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Abstract

α_2 -Macroglobulin (α_2 M) 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 biophysical techniques, this study establishes that *in vitro* at substoichiometric levels and under physiological conditions α_2 M and Hp both inhibit the formation of amyloid fibrils from a range of proteins. We also provide evidence that both α_2 M and Hp interact with prefibrillar species to maintain the solubility of amyloidogenic proteins. These findings suggest that both α_2 M and Hp are likely to play an important role in controlling the inappropriate aggregation of proteins in the extracellular environment.

Keywords

protein, amyloid, species, formation, haptoglobin, macroglobulin, suppress, interacting, prefibrillar, CMMB

Disciplines

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α_2 -Macroglobulin and Haptoglobin Suppress Amyloid Formation by Interacting with Prefibrillar Protein Species

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Running Title: α_2 -Macroglobulin and haptoglobin suppress amyloid formation

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The abbreviations used are: α_2 -macroglobulin (α_2 M); amyloid beta peptide (A β); bovine serum albumin (BSA); calcitonin (Calc); glutathione-S-transferase (GST); haptoglobin (Hp); I59T lysozyme (I59T); low density lipoprotein receptor related protein (LRP).

α_2 -Macroglobulin (α_2 M) 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 biophysical techniques, this study establishes that *in vitro*, at substoichiometric levels and under physiological conditions, α_2 M and Hp both inhibit the formation of amyloid fibrils from a range of proteins. We also provide evidence that both α_2 M and Hp interact with prefibrillar species to maintain the solubility of amyloidogenic proteins. These findings suggest that both α_2 M 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's (AD) and Parkinson's (PD) 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 nucleation event was observed to result in inhibition of fibril formation (8,9). Recently, two other secreted glycoproteins, haptoglobin (Hp) and α_2 -macroglobulin (α_2 M), 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.

α_2 M 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, α_2 M 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 α_2 M by reacting with an intramolecular thiol ester bond to yield a conformationally altered form known as "activated" or "fast" α_2 M (α_2 M*). Activated α_2 M exposes a receptor recognition site for low density lipoprotein receptor related protein (LRP) (13). Small nucleophiles such as methylamine can also activate α_2 M by directly interacting with the thiol ester bond (14).

Aside from its interactions with proteases, α_2 M binds to a wide range of ligands, including those associated with protein deposition disorders. Example of ligands include A β peptide (15), prion protein (16) and β_2 -microglobulin (17), which are strongly linked with Alzheimer's disease, the

spongiform encephalopathies and dialysis related amyloidosis, respectively. In addition, α_2 M 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, α_2 M is found associated with amyloid deposits in AD and spongiform encephalopathies (16,20). Previous work has indicated that α_2 M can inhibit the formation of amyloid fibrils by A β peptide (6) and protect cells from A β toxicity in an LRP dependent fashion (21). It was recently shown that α_2 M 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 α_2 M can inhibit amyloid formation by peptides and proteins other than A β 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 cross-over event is thought to have produced two principal alleles (Hp1 and Hp2), which results in individuals expressing one of three major Hp phenotypes (Hp 1-1, Hp 2-1, Hp 2-2). In its simplest form (Hp 1-1), Hp exists in a disulfide-linked ($\alpha^1\beta$)₂ structure with a molecular weight of ~100 kDa. However, in Hp 2-1 and Hp 2-2, an additional cysteine residue in the α^2 chain allows the formation of a series of disulfide-linked $\alpha\beta$ polymers (~100 to ~500 kDa). Hp is best known for its high affinity binding to hemoglobin (Hb) ($K_D \sim 10^{-15}$ 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 A β amyloid deposits *in vivo* (27).

The aims of this study were to determine if α_2 M 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 (A β_{1-42} , calcitonin, and lysozyme which are associated with Alzheimer's disease, a localized amyloidosis and a familial systemic amyloidosis, respectively) (1) and one other system, a designed peptide with no connections with disease (peptide cc β_w).

EXPERIMENTAL PROCEDURES

Materials

α_2 M and Hp (phenotype 2-1) were purified from human plasma obtained from Wollongong Hospital (Wollongong, NSW, Australia) as previously described (10,11). The concentration of Hp was determined by absorbance at 280 nm using the molar extinction coefficient of 5.1×10^4 (corresponding to a 50 kDa $\alpha\beta$ dimer) (28). α_2 M 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), bicinchoninic acid (BCA)

micro protein assay reagent, hexafluoroisopropanol (HFIP), and bovine serum albumin (BSA) were purchased from Sigma (MO, USA). Calcitonin (Calc) and the short coiled-coil β (cc β_w) peptide, which transforms from a helical conformation at 20 °C into amyloid fibrils at 37 °C (9), were purchased from Auspep (Melbourne, Australia). A β_{1-42} was purchased from Biopeptide (San Diego, CA, USA), resuspended in HFIP 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, I59T, was expressed and purified as described (29). The monoclonal anti-A β antibody WO2 (hybridoma culture supernatant) was a kind gift from Dr Kevin Barnham (Department of Pathology, University of Melbourne, Australia).

Fibril formation *in vitro*

Immediately before use, A β_{1-42} was resuspended in buffer (2 parts 20 mM NaOH, diluted in 7 parts Milli Q water and 1 part 10X 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 minutes to remove any aggregated material. A β_{1-42} (10 μ M) was shaken at 600 rpm for 8 h at 37 °C in PBS supplemented with 0.9 mM CaCl₂, 0.5 mM MgCl₂, 100 μ M CuCl₂, and 600 μ M glycine (PBSCu). cc β_w (60 μ M) and Calc (150 μ M) were shaken at 600 rpm for 300 min at 37 °C in 0.1 M Na₂HPO₄, pH 7.8, and 50 mM Na₂HPO₄, pH 7.4, respectively. Fibril formation by A β , cc β_w and Calc, in the presence or absence of either Hp or α_2 M, was measured *in situ* by Thio T fluorescence (see below) using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Australia). Variant human lysozyme (I59T) (6.8 μ M in 0.1 M 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 spectrofluorimeter (Varian Ltd., Oxford UK); the stirred cuvette was found to give more reproducible I59T aggregation than incubation in multiwell plates. Furthermore, co-incubation of Thio T with protein mixtures containing I59T was found to affect the rate of protein aggregation, thus Thio T analyses were

performed only at the endpoint 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 α_2 M tetramer and 50 kDa for Hp (based on the $\alpha\beta$ dimer). To confirm that Hp and α_2 M do not themselves form Thio T reactive aggregates under the conditions used, each protein (at 1.0–12.5 μ M) was treated as described above and the Thio T fluorescence then measured.

Effects of hemoglobin binding on the ability of Hp to inhibit amyloid formation

To generate Hp–hemoglobin (Hb) complexes (Hp-Hb), Hp (15 μ M) was incubated with Hb in PBS in an equimolar ratio for 2 h at room temperature. To form amyloid, 10 μ M A β ₁₋₄₂ was shaken at 600 rpm for 8 h at 37 °C in PBSCu; 60 μ M cc β _w 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, was measured as *in situ* Thio T fluorescence (see below) using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Australia). Molar ratios of 1:20 Hp:A β and 1:30 Hp:cc β _w 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 then measured.

Thioflavin T fluorescence assays

Thio T (50 μ M) was added to aggregation mixtures of A β ₁₋₄₂, cc β _w, or Calc and the fluorescence 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 endpoint Thio T fluorescence was measured using a Cary Eclipse spectrofluorimeter with 440 nm excitation (slit-width 10 nm) and an emission scan from 450–600 nm (slit-width 10 nm).

Effects of α_2 M and Hp on mature amyloid fibrils

Amyloid fibrils were formed using conditions outlined above. At the conclusion of *in vitro* fibril formation time courses, α_2 M or Hp were added to give molar ratios of α_2 M/Hp:A β = 1:10, α_2 M/Hp:Calc = 1:15, and α_2 M/Hp:cc β _w = 1:15, and incubated for a period of 500 min at 37°C. The samples were subsequently analysed using Thio T fluorescence (as described above) and then centrifuged for 30 min at 10,000 g. The supernatant was removed and the pellet resuspended and washed repeatedly in PBS. Insoluble material was then redissolved in DMSO before being diluted 10 fold in PBS. A BCA micro protein assay was conducted (as per the manufacturer's instructions) to quantify the protein content of the resulting samples.

Transmission electron microscopy (TEM)

Endpoint samples of aggregation reactions incubated as outlined above, in the presence or absence of α_2 M or Hp (at molar ratios of α_2 M/Hp:A β /I59T = 1:10 or α_2 M/Hp:cc β _w/Calc = 1:15), were applied to Formvar and carbon-coated nickel electron microscopy grids and stained with uranyl acetate (2% (w/v), Agar Scientific, UK). A Philips CM100 transmission electron microscope (FEI Company, USA) was used to view samples under a magnification of 20–125 K at an 80 kV excitation voltage. Images were analyzed using the SIS Megaview II Image Capture system (Olympus, Germany).

Effects of α_2 M and Hp on the sedimentation properties of substrate proteins

At the endpoints of the aggregation reactions, samples of A β , Calc and I59T, with or without α_2 M or Hp, were centrifuged for 30 min at 10,000 g. The supernatant was removed and the pellet resuspended and subsequently washed repeatedly in PBS. A β samples were analyzed by 15% SDS PAGE and subsequent immunoblotting using WO2 (an anti-A β monoclonal antibody). Electrophoretic blotting 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, FL, USA) before blocking with HDC/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, USA). Bound antibodies were detected with either HRP-conjugated sheep anti-mouse IgG or sheep anti-rabbit IgG antibodies (Silenus, Melbourne, Australia) followed by enhanced chemiluminescence (ECL) with Supersignal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

Immuno dot blots

Samples of A β (1 μ g), taken at various time points during fibril formation and stored frozen at - 20 °C were spotted on to nitrocellulose membranes (Pall, FL, USA) and allowed to air dry. The membranes were then blocked with 1% (w/v) heat denatured casein in PBS (HDC/PBS) and incubated for 2 h at 37 °C in PBS containing 10 μ g/ml α_2 M or Hp, or control protein GST, before being washed with PBS. Bound α_2 M and Hp were detected using specific rabbit polyclonal antibodies (Sigma and DAKO, respectively). A rabbit anti-GST antibody (Chemicon, Melbourne, Australia) was used to detect any bound control protein. Bound primary antibodies were detected as described above.

Sandwich ELISA

To determine if stable complexes are formed between α_2 M or Hp and amyloid-forming peptides, we sampled aggregation reactions containing sufficient α_2 M or Hp to inhibit most of the protein aggregation that is observed in their absence (i.e. molar ratios of α_2 M/Hp:A β /I59T = 1:10, α_2 M/Hp:Calc = 1:15). These samples contained almost all the substrate protein in the soluble fraction (see immunoblot analyses of corresponding samples in Figure 4A-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 mid-point 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 α_2 M/Hp to substrate of 1:10 (A β and I59T) or 1:15 (Calc and cc β_w). In the cases of A β and cc β_w , 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 10 μ g/ml of either anti- α_2 M (Dako, Glostrup, Denmark) or anti-Hp (Sigma, Sydney, Australia) antibodies raised in rabbit and then blocked with 1% (w/v) BSA in PBS. Samples of aggregation mixtures (with or without α_2 M or Hp), were next added to the wells at a concentration of 50 μ g/ml of amyloid forming protein. In other wells, solutions of α_2 M or Hp alone (at the same final concentrations as in the corresponding mixtures) were added. Next, a mixture of streptavidin and biotinylated-HRP (each at 2.5 μ g/ml in 1% (w/v) heat-denatured casein in PBS) was used to detect bound A β or cc β_w . Antibody detection of bound Calc was performed as described above. Bound I59T was detected using a mouse monoclonal anti-lysozyme antibody (Serotec, UK), followed by HRP-conjugated sheep anti-mouse IgG. Finally, plates were washed 3 times in PBS containing 0.1 % (v/v) Triton X-100 and then in PBS alone before being developed using 2.5 mg/ml o-phenylenediamine dihydrochloride and 0.03% (w/v) H₂O₂ in 0.05 M citric acid, 0.1M NaHPO₄ (pH 5) as substrate. The absorbance was measured at 490 nm in a SpectraMax Plus 384 microplate reader (Molecular Devices, USA). Negative controls included the use of an antibody that has no specificity to the proteins under investigation (rabbit anti-DNP), and wells incubated with only the mixture of streptavidin and biotinylated HRP described above.

Results

α_2 M and Hp inhibit fibril formation in vitro

In the absence of α_2 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 approximately 25-50, 5-20, 20-30 and 75-150 min for A β , cc β_w , Calc and I59T, respectively (see Figures 1A-D & 2A-D). The addition of α_2 M to all fibril-forming reactions produced a dose-dependent decrease in Thio T positive aggregation in each case (Figure 1). In the case of I59T, for which aggregation was followed by measuring absorbance at 500 nm, analysis of endpoint samples confirmed that Thio T fluorescence was reproducibly suppressed by α_2 M (Figure 1E; α_2 M:I59T = 1:10). α_2 M extended the lag phase in all reactions even though the final level of aggregation was not always greatly reduced (Figure 2A-D); this observation suggests that α_2 M affects the nucleation step in the aggregation reaction. A sub-stoichiometric ratio of α_2 M:substrate was sufficient to inhibit aggregation almost completely in each case (1:10 for A β and I59T; 1:15 for cc β_w and Calc). Hp also inhibited the aggregation of all substrates tested in a dose-dependent manner (Figure 2). Like α_2 M, at concentrations where aggregation was not completely suppressed, Hp extended the lag phase significantly (Figure 2A-D). As with α_2 M, a sub-stoichiometric ratio of Hp:substrate was sufficient to inhibit almost completely all the Thio T positive aggregation (1:10 for A β and I59T, 1:15 for cc β_w and Calc). In the case of I59T, endpoint Thio T analysis confirmed that Hp substantially suppressed Thio T fluorescence (Figure 2E; Hp: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 α_2 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 A β and cc β_w , however this ability was less than that of uncomplexed Hp (supplemental Fig. S2).

To verify that both α_2 M and Hp can inhibit the formation of amyloid fibrils we examined protein aggregation mixtures with and without α_2 M and Hp using transmission electron microscopy (TEM). In the absence of either α_2 M or Hp, samples from all substrate proteins tested contained fibrillar aggregates (Figure 3). Addition of α_2 M 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_2 M:A β and 1:15 α_2 M:cc β_w samples (Figure 3). The addition of Hp to A β , cc β_w and I59T aggregation reactions (at ratios of Hp:substrate of 1:10, 1:15 & 1:10, respectively) significantly inhibited the formation of fibrils; only small amounts of amorphous material were detected in these samples (Figure 3). However, although a 1:15 ratio of Hp:Calc substantially reduced Thio T fluorescence (Figure 2C), a variety of short fibril-like structures were detected in these cases.

Both α_2 M and Hp affect the sedimentation properties of amyloid forming proteins

α_2 M and Hp were added at various concentrations to A β , 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 A β and I59T samples were then analyzed by immunoblotting. Due to the low efficiency with which relatively small peptides are electrophoretically transferred to nitrocellulose, Calc samples were analyzed by immuno dot blotting. cc β_w samples could not be analysed 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_2 M or Hp, A β was found exclusively in the pellet fraction and consisted of high molecular mass aggregates (Figure 4A; A β alone). When α_2 M was added to the reaction to give a 1:10 molar ratio of α_2 M:A β , all of the A β was found in the supernatant fraction; subsequent decreases in the amount of α_2 M added to the reaction resulted in a progressive shift of A β from the supernatant fraction to the pellet fraction (Figure 4Ai). Similarly, the addition of a 1:10 molar ratio of Hp:A β resulted in the presence of A β in the supernatant fraction; as the amount of Hp added was decreased, progressively more A β was found in the pellet fraction (Figure 4Aii). In the absence of either α_2 M or Hp, Calc was detected only in the pellet fraction (Figure 4B). In contrast, when α_2 M was present in the reaction (at α_2 M: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_2 M in the reaction decreased (Figure 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 (Figure 4B). In the absence of either α_2 M or Hp, I59T was detected only in the pellet fraction (Figure 4C). In contrast, when incubated in the presence of either α_2 M or Hp (α_2 M/Hp:I59T = 1:10), I59T was detected primarily in the supernatant fraction (Figure 4C).

α_2 M and Hp do not influence fibril elongation or disrupt pre-formed fibrils

To determine whether α_2 M and Hp inhibit fibril formation by affecting the nucleation and/or fibril elongation phases, α_2 M or Hp was added either at the beginning of the reaction (0 min) or mid-way through the elongation phase of aggregating A β , cc β_w , calc or I59T (at 150, 50, 75 and 100 min, respectively). Addition of either α_2 M or Hp at zero time (Hp/ α_2 M:substrate = 1:10) resulted in almost total inhibition of aggregation over the entire reaction (Figure 5). In contrast, when the same molar ratios of α_2 M or Hp were added to the reactions during the elongation phase, there were little or no measurable effects on aggregation (Figure 5A and 5B). It was also shown that, over a 500 min time frame, the addition of α_2 M or Hp to pre-formed fibrils of A β , Calc or cc β_w had no measurable effect on the integrity of the fibrils (supplemental Fig. S3).

α_2 M and Hp bind transient prefibrillar species on the A β amyloid formation pathway

Samples taken at various points in time during A β fibril formation reactions in the absence of Hp and α_2 M were spotted on to nitrocellulose membranes. Maximum binding of both α_2 M and Hp was detected to A β species present in samples taken 0.5 and 1 hour after the initiation of aggregation (Figure 6). These times correspond to the transition between the lag and growth phases (Figure 6). α_2 M and Hp were not found to bind to the native substrates present in samples at the zero

timepoint, nor to the final timepoint samples (Figure 6). There was also no detectable binding of the control protein GST to any of the A β samples tested (data not shown). In addition when assayed by immuno dot blotting there was no detectable binding of α_2 M 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 α_2 M/Hp-substrate protein complexes in the aggregation reactions.

α_2 M and Hp form stable complexes with species on the amyloid forming pathway

A sandwich ELISA was used to determine whether or not α_2 M 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 α_2 M and A β , cc β_w or calc were compared with samples containing substrate alone and with negative controls (Figure 7A). We were unable to detect a corresponding increase in absorbance for samples taken from mixtures of α_2 M and I59T (Figure 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 ($p < 0.05$; Figure 7B).

Discussion

Previous work has indicated that α_2 M can inhibit the *in vitro* formation of amyloid fibrils by A β (6). In addition, both α_2 M and Hp have been found associated with A β plaques in Alzheimer's patients (20,27). The results presented here demonstrate that both α_2 M and Hp can potentially inhibit the *in vitro* formation of amyloid fibrils by a diverse panel of proteins under physiologically relevant conditions. For A β , cc β_w and Calc these effects were observed at 37 °C and pH 7.5. In the case of A β , fibril formation was induced by the

addition of 100 μM Cu^{2+} (30), the concentration that Cu^{2+} can reach in the normal human neocortex (31); 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 $\alpha_2\text{M}$ both exert similar activity under the non-physiological conditions used here to form fibrils from I59T (60 $^\circ\text{C}$, pH 5). We have previously shown that both $\alpha_2\text{M}$ and Hp remain fully functionally active at this elevated temperature and do not aggregate even when incubated at 60 $^\circ\text{C}$ for extended periods (10,11). These data suggest that the anti-amyloidogenic activities of Hp and $\alpha_2\text{M}$ 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 since 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 to 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 previously reported that activation of $\alpha_2\text{M}$ 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 $\alpha_2\text{M}$ to inhibit amyloid formation are complex and are influenced by the facts that the bound protease remains catalytically active and sterically accessible to small protein substrates of less than 8 kDa in mass (but not to those of greater mass) (34). The consequences of this for the anti-amyloidogenic activity of $\alpha_2\text{M}$ will be dealt with in detail in a separate report.

In all cases examined in this study, significantly sub-stoichiometric molar ratios of $\alpha_2\text{M}$ 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 (Figures 1-3). When present at sufficient levels, both $\alpha_2\text{M}$ and Hp reduced the sedimentability of species present in aggregation reactions of A β , Calc and I59T (Figure 4). In some

experiments, lower levels of $\alpha_2\text{M}$ and Hp (Hp/ $\alpha_2\text{M}$:substrate of 1:500 to 1:30, Figures 1 & 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 $\alpha_2\text{M}$ and Hp are eventually overwhelmed, but that even under these conditions they can delay nucleation and thus defer the subsequent elongation phase. Both $\alpha_2\text{M}$ 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 (Figure 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 $\alpha_2\text{M}$ 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. Immuno dot blot analyses show $\alpha_2\text{M}$ and Hp bind to transient species of A β present early in the aggregation time course (Figure 6). $\alpha_2\text{M}$ and Hp appear not to bind to samples taken before the start of the reaction or those taken at the end of the reaction (Figure 6). This result demonstrates that, at least in the case of A β , $\alpha_2\text{M}$ 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, $\alpha_2\text{M}$ and Hp did not affect the stability of mature fibrils formed from A β , Calc or cc β_w (supplemental Fig. S3). These data are all consistent with the idea that both $\alpha_2\text{M}$ and Hp specifically bind to species that are particularly abundant during fibril nucleation.

The above suggests that $\alpha_2\text{M}$ and Hp form complexes with conformations of substrate proteins which 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 suggest that stable complexes are formed between Hp and all four substrate proteins tested. The fact that complexes between $\alpha_2\text{M}$ and I59T were not detected suggests that the interacting species are

present in this case at very low levels. Nevertheless, it seems likely that α_2 M binds to aggregation-prone prefibrillar species formed early in aggregation reactions.

Collectively, the results indicate that sub-stoichiometric levels of α_2 M and Hp effectively suppress amyloid fibril formation *in vitro* by a broad range of protein substrates. The data suggest that both α_2 M 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, α_2 M and Hp bind to species sharing common structural features present during amyloid formation of a range of substrates (9). The interactions between α_2 M/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 α_2 M and Hp have chaperone activity similar to that of the small heat shock proteins. Both α_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, α_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 radiolabelled A β from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against the α_2 M receptor (LRP-1) and α_2 M (36). This hypothesis is also supported by the demonstration that, when complexed with clusterin, the rate of clearance of A β_{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 α_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 defence is overwhelmed and disease results. The increasing number of known extracellular deposition disorders, which include conditions such as Alzheimer's 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.

FOOTNOTES

* Wollongong Hospital kindly donated human blood for use in this study. JJY is grateful for an Australian Postgraduate Award. This work was partly funded by a Rosemary Foundation Leader Research Grant through Alzheimer's Australia Research. The research of CMD is supported in part by Programme Grants from the Leverhulme and Wellcome Trusts and by the BBSRC. MRW acknowledges support from grants from the Australian Research Council.

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FIGURE LEGENDS

Figure 1 α_2M suppresses the aggregation of amyloidogenic proteins. Samples of A β (10 μ M), cc β_w (60 μ M), Calc (50 μ M), and I59T (6.8 μ M) were incubated as described in Experimental Procedures, and amyloid formation monitored by *in situ* measurements of Thio T fluorescence (A-C), or for I59T by measuring turbidity (absorbance at 500 nm; D) and endpoint measurement of Thio T fluorescence emission spectra (E). In A-E, the open circles represent data for the proteins aggregating in the absence of α_2M ; the molar ratio of α_2M to substrate protein is indicated next to the variously shaded circles on each panel. In (E), the spectrum of Thio T alone (in the absence of any protein) is also shown. The data points shown are representative of at least three independent experiments.

Figure 2 Hp suppresses the aggregation of amyloidogenic proteins. The experiments with A β (10 μ M), cc β_w (60 μ M), Calc (50 μ M), and I59T (6.8 μ M) are analogous to those described for α_2M in the legend for Figure 1.

Figure 3 α_2M and Hp suppress the formation of fibrillar structures. TEM images of samples taken at the conclusion of aggregation reactions containing substrate proteins either alone or in the presence of α_2M or Hp (as indicated above the panels). The molar ratio of Hp/ α_2M :substrate used was 1:10 for

A β and I59T, and 1:15 for cc β_w and Calc. In all cases the results shown are representative of two or more individual experiments. The scale bar in the lower right panel applies to all TEM images shown here and represents 500 nm.

Figure 4 α_2M and Hp form stable complexes with A β and maintain its solubility. (A) A β immunoblot showing supernatant (S) and pellet (P) fractions prepared by centrifugation of aggregation reactions containing various ratios of α_2M /Hp:A β (indicated above the corresponding lanes). (B) Images of immuno dot blots showing supernatant (S) and pellet (P) fractions resulting from centrifugation of samples from aggregation reactions containing various ratios of α_2M /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 α_2M /Hp:I59T (indicated above the corresponding lanes). The results shown are representative of at least 2 independent experiments.

Figure 5 α_2M and Hp are most effective when added at the start of the aggregation reaction. Samples of A β (10 μM), cc β_w (60 μM), Calc (50 μM) and I59T (6.8 μM) were incubated as described in Experimental Procedures and amyloid formation was monitored by *in situ* measurements of Thio T fluorescence (i-iii), or for I59T by measuring turbidity (iv). 1:10 ratios of (A) α_2M :substrate or (B) Hp:substrate were added at either time zero (grey diamonds) or at the mid-point of the elongation phase (grey circles; addition of α_2M and Hp indicated by arrows). Each symbol represents an individual measurement and the results shown are representative of at least 2 independent experiments.

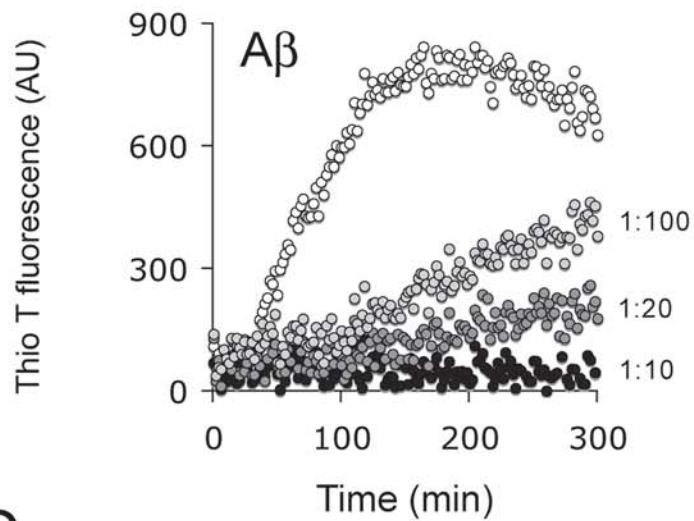
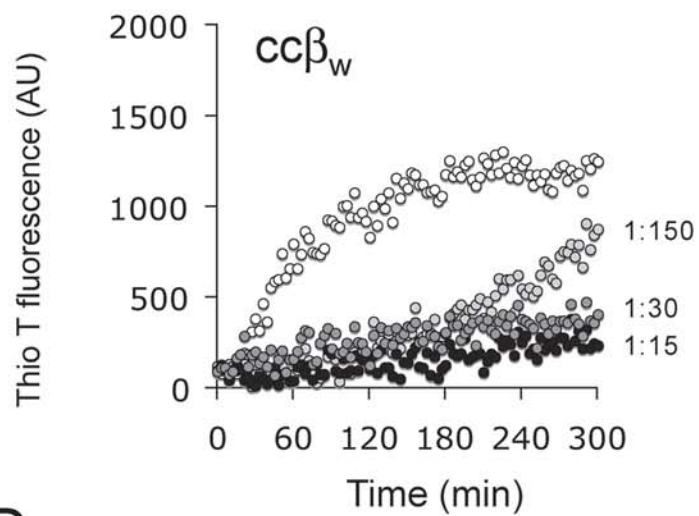
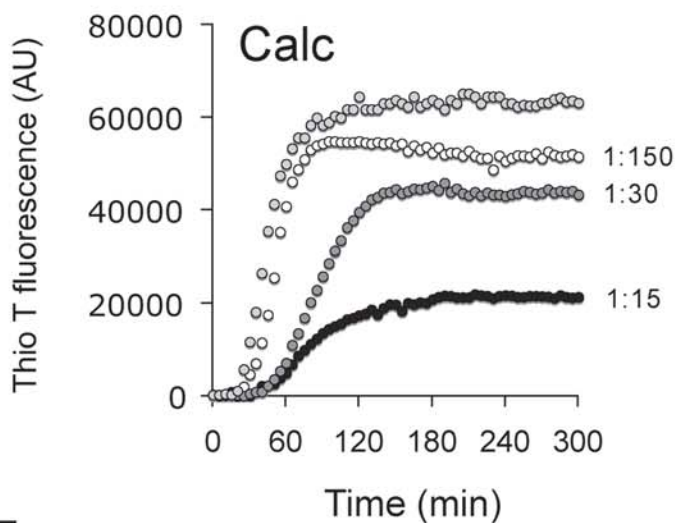
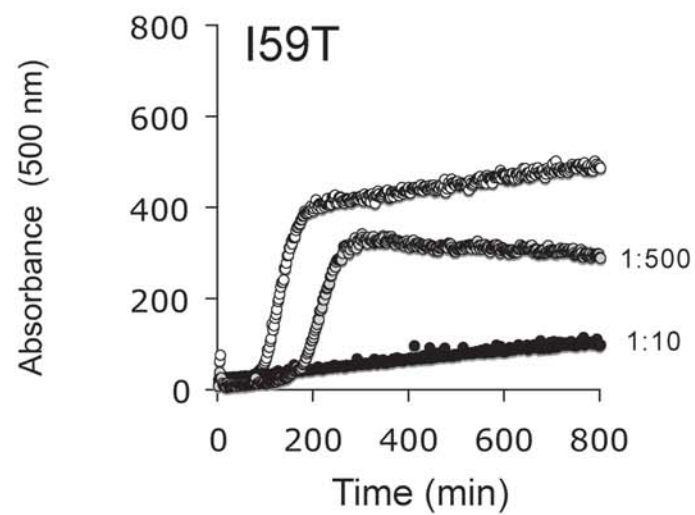
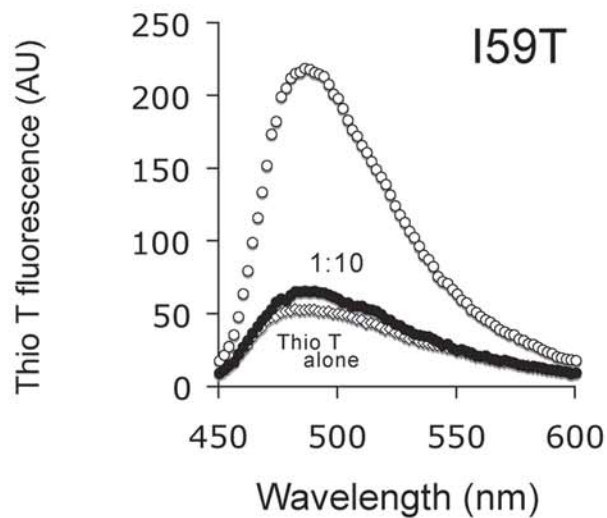
Figure 6 α_2M and Hp bind transient species early in A β fibril formation. Results of immuno dot blot assay measuring the binding of α_2M and Hp to protein species present at different times during A β fibril formation. A β (10 μM) was incubated as described in Experimental Procedures and aliquots taken 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. A β (~ 1 μg) from each sample was then spotted onto nitrocellulose and the membrane blocked and subsequently incubated with 10 $\mu g/ml$ of either α_2M or Hp. The presence of α_2M and Hp was detected using rabbit anti- α_2M and anti-Hp antibodies.

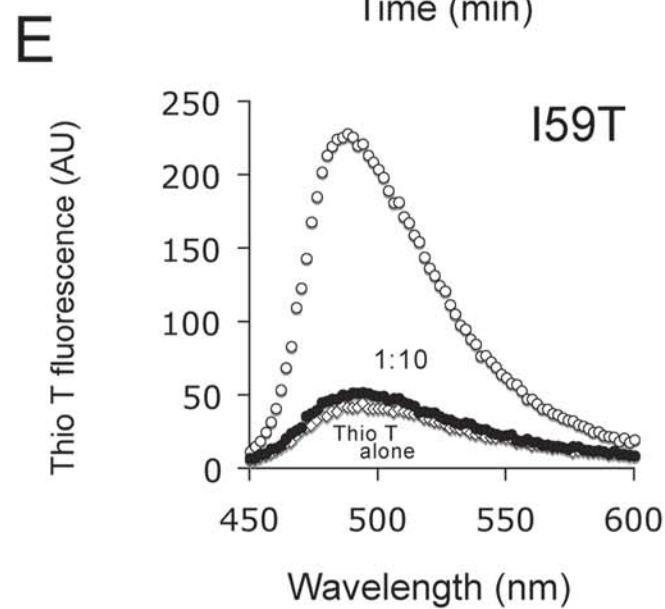
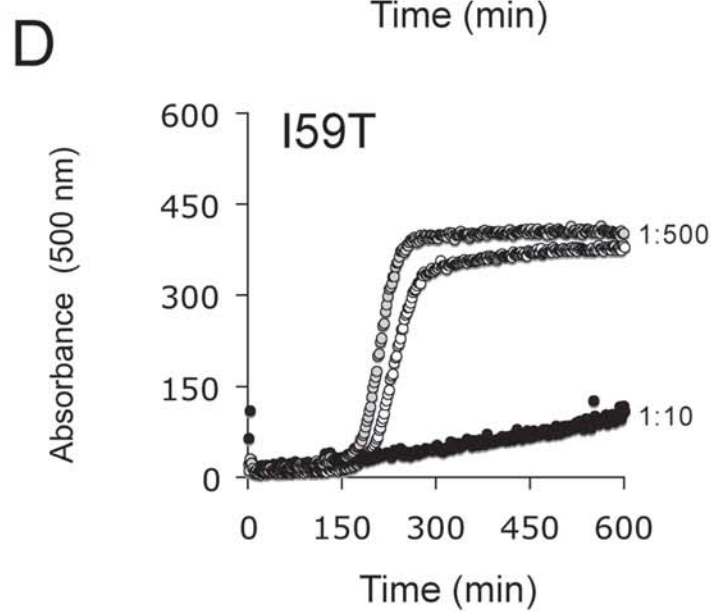
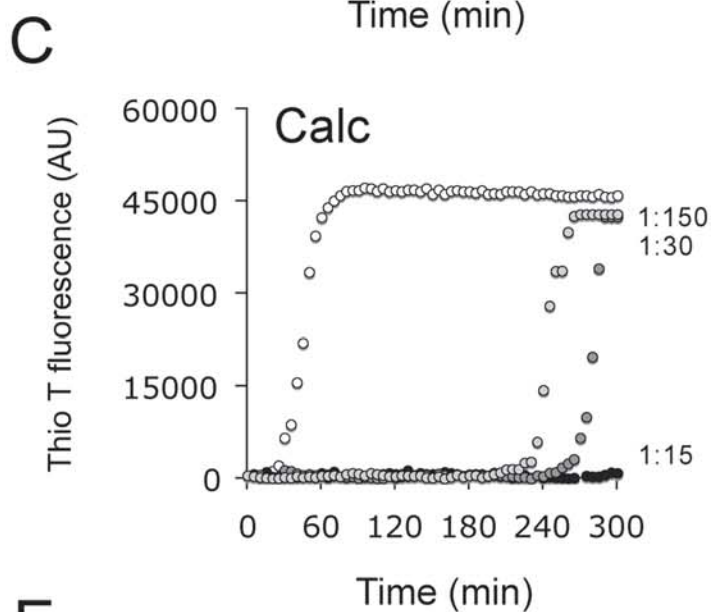
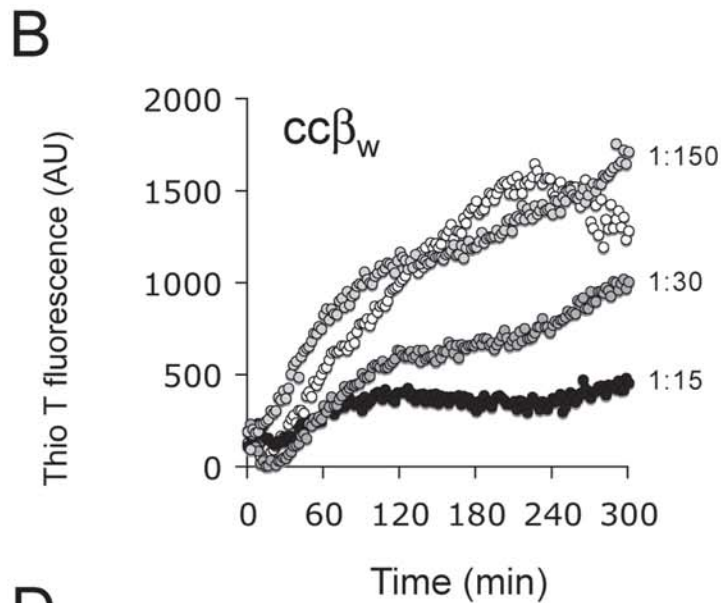
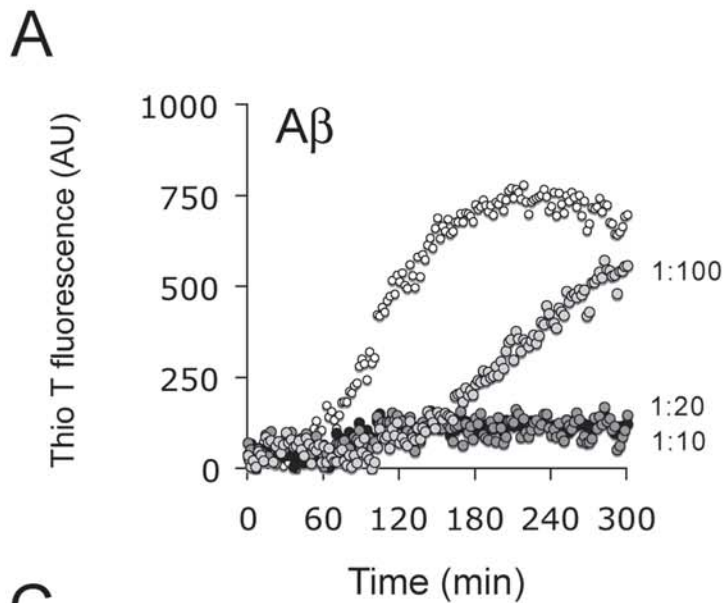
Figure 7 α_2M and Hp form stable complexes with amyloid forming proteins. Histograms showing the results of sandwich ELISA detecting complexes formed between α_2M /Hp and amyloid forming proteins. Samples taken from aggregation reactions at times corresponding to the midpoint of the elongation phase (in the absence of α_2M /Hp) were analyzed by ELISA using plate bound anti- α_2M (A) or anti-Hp (B) antibodies to capture α_2M and Hp respectively. Bound amyloid forming proteins were subsequently detected using specific antibodies, or (in the case of biotinylated A β and cc β_w) a streptavidin/biotinylated-horseradish (b-HRP) peroxidase complex. Non-specific binding of substrate was tested using samples taken from aggregation reactions of substrate alone at the same time points. For biotinylated A β and cc β_w , the negative controls were anti- α_2M /anti-Hp coated wells incubated with only streptavidin/biotinylated-horseradish peroxidase 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 3 wells and error bars represent standard error. Significant differences to controls are indicated by * (Student's t-test; $p < 0.05$). Results shown are representative of at least 2 independent experiments.

Supplementary Figure S1 Control protein BSA does not inhibit aggregation of amyloid forming proteins. A β (10 μ M), cc β_w (60 μ M), Calc (50 μ M), and I59T (6.8 μ M) were incubated as described in Experimental Procedures and amyloid formation monitored by *in situ* measurements of Thio T fluorescence (A-C), or for I59T by measuring turbidity (absorbance at 500 nm; D). In A-E, the empty circles represent data for the protein aggregating in the absence of BSA; when present, the molar ratio of BSA to substrate protein is 1:10. Data points shown are individual measurements and are representative of at least three 3 independent experiments.

Supplemental Figure S2 Haptoglobin suppression of amyloid forming protein aggregation is affected by binding of Hb. A β (10 μ M) and cc β_w (60 μ M) were incubated as described in Experimental Procedures and amyloid formation monitored by *in situ* measurements of Thio T fluorescence. The empty circles represent data for the protein aggregating in the absence of Hp, while shaded symbols represent amyloid forming protein incubated with Hp or a preincubated equimolar mixture of Hp and Hb (as indicated on panels). In all cases when present, the molar ratio of Hp to A β and cc β_w is 1:10 and 1:15, respectively. Data points shown are individual measurements and are representative of at least three 3 independent experiments.

Supplemental Figure S3 α_2 M and Hp do not dissociate mature amyloid fibrils. (A) Histogram showing the end point Thio T fluorescence of mature fibrils incubated with either α_2 M or Hp (as described in Experimental Procedures) for 500 minutes at 37°C in PBS. (B) Protein content of insoluble material retrieved by centrifugation at the endpoint of mixtures containing mature fibrils and either α_2 M or Hp (molar ratios as above). Values shown represent the mean of at least 3 determinations and error bars represent standard error. Results shown are representative of at least 2 independent experiments.

A**B****C****D****E**

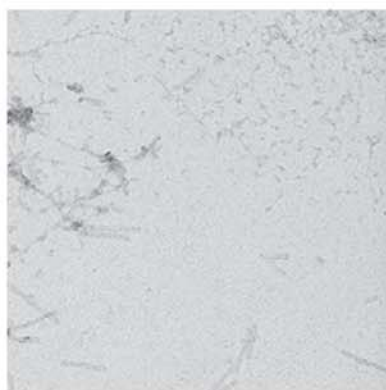
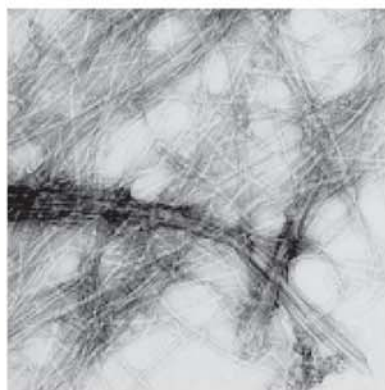


Alone

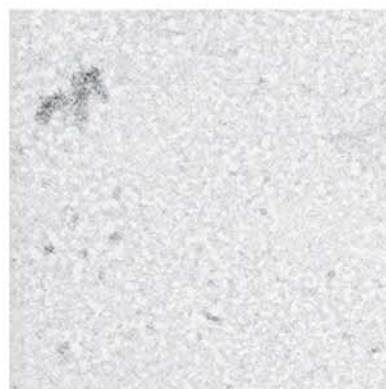
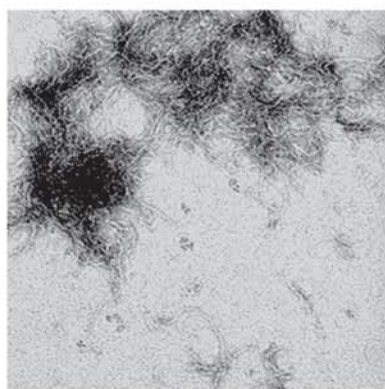
+ α_2 M

+ Hp

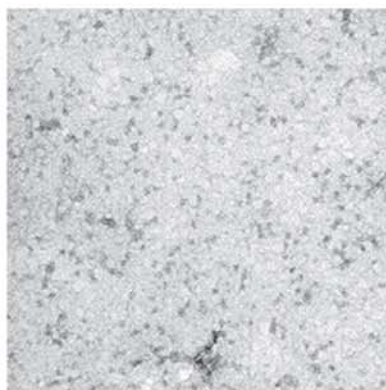
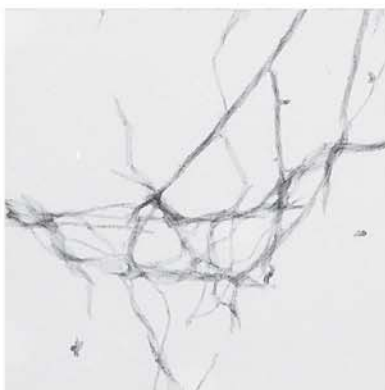
A β



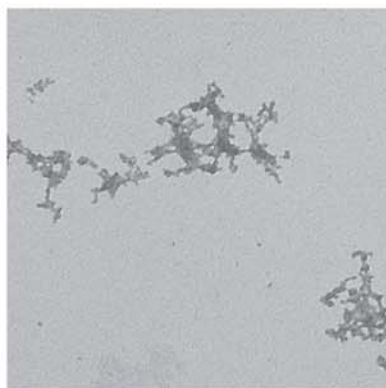
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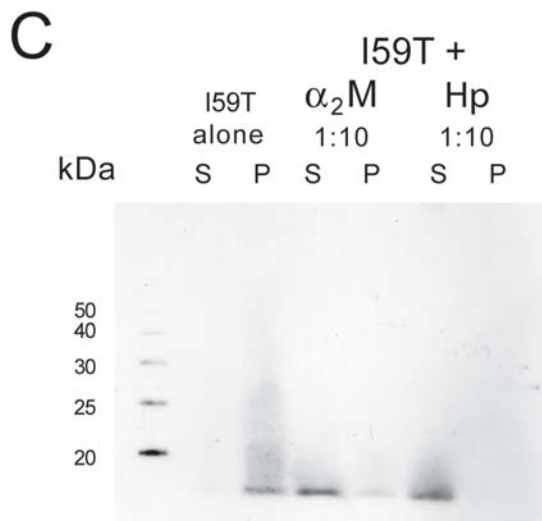
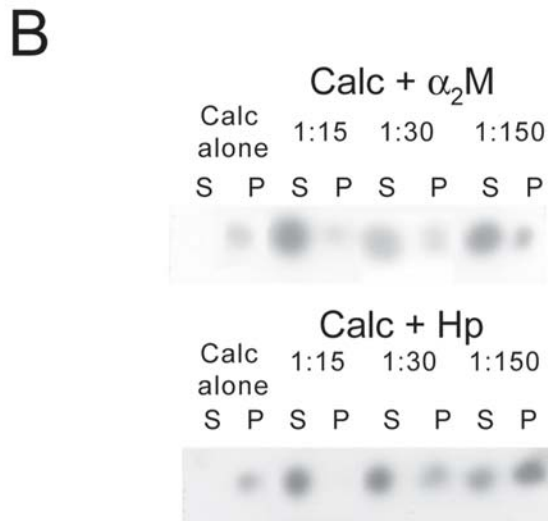
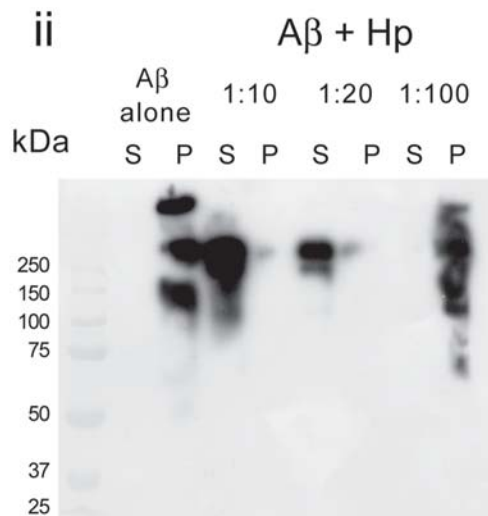
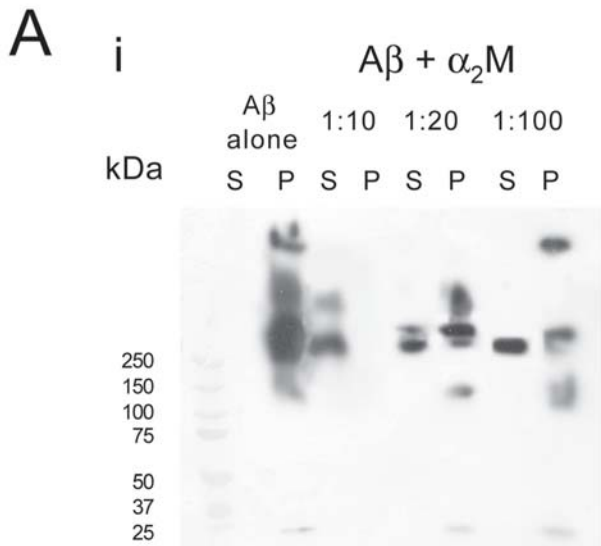
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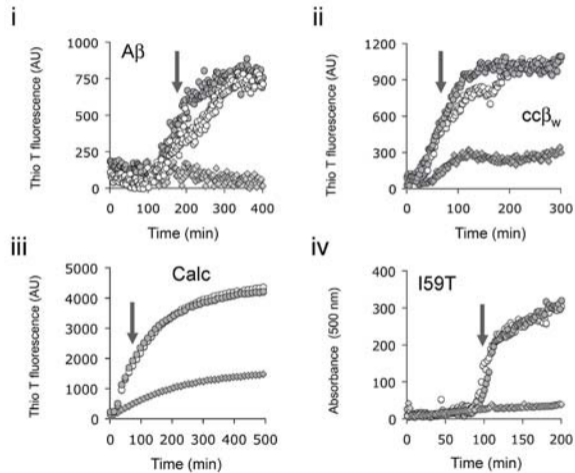
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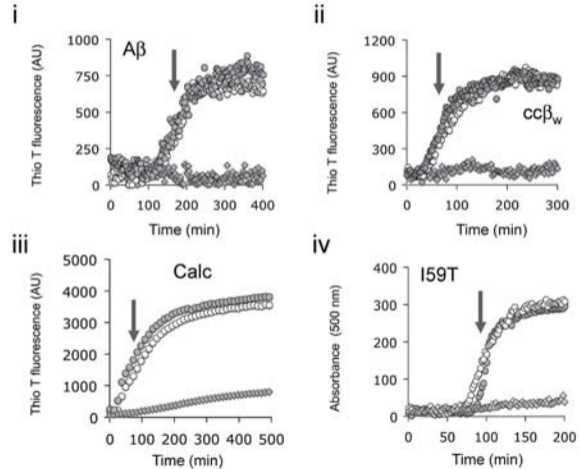
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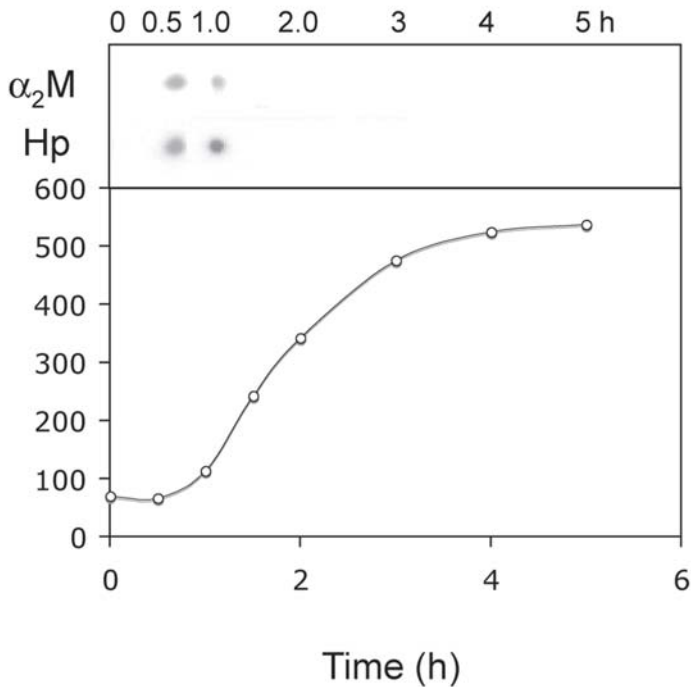
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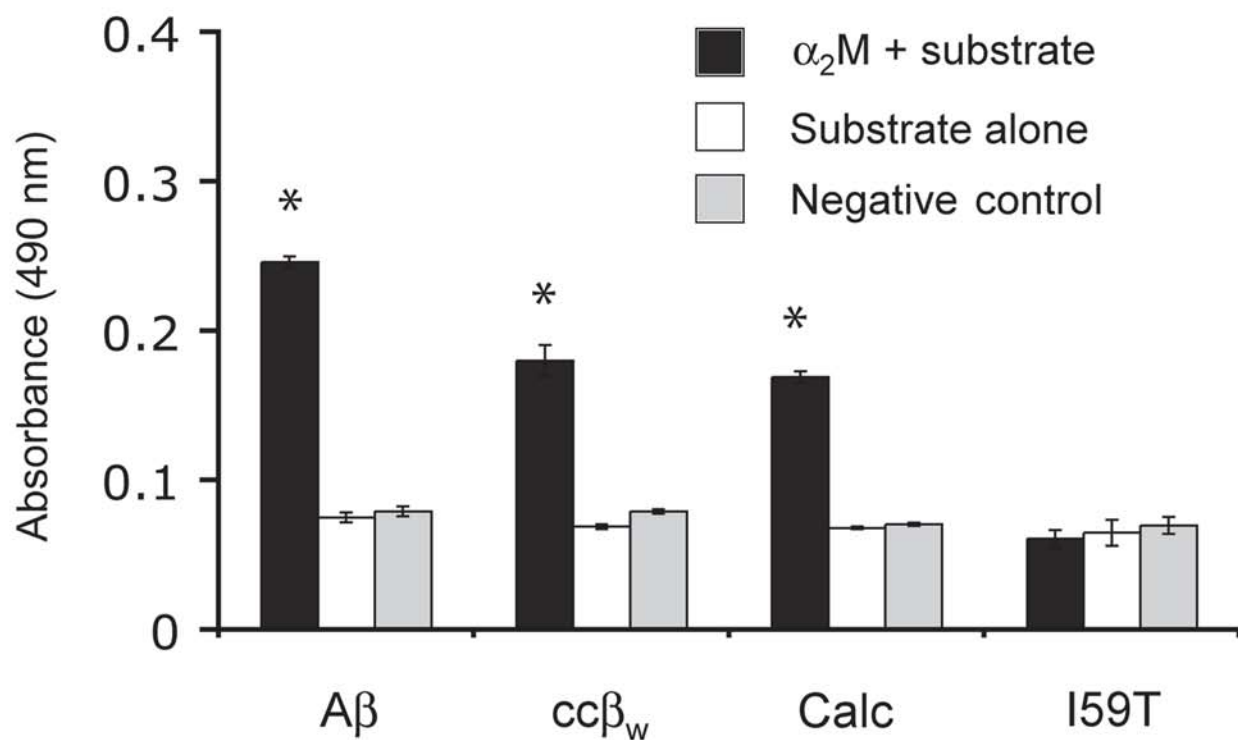
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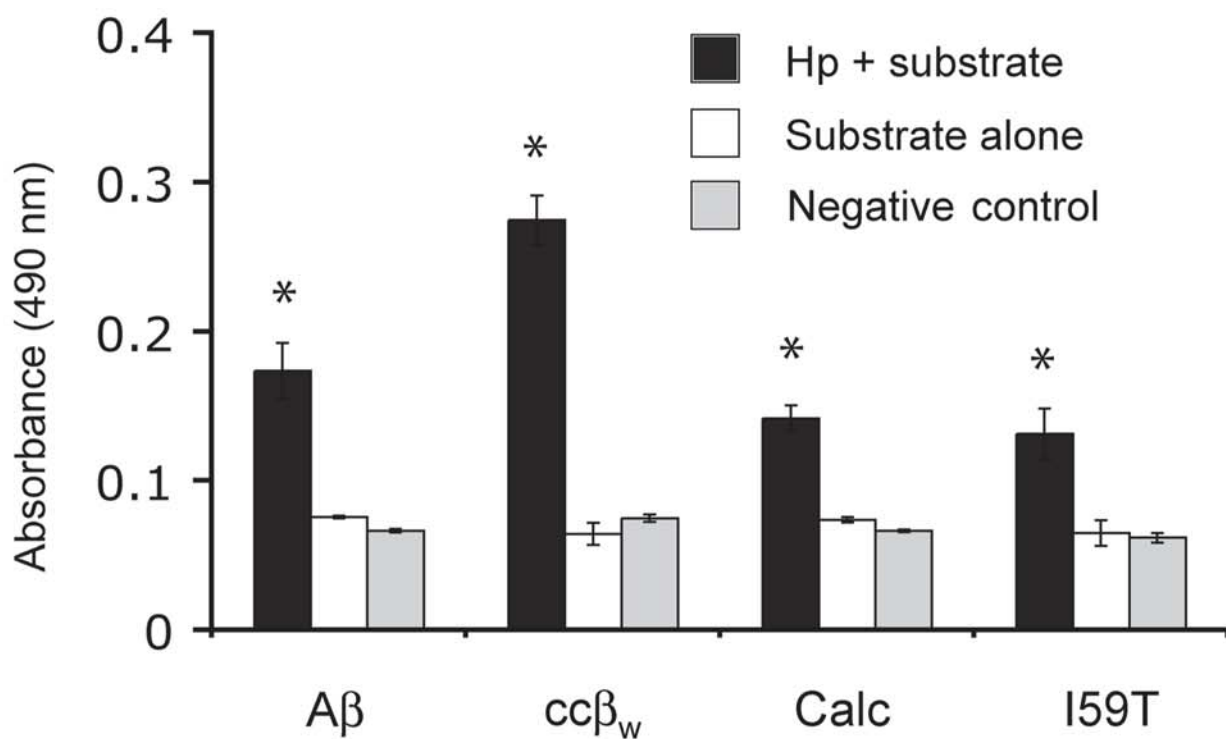
Thio T fluorescence (AU)

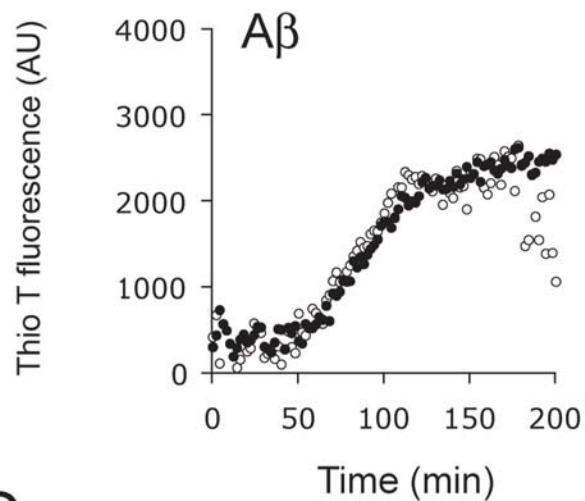
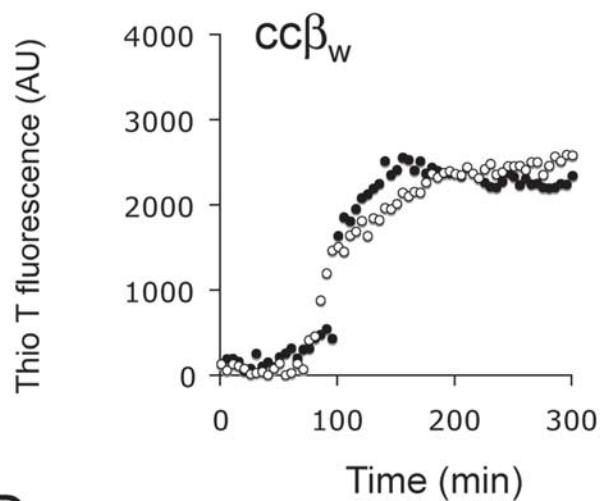
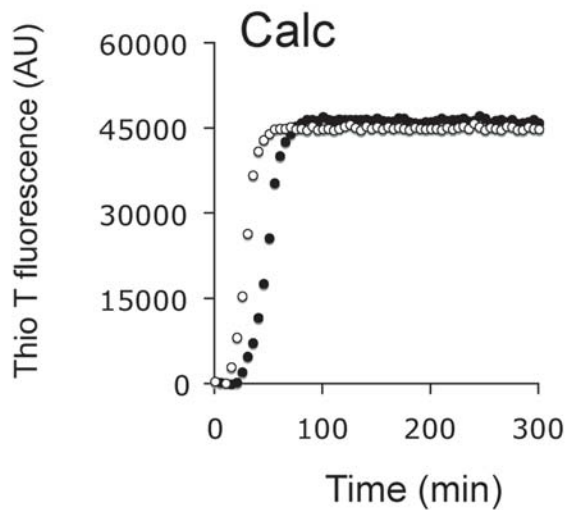
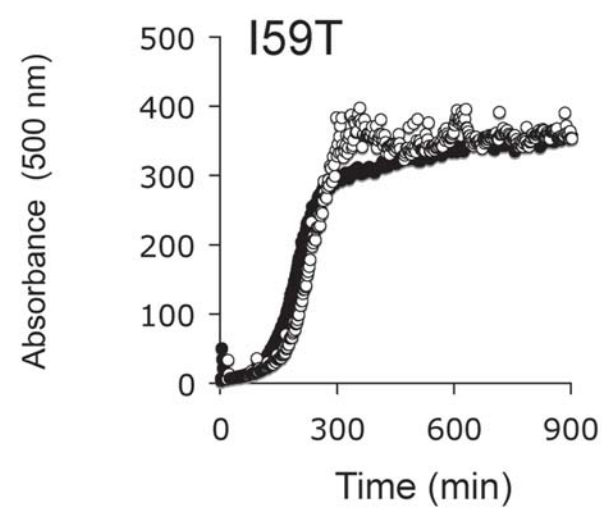


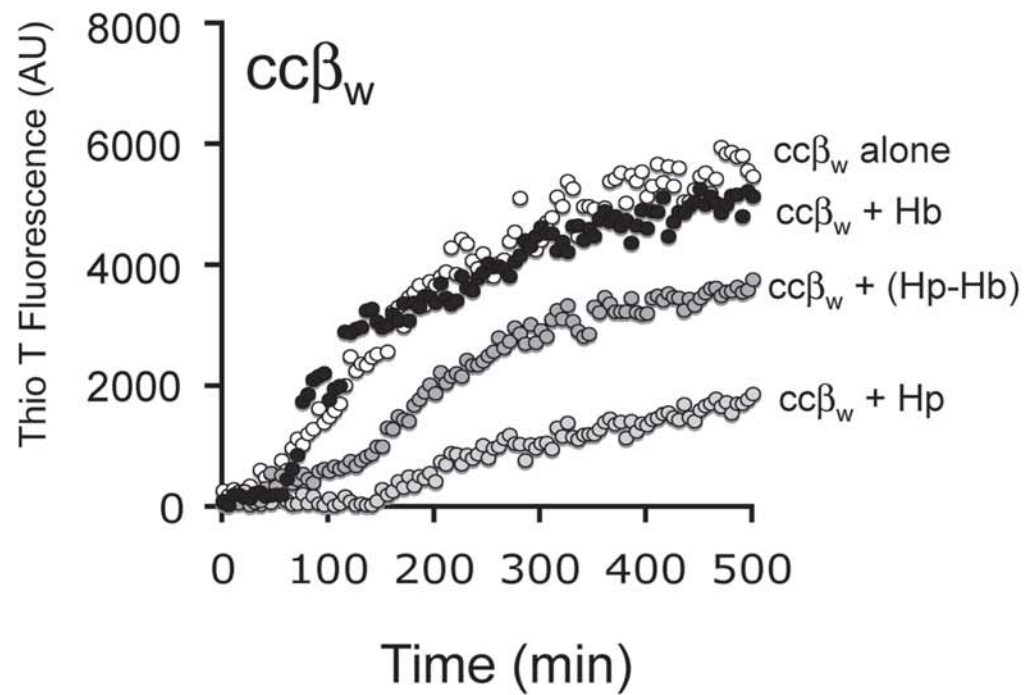
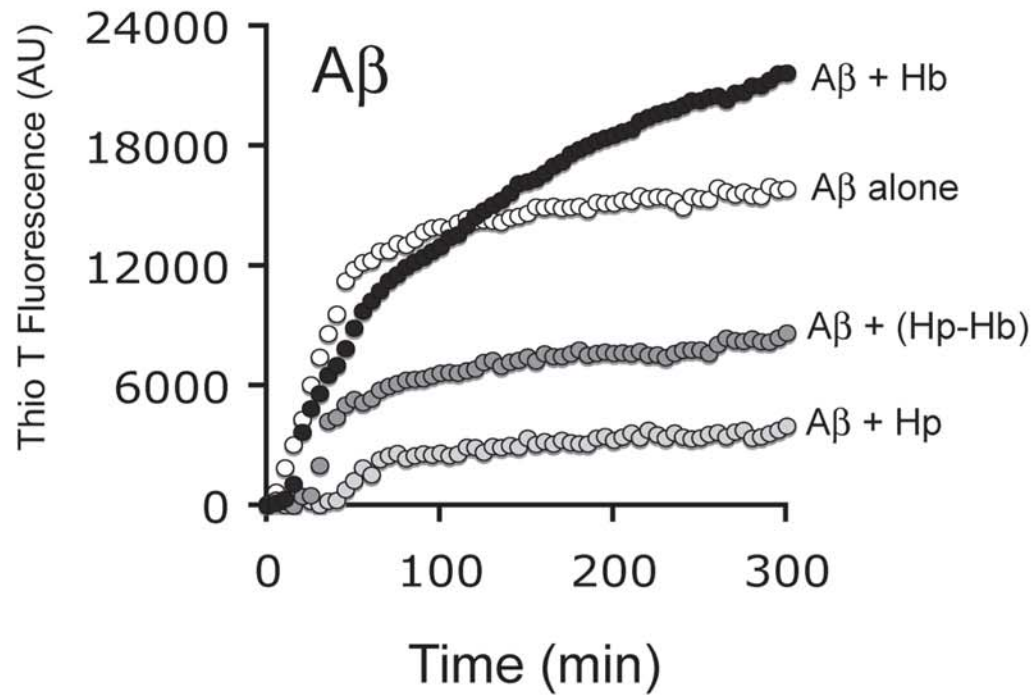
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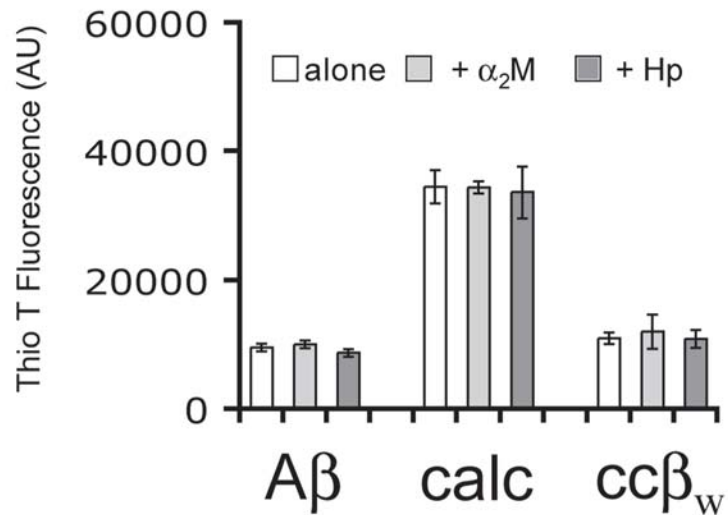


B



A**B****C****D**



A**B**