fibrils; only small amounts of amorphous material were detected in these samples (Fig. 3). However, although a 1:15 ratio of Hp:Calc substantially reduced Thio T fluorescence (Fig. 2C), a variety of short fibril-like structures were detected in these cases.

Both α,2M and Hp Affect the Sedimentation Properties of Amyloid-forming Proteins—α,2M and Hp were added at various concentrations to Aβ, Calc, and I59T fibril formation reactions, and at the end of the time course the samples were centrifuged to obtain supernatant and pellet fractions. The Aβ and I59T samples were then analyzed by immunoblotting. Because of the low efficiency with which relatively small peptides are electrophoretically transferred to nitrocellulose, Calc samples were analyzed by immunodot blotting. ccβw samples could not be analyzed in either of these ways because there are no suitable commercially available antibodies; furthermore it stains very poorly with Coomassie Blue making SDS-PAGE analysis difficult. In the absence of α,2M or Hp, Aβ was found exclusively in the pellet fraction and consisted of high molecular mass aggregates (Fig. 4A, Aβ alone). When α,2M was added to the reaction to give a 1:10 molar ratio of α,2M:Aβ, all of the Aβ was found in the supernatant fraction; subsequent decreases in the amount of α,2M added to the reaction resulted in a progressive shift of Aβ from the supernatant fraction to the pellet fraction (Fig. 4A, panel i). Similarly the addition of a 1:10 molar ratio of Hp:Aβ resulted in the presence of Aβ in the supernatant fraction; as the amount of Hp added was decreased, progressively more Aβ was found in the pellet fraction (Fig. 4A, panel ii). In the absence of either α,2M or Hp, Calc was detected only in the pellet fraction (Fig. 4B). In contrast, when α,2M was present in the reaction (at α,2M:Calc = 1:15), most of the Calc was detected in the supernatant fraction, and the proportion of Calc in the pellet fraction increased as the amount of α,2M in the reaction decreased (Fig. 4B). Similarly in the presence of Hp (Hp:Calc = 1:15) all of the Calc was detected in the supernatant fraction and subsequent decreases in Hp resulted in more Calc detected in the pellet fraction (Fig. 4B). In the absence of either α,2M or Hp, I59T was detected only in the pellet fraction (Fig. 4C). In contrast, when incubated in the presence of either α,2M or Hp (α,2M/Hp:I59T = 1:10), I59T was detected primarily in the supernatant fraction (Fig. 4C).

α,2M and Hp Do Not Influence Fibril Elongation or Disrupt Preformed Fibrils—To determine whether α,2M and Hp inhibit fibril formation by affecting the nucleation and/or fibril elongation phases, α,2M or Hp was added either at the beginning of the reaction (0 min) or midway through the elongation phase of
aggregating Aβ, ccβw, Calc, or I59T (at 150, 50, 75, and 100 min, respectively). Addition of either α2M or Hp at zero time (Hp/α2M:substrate = 1:10) resulted in almost total inhibition of aggregation over the entire reaction (Fig. 5). In contrast, when the same molar ratios of α2M or Hp were added to the reactions during the elongation phase, there were little or no measurable effects on aggregation (Fig. 5, A and B). It was also shown that, over a 500-min time frame, the addition of α2M or Hp to preformed fibrils of Aβ, Calc, or ccβw had no measurable effect on the integrity of the fibrils (supplemental Fig. S3).

α2M and Hp Bind Transient Prefibrillar Species on the Aβ Amyloid Formation Pathway—Samples taken at various points in time during Aβ fibril formation reactions in the absence of Hp and α2M were spotted on to nitrocellulose membranes. Maximum binding of both α2M and Hp was detected to Aβ species present in samples taken 0.5 and 1 h after the initiation of aggregation (Fig. 6). These times correspond to the transition between the lag and growth phases (Fig. 6). α2M and Hp were not found to bind to the native substrates present in samples at the zero time point or to the final time point samples (Fig. 6). There was also no detectable binding of the control protein glutathione S-transferase to any of the Aβ samples tested (data not shown). In addition when assayed by immunodot blotting there was no detectable binding of α2M or Hp to species present at any time in the aggregation reactions of any of the other substrates tested (data not shown). The reasons for this observation are unclear but might result from the interacting species being present at levels too low to detect using this assay procedure. We therefore used a sandwich ELISA method to demonstrate the formation of α2M/Hp-substrate protein complexes in the aggregation reactions.

α2M and Hp Form Stable Complexes with Species on the Amyloid-forming Pathway—A sandwich ELISA was used to determine whether or not α2M and Hp form stable complexes with substrate proteins during aggregation reactions. We observed a statistically significant increase in absorbance (p < 0.05) when aggregation mixtures of α2M and Aβ, ccβw, or Calc were compared with samples containing substrate alone and with negative controls (Fig. 7A). We were unable to detect a corresponding increase in absorbance for samples taken from mixtures of α2M and I59T (Fig. 7A). Samples from aggregation mixtures containing Hp and any of the four substrate proteins we studied also gave significantly higher absorbances than corresponding samples of aggregation reactions containing substrate alone or of negative controls (p < 0.05; Fig. 7B).

DISCUSSION

Previous work has indicated that α2M can inhibit the in vitro formation of amyloid fibrils by Aβ (6). In addition, both α2M and Hp have been found associated with Aβ plaques in Alzheimer disease patients (20, 27). The results presented here demonstrate that both α2M and Hp can potently inhibit the in vitro formation of amyloid fibrils by a diverse panel of proteins under physiologically relevant conditions. For Aβ, ccβw, and Calc these effects were observed at 37 °C and pH 7.5. In the case of Aβ, fibril formation was induced by the addition of 1 μM Cu2+ (30). Cu2+ can reach 100 μM in the normal human neocortex (31), and in the neuropil of the cortical and accessory basal nuclei of the amygdala of Alzheimer diseased brains it has been reported to reach several times this level (32). Hp and α2M both exert similar activity under the non-physiological conditions used here to form fibrils from I59T (60 °C, pH 5). We have shown previously that both α2M and Hp remain fully functionally active at this elevated temperature and do not aggregate even when incubated at 60 °C for extended periods (10, 11). These data suggest that the antiamyloidigenic activities of Hp and α2M are not dependent on the identity of the polypeptide substrate or the conditions under which it is incubated. This activity was found to be specific because similar levels of the control protein BSA (molar ratio of BSA:substrate = 1:10) had no detectable effect on the aggregation of any of the substrates tested here (supplemental Fig. S1). Hp retained the ability to inhibit amyloid formation even when complexed with hemoglobin, although this activity was reduced when compared with that of uncomplexed Hp. This is consistent with a previous report that complexation with Hb reduces but does not abolish the ability of Hp to inhibit the amorphous aggregation of protein (33). We have reported previously that activation of α2M by interaction with trypsin abolished its ability to inhibit the amorphous heat-induced aggregation of two globular proteins (11).
The effects of protease activation on the ability of \( \alpha_2 \)-M to inhibit amyloid formation are complex and are influenced by the facts that the bound protease remains catalytically active and sterically accessible to small proteins of less than about 8 kDa in mass (but not to those of greater mass) (34). The consequences of this for the antiamyloidogenic activity of \( \alpha_2 \)-M will be dealt with in detail in a separate report.

In all cases examined in this study, significantly substoichiometric molar ratios of \( \alpha_2 \)-M or Hp to substrate (from 1:10 to 1:15) were enough to substantially inhibit the formation of amyloid fibrils as judged by thioflavin T fluorescence, light scattering, and TEM (Figs. 1–3). When present at sufficient levels, both \( \alpha_2 \)-M and Hp reduced the sedimentability of species present in aggregation reactions of A\( \beta \), Calc, and I59T (Fig. 4). In some experiments, lower levels of \( \alpha_2 \)-M and Hp (Hp/\( \alpha_2 \)-M:substrate of 1:500 to 1:30; Figs. 1 and 2) failed to reduce the final level of protein aggregation. However, in these cases the lag phase of the reaction was significantly extended. This suggests that when present at low levels the effects of \( \alpha_2 \)-M and Hp are

\begin{figure}
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\includegraphics[width=\textwidth]{image1}
\caption{\( \alpha_2 \)-M and Hp maintain the solubility of Ab, Calc and I59T. A, A\( \beta \) immunoblot showing supernatant (S) and pellet (P) fractions prepared by centrifugation of aggregation reactions containing various ratios of \( \alpha_2 \)-M (panel i) or Hp (panel ii); A\( \beta \) (indicated above the corresponding lanes). B, images of immunoblot blots showing supernatant (S) and pellet (P) fractions resulting from centrifugation of samples from aggregation reactions containing various ratios of \( \alpha_2 \)-M/Hp:Calc (indicated above the corresponding spots). C, I59T immunoblot showing supernatant (S) and pellet (P) fractions prepared by centrifugation of samples from aggregation reactions containing I59T alone or a 1:10 ratio of \( \alpha_2 \)-M/Hp:I59T (indicated above the corresponding lanes). The results shown are representative of at least two independent experiments.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image2}
\caption{\( \alpha_2 \)-M and Hp are most effective when added at the start of the aggregation reaction. Samples of A\( \beta \) (10 \( \mu \)M), cc\( \beta \)_w (60 \( \mu \)M), Calc (50 \( \mu \)M), and I59T (6.8 \( \mu \)M) were incubated as described under “Experimental Procedures,” and amyloid formation was monitored by \textit{in situ} measurements of Thio T fluorescence (panels i–iii) or for I59T by measuring turbidity (panel iv). 1:10 ratios of \( \alpha_2 \)-M:substrate (A) or Hp:substrate (B) were added at either time 0 (gray diamonds) or at the midpoint of the elongation phase (gray circles; addition of \( \alpha_2 \)-M and Hp indicated by arrows). Each symbol represents an individual measurement, and the results shown are representative of at least two independent experiments. AU, arbitrary units.}
\end{figure}
eventually overwhelmed but that even under these conditions they can delay nucleation and thus defer the subsequent elongation phase. Both α₂M and Hp were more effective at suppressing aggregation of amyloidogenic substrates when added at the initiation of the reaction rather than when added during the elongation phase (Fig. 5). This finding suggests that both proteins exert their effects on amyloid formation primarily by interacting with species on the amyloid-forming pathway that are more abundant prior to fibril elongation. Together with the observation that both α₂M and Hp effectively extend the lag phase even when present at low concentrations, these data suggest that they interact with protein species that are either functional nuclei or their precursors. Immunodot blot analyses show α₂M and Hp bind to transient species of Aβ present early in the aggregation time course (Fig. 6). α₂M and Hp appear not to bind to samples taken before the start of the reaction or those taken at the end of the reaction (Fig. 6). This result demonstrates that, at least in the case of Aβ, α₂M and Hp do not detectably bind to the native monomer or to mature fibrils formed from them. It was also shown that, at least over a 500-min co-incubation, α₂M and Hp did not affect the stability of mature fibrils formed from Aβ, Calc, or ccβW (supplemental Fig. S3). These data are all consistent with the idea that both α₂M and Hp specifically bind to species that are particularly abundant during fibril nucleation.

The above suggest that α₂M and Hp form complexes with conformations of substrate proteins that are intermediate on the amyloid-forming pathway; these complexes could be either short lived or more persistent. Analysis of aggregation mixtures by sandwich ELISA strongly suggests that stable complexes are formed between Hp and all four substrate proteins tested. The fact that complexes between α₂M and I59T were not detected suggests that the interacting species are present in this case at very low levels. Nevertheless it seems likely that α₂M binds to aggregation-prone prefibrillar species formed early in aggregation reactions.

Collectively the results indicate that stoichiometric levels of α₂M and Hp effectively suppress amyloid fibril formation in vitro by a broad range of protein substrates. The data suggest that both α₂M and Hp exert this activity by interacting with one or more transient species on the amyloid-forming pathway that is populated most heavily before fibril elongation begins. This conclusion is consistent with the hypothesis that, like clusterin, α₂M and Hp bind to species sharing common structural features present during amyloid formation of a range of substrates (9). The interactions between α₂M/Hp and substrate proteins appear to be stable and act to preserve the solubility of the substrates. The current findings are in accord with previous work demonstrating that both α₂M and Hp have chaperone

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**FIGURE 6.** α₂M and Hp bind transient species early in Aβ fibril formation. Results of immunodot blot assay measuring the binding of α₂M and Hp to protein species present at different times during Aβ fibril formation are shown. Aβ (10 μg) was incubated as described under “Experimental Procedures,” and aliquots were taken at various time points. The times at which individual samples were taken are indicated above the images, and the Thio T fluorescence of each aliquot was measured. Aβ (~1 μg) from each sample was then spotted onto nitrocellulose, and the membrane was blocked and subsequently incubated with a 10 μg/ml concentration of either α₂M or Hp. The presence of α₂M and Hp was detected using rabbit anti-α₂M and anti-Hp antibodies.

**FIGURE 7.** α₂M and Hp form stable complexes with amyloid-forming proteins. Histograms show the results of sandwich ELISA detecting complexes formed between α₂M/Hp and amyloid-forming proteins. Samples taken from aggregation reactions at times corresponding to the midpoint of the elongation phase (in the absence of α₂M/Hp) were analyzed by ELISA using plate-bound anti-α₂M (A) or anti-Hp (B) antibodies to capture α₂M and Hp, respectively. Bound amyloid-forming proteins were subsequently detected using specific antibodies or (in the case of biotinylated Aβ and ccβW) a streptavidin-biotinylated HRP complex. Nonspecific binding of substrate was tested using samples taken from aggregation reactions of substrate alone at the same time points. For biotinylated Aβ and ccβW, the negative controls were anti-α₂M/anti-Hp-coated wells incubated with only streptavidin-biotinylated HRP complex. For Calc and I59T, the negative controls were provided by substituting appropriate antibodies of irrelevant specificity for the anti-Calc or anti-lysozyme antibodies (see “Experimental Procedures” for details). Values shown represent the mean of at least three wells, and error bars represent S.E. Significant differences to controls are indicated by * (Student’s t test; p < 0.05). Results shown are representative of at least two independent experiments.
activity similar to that of the small heat shock proteins. Both \( \alpha_2 \)-M and Hp inhibit the formation of amorphous aggregation by a range of proteins by forming soluble complexes (10, 11). Together with previous findings (8–11, 35), the work presented here suggests that clusterin, \( \alpha_2 \)-M, and Hp make up a small family of extracellular chaperones that may be an important part of an in vivo quality control system for extracellular proteins. It has been proposed that extracellular chaperones respond to misfolded or aggregating proteins in extracellular space by binding to exposed hydrophobic regions, maintaining the solubility of the substrate and promoting its removal from the extracellular space via receptor-mediated endocytosis. This proposal is fully consistent with all the data presented here and with data that show that the removal of radiolabeled A\( \beta \) from mouse brain is significantly inhibited by the low density lipoprotein family inhibitor receptor-associated protein and antibodies against the \( \alpha_2 \)-M receptor (low density lipoprotein receptor-related protein-1) and \( \alpha_2 \)-M (36). This hypothesis is also supported by the demonstration that, when complexed with clusterin, the rate of clearance of A\( \beta \) (1–42) from the mouse brain (across the blood-brain barrier into plasma) is increased by more than 80% and that this transport is significantly inhibited by anti-megalin antibodies (37).

Clusterin (8, 9) and both \( \alpha_2 \)-M and Hp (this study) appear to inhibit amyloid formation and maintain substrate protein solubility by interacting with prefibrillar species. These extracellular chaperones are also known to potently inhibit amorphous aggregation of proteins (10, 11, 35, 38, 39). We suggest that this small family of chaperones is likely to be an important part of a system that defends the human body against inappropriate extracellular protein aggregation, which can be either amorphous or amyloid in character. Presumably it is only during exceptional circumstances (e.g., the result of a mutation, age-related loss of function, or acute stress) that this system of defense is overwhelmed and disease results. The increasing number of known extracellular deposition disorders, which include conditions such as Alzheimer disease and Type II diabetes, has made the need for understanding the mechanisms controlling extracellular protein folding of major importance to modern health care. Greater understanding of these processes will ultimately lead to the development of new therapeutic strategies.

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REFERENCES

1. Chiti, F., and Dobson, C. M. (2006) Annu. Rev. Biochem. 75, 333–366
2. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) Cell 73, 1055–1058
3. Dobson, C. M. (1999) Trends Biochem. Sci. 24, 329–332
4. Muchowski, P. J., and Wacker, J. L. (2005) Nat. Rev. Neurosci. 6, 11–22
5. Yerbury, J. J., Stewart, E. M., Wyatt, A. R., and Wilson, M. R. (2005) EMBO Rep. 6, 1131–1136
6. Hughes, S. R., Khorkova, O., Goyal, S., Knaebel, J., Heroux, J., Riedel, N. G., and Sahasrabudhe, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3275–3280
7. Hatters, C. M., Wilson, M. R., Easterbrook-Smith, S. B., and Howlett, G. J. (2002) Eur. J. Biochem. 269, 2789–2794
8. Kumita, J. R., Poon, S., Caddy, G. L., Hagan, C. L., Dumoulin, M., Yerbury, J. J., Stewart, E. M., Robinson, C. V., Wilson, M. R., and Dobson, C. M. (2007) J. Mol. Biol. 369, 157–167
9. Yerbury, J. J., Poon, S., Meehan, S., Thompson, B., Kumita, J. R., Dobson, C. M., and Wilson, M. R. (2007) FASEB J. 21, 2312–2322
10. Yerbury, J. J., Rybchyn, M. S., Easterbrook-Smith, S. B., Henriques, C., and Wilson, M. R. (2005) Biochemistry 44, 10914–10925
11. French, K., Yerbury, J. J., and Wilson, M. R. (2008) Biochemistry 47, 1176–1185
12. Bowman, B. H., and Kurosky, A. (1982) Adv. Hum. Genet. 12, 189–261
13. Sottrop-Jensen, L. (1989) J. Biol. Chem. 264, 11539–11542
14. Imber, M. J., and Pizzo, S. V. (1981) J. Biol. Chem. 256, 8134–8139
15. Narita, M., Holtzman, D. M., Schwartz, A. L., and Bu, G. (1997) J. Neurochem. 69, 1904–1911
16. Adler, V., and Kryukov, V. (2007) Neurochem. J. 1, 43–52
17. Motomiya, Y., Ando, Y., Haraoka, K., Sun, X., Iwamoto, H., Uchimura, T., and Maruyama, I. (2003) Kidney Int. 64, 2244–2252
18. Mettenburg, J. M., Webb, D. J., and Gonias, S. L. (2002) J. Biol. Chem. 277, 13238–13245
19. Barrett, A. J. (1981) Method. Enzymol. 80, 737–754
20. Fabrizi, C., Businaro, R., Lauro, G. M., and Fumagalli, L. (2001) J. Neurochem. 78, 406–412
21. Du, Y., Ni, B., Glinn, M., Dodel, R. C., Bales, K. R., Zhang, Z., Hyslop, P. A., and Paul, S. M. (1997) J. Neurochem. 69, 299–305
22. Dobryszycza, W. (1997) Eur. J. Clin. Chem. Clin. Biochem. 35, 647–654
23. Graversen, J. H., Madsen, M., and Moestrup, S. K. (2002) Int. J. Biochem. Cell. Biol. 34, 309–314
24. Louagie, H., Delanghe, J., Desombere, I., De Buyzere, M., Hauser, P., and Leroux-Roels, G. (1993) Vaccine 11, 1188–1190
25. Snellman, O., and Sylven, B. (1967) Nature 216, 1033
26. Pavlicek, Z., and Eitrich, R. (1999) Coll. Czech. Chem. Commun. 64, 717–725
27. Powers, J. M., Schlaper, W. W., Willingham, M. C., and Hall, B. I. (1981) J. Neuropathol. Exp. Neurol. 40, 592–612
28. El-Ghmati, S. M., Arredouni, M., Van Hoyeveld, E. M., Ceuppens, J. L., and Stevens, E. A. (2002) Scand. J. Immunol. 55, 352–358
29. Kumita, J. R., Johnson, R. J., Alcocer, M. J., Dumoulin, M., Holmqvist, F., McCammon, M. G., Robinson, C. V., Archer, D. B., and Dobson, C. M. (2006) FEBS J. 273, 711–720
30. Atwood, C. S., Moir, R. D., Huang, X. D., Scarpa, R. C., Bacarra, N. M. E., Romano, D. M., Hartshorn, M. K., Tanzi, R. E., and Bush, A. I. (1998) J. Biol. Chem. 273, 12817–12826
31. Strausak, D., Mercer, J. F., Alcocer, M. J., Dumoulin, M., Holmqvist, F., McCammon, M. G., Robinson, C. V., Archer, D. B., and Dobson, C. M. (2006) FEBS J. 273, 711–720
32. Lovell, M., Robertson, I., Teesdale, W., Campbell, J., and Markesbery, W. (1998) J. Neurol. Sci. 158, 47–52
33. Eitrich, R., Brandt, W. J., Kopecky, V., Baumruk, W., Hofbauerova, K., and Pavlicek, Z. (2002) Biochem. Biophys. 383, 1667–1676
34. Starkey, P. M., and Barrett, A. J. (1977) in Proteins in Mammalian Cells and Tissues (Barrett, A. J., ed) pp. 663–696, Elsevier, Cambridge, MA
35. Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B., and Wilson, M. R. (1999) J. Biol. Chem. 274, 6875–6881
36. Shibata, M., Yamaeda, S., Kumar, S. R., Calero, M., Bading, J., Frangione, B., Holtzman, D. M., Miller, C. A., Strickland, D. K., Ghiso, J., and Zlokovic, B. V. (2000) J. Clin. Invest. 106, 1489–1499
37. Bell, R. D., Sagare, A. P., Friedman, A. E., Bedi, G. S., Holtzman, D. M., Deane, R., and Zlokovic, B. V. (2007) J. Cereb. Blood Flow Metab. 27, 909–918
38. Poon, S., Rybchyn, M. S., Easterbrook-Smith, S. B., Carver, J. A., and Wilson, M. R. (2000) Biochemistry 39, 15953–15960
39. Poon, S., Treweek, T. M., Wilson, M. R., Easterbrook-Smith, S. B., and Carver, J. A. (2002) FEBS Lett. 513, 259–266