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Characterisation of novel extracellular  
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amyloid formation

Justin J. Yerbury  
University of Wollongong

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Characterisation of Novel Extracellular Molecular Chaperones and Their  
Effects on Amyloid Formation.

A thesis submitted in fulfilment of the requirements for the award of the degree

Doctor of Philosophy

from

the UNIVERSITY of WOLLONGONG

by

Justin J. Yerbury, BSc, BCom

School of Biological Sciences  
University of Wollongong, Wollongong, Australia

2007



## Certification

I, Justin J. Yerbury, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Justin J. Yerbury

10 January 2008

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## List of Abbreviations

A $\beta$	amyloid $\beta$ peptide
ALS	amyotrophic lateral sclerosis
ADP	adenosine diphosphate
ATP	adenosine triphosphate
b	biotin
BAG-1	Bcl-2-associated anti-death gene-1
bisANS	4,4'-bis(1-anilinonaphthalene-8-sulfonate)
BSA	bovine serum albumin
Calc	calcitonin
$\kappa$ -cas	$\kappa$ -casein
cc $\beta$	coiled-coil $\beta$ peptide
CD	circular dichroism
CDS	clusterin depleted serum
CHIP	C-terminus Hsp70 interacting protein
CON	control
CPK	creatine phosphokinase
CS	citrate synthase
CSF	cerebrospinal fluid
Da	daltons
DDS	double depleted serum
DF	disturbing factors
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
fMLP	formyl-met-Leu-Phe
GSH	reduced glutathione

GST	glutathione-S-transferase
Hb	haemoglobin
HBB	Hank's binding buffer
HDC	heat denatured casein
HDS	haptoglobin depleted serum
Hip	Hsp70 interacting protein
HMW	high molecular weight
Hop	Hsp organizing protein
Hp	haptoglobin
HRP	horseradish peroxidase
Hsc	heat shock cognate
HSF	heat shock factor
Hsp	heat shock protein
Ig	Immunoglobulin
K <sub>D</sub>	dissociation constant
LDLR	low density lipoprotein receptor
LRP	low density lipoprotein receptor related protein
Lys	lysozyme
$\alpha_2$ M	$\alpha_2$ -macroglobulin
$\alpha_2$ M*	activated $\alpha_2$ -macroglobulin
$\alpha_2$ MDS	$\alpha_2$ -macroglobulin depleted serum
$\beta_2$ M	$\beta_2$ -microglobulin
NADH	reduced nicotinamide adenine dinucleotide
NHS	normal human serum
NHS-LC-b	succinimidyl-6-[biotin-amido]hexanoate
OSB	oxidative stress buffer
Ovo	ovotransferrin
OX	oxidative
P	pellet
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PDAPP	PDGF promoter expressing amyloid precursor protein
PI	propidium iodide
PrP	prion protein
RAP	receptor associated protein
RT	room temperature
S	supernatant
SAP	serum amyloid P component
SaRIgG	sheep anti rabbit IgG
SDS	sodium dodecyl sulphate
SE	standard error
SEC	size exclusion chromatography
SH3	SH3 domain of the p85 alpha subunit of phosphatidylinositol 3 kinase
sHSP	small heat shock protein
SMR	subunit molar ratio
SOD	superoxide dismutase
$\tilde{\alpha}$ syn	$\alpha$ -synuclein
TEM	transmission electron microscopy
TGF $\beta$	transforming growth factor $\beta$
Thio T	thioflavin T
TNF $\alpha$	tumor necrosis factor $\alpha$
TPR	tetratricopeptide repeats
tRNA	transfer ribonucleic acid
UV	ultra violet
VLDLR	very low density lipoprotein receptor

## List of Publications

**Yerbury JJ**, Rybchyn MS, Easterbrook-Smith SB, Henriques C, Wilson MR. (2005) The acute phase protein haptoglobin is a mammalian extracellular chaperone with an action similar to clusterin. *Biochemistry* 44: 10914-10925

**Yerbury JJ**, Stewart EM, Wyatt AR, Wilson MR. (2005) Quality control of protein folding in extracellular space. *EMBO Reports* 6: 1131-1136

Kumita, JR, Poon, S, Caddy, GL, Hagan, CL, Dumoulin, M, **Yerbury, JJ**, Stewart, EM, Robinson, CV, Wilson, MR and Dobson, CM. (2007) The extracellular chaperone clusterin potently inhibits human lysozyme amyloid formation by interacting with prefibrillar species. *Journal of Molecular Biology* 369: 157-167

**Yerbury, JJ**, Poon, S, Meehan, S, Thompson, B, Kumita, JR, Dobson, CM and Wilson, MR. (2007) The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with pre-fibrillar structures. *FASEB Journal* 21: 2312-22

Wilson MR., **Yerbury JJ.**, Poon S. (2008) The role of extracellular chaperones in amyloid formation. *Molecular BioSystems* 4: 42–52

Wilson MR. and **Yerbury JJ**, (2008) Instant insight: Think outside the cell. *Chemical Biology* 3: B15.

French K, **Yerbury JJ**, Wilson MR. (2008) Protease activation of  $\alpha_2$ -macroglobulin modulates a chaperone-like action with broad specificity. *Biochemistry* 47: 1176-1185.

Wilson MR, **Yerbury JJ**, Poon S. (2008) “Extracellular chaperones and amyloids” in the book titled “*Heat shock proteins and the brain: Implications for Neurodegenerative disease and Neuroprotection*”. Series: Heat Shock Proteins, Volume 3. Springer publications, New York, USA. Edited by Alexander Asea and Ian R. Brown.

Park DC, Yeo SG, Wong K, **Yerbury JJ**, Wilson MR, Bandera CA, Welch R, Choi YK, Birrer MJ, Berkowitz RS, and Mok SC. Overexpression of clusterin confers paclitaxel resistance in ovarian cancer. *Manuscript submitted*.

## List of Conference Presentations

Poster presentation “Haptoglobin is an extracellular chaperone with an action similar to that of clusterin” at the 29th Annual Lorne Conference on Protein Structure and Function. Lorne, Victoria, Australia, February 8-12, 2004.

Oral Presentation “The effects of clusterin on amyloid formation” at the 4th International Workshop on Clusterin, Villars-sur-Ollon, Switzerland, June 16-18, 2005.

Poster presentation "Does the extracellular chaperone clusterin affect amyloidogenesis" at the FASEB Summer Research Conference entitled "Amyloid fibril formation, protein misfolding and aggregation" Snowmass Village, CO, USA, June 10-15, 2006.

Poster presentation “The extracellular protease inhibitor  $\alpha_2$ -macroglobulin has chaperone-like properties” at the World Conference of Stress, Budapest, Hungary 23-26 August 2007.

Invited oral presentation “Quality control of extracellular protein folding: An emerging field” at the World Conference of Stress, Budapest, Hungary 23-26 August 2007.

## Abstract

Individual proteins have a specific three-dimensional structure that gives them their unique function. However, a protein must be folded from a linear string of amino acids in order to gain this native conformation and thus function. There are many hurdles to a protein attaining and maintaining its native conformation. Stresses that are encountered in the life of a protein, such as changes in pH, temperature and oxidative stress, can promote protein misfolding or unfolding. In addition, some genetic mutations can modify a protein such that a non-native conformation is more energetically favourable than the native state. The unfolding or misfolding of a protein makes it more likely that it will aggregate with itself. There are more than 40 human diseases associated with the inappropriate deposition of aggregated protein. It is well known that there is a well-defined and efficient quality control system to deal with intracellular proteins that have either unfolded or are misfolded. Cells have a range of molecular chaperones to inhibit inappropriate aggregation and if this fails the cell labels the proteins with ubiquitin for degradation via the proteasome. However, many proteins are secreted from cells into the extracellular environment and there are many protein deposition disorders associated with extracellular protein deposits, outside the reach of the well-characterised intracellular quality control system.

This thesis reports that the secreted proteins haptoglobin and  $\alpha_2$ -macroglobulin have small heat shock protein-like chaperone activity. Both haptoglobin and  $\alpha_2$ -macroglobulin specifically inhibited the precipitation of a variety of proteins induced by either heat or oxidation, including proteins in unfractionated human serum. In addition, it was demonstrated that haptoglobin and  $\alpha_2$ -macroglobulin inhibit the precipitation of stressed proteins by forming solubilized complexes with them, cannot protect enzymes from heat-induced loss of function, and lack ATPase activity and the ability to independently refold proteins following stresses. In addition, data presented here shows that clusterin, haptoglobin and  $\alpha_2$ -macroglobulin exert potent effects on amyloid formation, and provide evidence to suggest that these effects are exerted via interactions with pre-fibrillar species. These findings suggest that clusterin, haptoglobin and  $\alpha_2$ -macroglobulin are an important element in the control of extracellular protein misfolding.

## Chapter 1

# Introduction

### **Preface**

This thesis has been prepared as a series of manuscripts written for publication. As a consequence, each chapter stands alone and, therefore, their introductions are detailed, citing relevant literature and setting the context for each of the aims addressed. However, the reader will find that this results in the inevitable overlap of some material between the Introduction sections of each individual chapter and that contained in the overview presented in this Chapter. The material in this chapter, therefore, only briefly touches on certain subject matters while attempting to give a general background to concepts arising in the subsequent chapters.

## 1.1 General introduction

It has been estimated that about 400 grams of protein is synthesised and degraded each day in the human body; the liver alone can produce up to 100 grams per day of plasma protein (Guyton 1976). Individual proteins are degraded at extremely varied rates, with half-lives ranging from several minutes to many hours. Inside cells this variation in half-life has been attributed to the differences in intrinsic stability of proteins and the recognition of non-native structures by a highly selective and precisely regulated intracellular protein quality control system (Wickner *et al.*, 1999). Although extracellular proteins are also known to have extremely varied half-lives (Bouma 1982) the existence of a system that selectively degrades proteins in the extracellular space has not been demonstrated. This chapter briefly introduces the concept of protein folding, misfolding and its consequences and examines known protein quality control machinery.

## 1.2 Protein structure and function

The central dogma of structural biology states that a protein's function depends on the exact three-dimensional structure of its mature folded form (Hohfeld *et al.*, 2001; Slavotinek and Biesecker 2001; Ellis and Pinheiro 2002). This relationship between tertiary structure and function has long been established (Dixon and Webb 1965). Early experiments showed that proteins could be “scrambled” or “denatured” by various stresses, such as extreme pH, temperature and chemical denaturants (Dixon and Webb 1965; Anfinsen 1973). Under these “denaturing” conditions it was found that the proteins abandoned their function and were essentially devoid of the various aspects of regular structure that characterised their individual native states (Anfinsen 1973). However, some proteins were found to spontaneously refold upon removal from the denaturing conditions, “unscrambling” them. It was thus theorised by Anfinsen that under certain conditions the fully folded three-dimensional structures were solely determined by the amino-acid sequence of the protein in question. This posed the



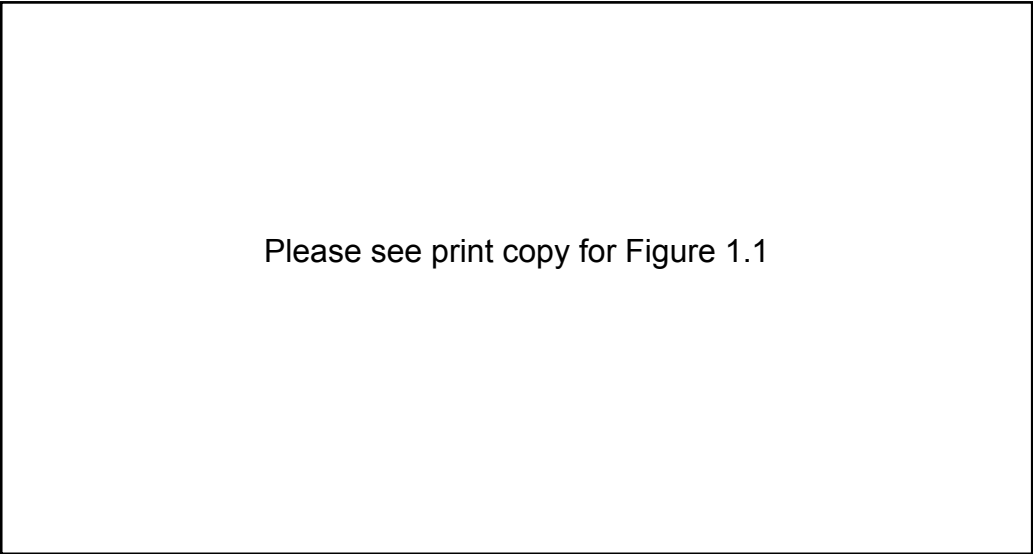
question; with only the amino acid sequence as information, how does a protein self assemble? This quandary is known as the *protein folding problem*.

### 1.2.1 The protein folding problem

The native conformation of proteins almost always corresponds to the structures that are most thermodynamically stable under physiological conditions (Dobson 2003). This stability stems from the thousands of weak non-covalent interactions between amino acid side chains. Two of the most important of these stabilising interactions are hydrogen bonds and van der Waals interactions between non-polar atoms (also called hydrophobic interactions) (Creighton 1994). In stark contrast to the idea of a protein having one specific, stable, native state is the fact that a protein may adopt many different conformations in the non-native state. This is due to the fact that there is relatively free rotation about the single bonds (excluding the peptide bond) on the peptide backbone, thus polypeptide chains have the potential to form a plethora of conformations (Creighton 1994). Moreover, random searching through these apparently endless conformations to find the most thermodynamically favourable would take an infinite amount of time (Daggett and Fersht 2003). That proteins *can* fold into their native state in a relatively small amount of time (typically seconds or minutes) has become known as the ‘Levinthal Paradox’ (Fersht 1997).

The new view of protein folding suggests that there is not a single specific folding pathway for a given protein as was suggested in earlier models (Radford 2000). Recent thinking suggests that the fundamental mechanism of protein folding involves the interaction of a relatively small number of residues to form a folding nucleus, about which the remainder of the structure rapidly condenses (Daggett and Fersht 2003; Dobson 2003) (see Figure 1.1A). A useful analogy is to that of a large jigsaw puzzle (Creighton 1994). The pieces are joined together in a completely different sequence each time the puzzle is completed, yet the final result is always the same. Certain parts of the puzzle (the more recognisable bits) are usually made first, and from these puzzle “nuclei” the other more difficult parts are assembled. Due to the fact that native-like interactions in the folding protein are more stable than non-native ones, they are more persistent and the polypeptide is able to find its lowest-energy structure by a process of

trial and error. This can be represented by what has been termed an energy landscape. The energy landscape (Figure 1.1B) dictates that a protein has a plethora of potential routes to the native state, but energy restraints in the landscape make it more likely that the folding protein will populate certain intermediates (Fersht 1999; Radford 2000; Dobson 2003). Accordingly, the energy landscape for a given protein describes the folding pathway as a progressive collection of geometrically similar collapsed structures, of varying energy states, one of which is more thermodynamically favourable than the rest (Fersht 1999).



Please see print copy for Figure 1.1

**Figure 1.1 The theory of protein folding.** (A) A hydrophobic collapse can direct a folding protein into 2 (for small proteins) and 3 stage folding pathways. For small proteins (a) it is thought that a two stage folding pathway exists with no intermediate structures. Larger proteins (more than 100 residues in size) are thought to fold through a series of intermediate structures driven by either (b) a hydrophobic collapse into a molten globule state, which shortens the search for a favourable thermodynamic structure, or (c) nucleation of a folding domain which then promotes favourable interactions for further folding (reproduced from Radford (2000)). (B) A three-dimensional folding funnel best describes the protein folding process. Shown is the energy surface landscape of a folding funnel generated from experimental data for lysozyme. The axes are defined as follows: E represents the energy of the system, Q is the proportion of native contacts formed, and P is a measure of the available conformational space. Three possible pathways are shown; a fast folding pathway (yellow), a slow folding pathway that crosses the high energy barrier (green), and a slow folding pathway (red) which passes through a less folded state before traveling the fast folding pathway (reproduced from Matagne and Dobson (1998)).

## 1.3 The consequences of protein misfolding and unfolding

### 1.3.1 Protein misfolding and unfolding

Protein folding is essential to living organisms because it provides the functional machinery for the genetic blueprint from which it is made. Consequently much energy is invested in ensuring that the transfer of structural information from genes to folded proteins is correctly executed. Individual cells employ mechanisms such as the exonuclease activity of DNA polymerases and the proof-reading ability of tRNA synthetases to control fidelity during transferral of genetic information (Zubay *et al.*, 1995). This high fidelity transferral of information is vital since the amino acid sequence is the only information used to direct the proper folding of a protein (Soto 2001). Nevertheless, correct protein folding is still not assured. Mistakes in transcription or translation that result in errors as small as an amino acid substitution can destabilise, and thus prevent, normal folding causing a protein to misfold. In addition, certain environmental conditions, such as macromolecular crowding, inappropriate ionic strength, oxidative stress and extremes of pH and temperature are known to promote the formation of misfolded states or slowly reacting intermediates trapped on the folding pathway. These same environmental conditions are also known to promote partial protein unfolding in fully folded native protein (Sherman and Goldberg 2001).

### 1.3.2 The fate of misfolded proteins

If left unchecked, unfolded or misfolded proteins will aggregate; exposed hydrophobic regions on unfolded or misfolded proteins are thought to bind (by highly specific self-association (Rajan *et al.*, 2001)) to similar regions on nearby proteins instead of becoming internalised in the final globular structure (Figure 1.2). The aggregating structure can continue binding exposed hydrophobic regions of nearby proteins and thus has the potential to form various stable structures, such as soluble aggregates, insoluble amorphous aggregates and insoluble fibrillar structures (Ellis and Pinheiro 2002). Many disease states are associated with abnormal protein deposits comprised of aggregated protein (Table 1.1), including an insoluble fibrillar aggregate known as amyloid. The

mechanism of toxicity of protein aggregates is a topic of contention, with views differing on the types of structures that are toxic (Wang *et al.*, 2002).

**Table 1.1 Aggregated proteins associated with disease**

Protein involved	Disease(s)
Amyloid- $\beta$ *	Alzheimer's disease
Tau	Alzheimer's disease
$\alpha$ -Synuclein	Parkinson's disease, Lewy body dementia
Amylin *	Diabetes type 2
SOD1	Amyotrophic Lateral Sclerosis
$\beta_2$ -Microglobulin *	Haemodialysis-related amyloidosis
Amyloid-A *	Reactive amyloidosis
Haemoglobin	Sickle cell anaemia
Huntingtin	Huntington's disease
PrP *	Creutzfeldt-Jakob disease
Androgen receptor	Spinobulbar muscular atrophy
Ataxins 1,2 & 3	Spinocerebellar ataxia's 1,2 & 3
Ten other proteins*	Systemic and cerebral hereditary amyloidosis

\* indicates the protein is deposited extracellularly

Source: (Sherman and Goldberg 2001; Soto 2001)

### 1.3.3 Amyloid

More than 20 structurally unrelated proteins have been found to form amyloid deposits *in vivo*. Amyloid fibrils found associated with different pathologies display common structural features despite variation in the individual amino acid sequence and native structures of the amyloid forming proteins. It has been suggested that these structural similarities arise because amyloid fibrils are formed from interactions between polypeptide backbones, which are common to all proteins (Dobson 2001). Typically, amyloid fibrils are composed of 2-6 unbranched "protofilaments" which are 2-5 nm wide and that twist together in a rope like structure to form fibrils (Serpell 2000). The formation of fibrils with this morphology has also been widely observed *in vitro*, both from proteins associated with disease and from other unrelated proteins. X-ray diffraction studies have confirmed that a range of amyloid fibrils share a common core

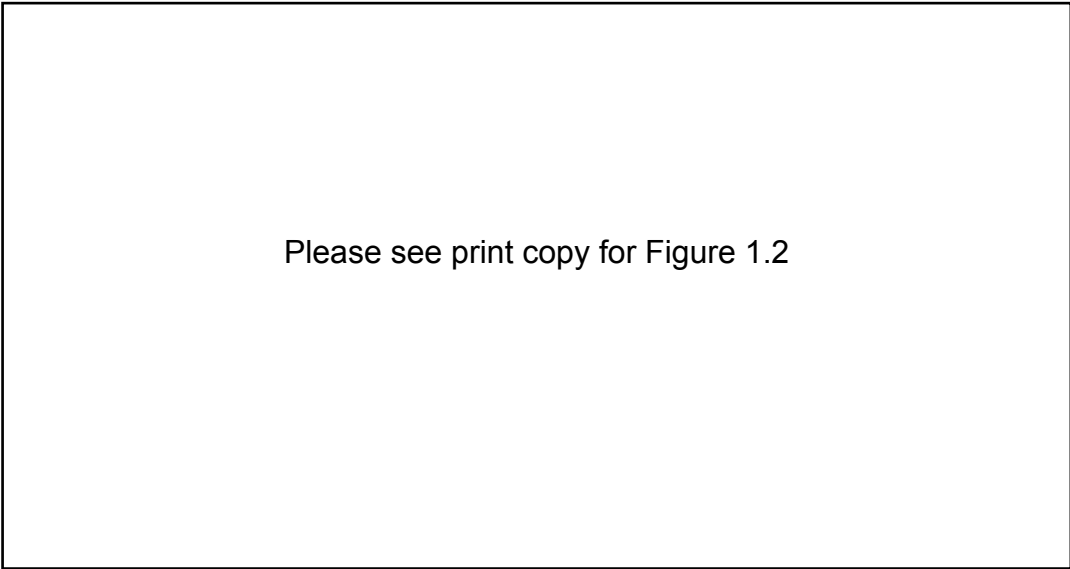
structure consisting of  $\beta$ -strands lying with their axis perpendicular to the axis of the fibrils (Sunde *et al.*, 1997). This cross  $\beta$ -structure gives rise to characteristic spectroscopic properties when dyes such as Congo Red and Thioflavin T (or S) bind; when bound to amyloid, these dyes produce green birefringence under polarised light, and fluorescence, respectively (Puchtler and Sweat 1965; Naiki *et al.*, 1989).

#### *1.3.3.1 How is amyloid formed?*

It has been postulated that the ability of proteins or peptides to form amyloid fibrils is a generic feature of the polypeptide chains (Dobson 1999). However, it is clear that some proteins or peptides have a greater propensity to form amyloid compared to others. It is thought that hydrophobic residues, net charge and relative ease of  $\beta$ -sheet formation all contribute to the propensity of a protein to form amyloid (Chiti and Dobson 2006). Regardless, the process of amyloid fibrillogenesis must involve marked refolding of the native precursor proteins to generate intermediate structures with a high proportion of  $\beta$ -structure that can assemble together into mature fibrils (Figure 1.2). This is consistent with the observation that amyloid fibrils typically form under conditions in which the native state is destabilised, such as low pH or elevated temperature (Dobson 2001). Moreover, there are many genetically inherited mutations that destabilise specific proteins associated with amyloid deposits *in vivo*. All amyloidogenic proteins investigated have been shown, *in vitro*, to be capable of partly unfolding and refolding into structures that favour aggregating to form typical amyloid fibrils. In general, amyloid formation follows a kinetic pathway characteristic of crystallization; there is an initial ‘lag’ or nucleation phase, followed by a rapid exponential ‘growth’ or polymerization phase (Jarrett and Lansbury 1993), lastly there is a plateau phase in which no further fibril growth occurs (Figure 1.2). The lag phase represents the time required for the formation of the soluble (prefibrillar) oligomers or nuclei that are required to seed fibril growth. There is no clear consensus on the physiological conditions that induce partial unfolding of amyloid forming proteins and their assembly into amyloid fibrils. Since specific protein structure determines stability (Goldberg 2003) the inducing conditions may differ for different precursor proteins.

### 1.3.3.2 Amyloid toxicity

Amyloid deposits can eventually become so large that they disrupt the normal structure and function of affected tissues or organs which can lead to organ failure and death (Pepys 2001). However, it is thought that cell damage and disease pathology, particularly in the brain, can also arise from direct toxic effects of amyloid species on cells. In some cases mature amyloid fibrils have been shown to be cytotoxic to cells *in vitro* (Lorenzo *et al.*, 1994; Su and Chang 2001). However, non-fibrillar aggregates on the amyloid-forming pathway of both A $\beta$  and transthyretin are cytotoxic, while mature fibrils formed from the same proteins are less toxic (Walsh *et al.*, 1999; Sousa *et al.*, 2001).



Please see print copy for Figure 1.2

**Figure 1.2 Schematic diagram of protein aggregation.** There is thought to be an ensemble of possible intermediate structures of any one protein on the protein folding/unfolding pathway. Partly folded proteins may sometimes associate with similar chains to form aggregates. Amorphous aggregates are depicted here as resulting from non-specific interactions between many different conformations, while fibrillar structures can be formed by certain intermediates high in  $\beta$  structure (reproduced from Wilson *et al.*, (2008)).

In addition, it was recently shown that pre-fibrillar aggregates of two small protein fragments, PI3-SH3 and HypF-N, are highly cytotoxic while the mature fibrils formed from these are not (Bucciantini *et al.*, 2002). Similar results have also been reported for

$\alpha$ -synuclein (Conway *et al.*, 2000). Moreover, in many protein deposition diseases the presence of protein deposits does not correlate well with disease progression (e.g. Alzheimer's (Kirkitadze *et al.*, 2002), ALS (Lee *et al.*, 2002), Parkinson's (Volles and Lansbury 2003) and familial amyloidotic polyneuropathy (Sousa *et al.*, 2001)). Thus it would seem that non-fibrillar aggregates which precede the formation of mature fibrils may be the primary clinical toxic species in amyloid diseases. A recent study has shown that there is a common recognisable pre-fibrillar structure detectable on the amyloid forming pathway of IAPP, lysozyme,  $\alpha$ -synuclein, A $\beta$ , Prion<sub>106-126</sub>, insulin and poly-L-glutamine (Kayed *et al.*, 2003). This suggests that the pre-fibrillar toxic species may be comprised of small oligomeric aggregates of the  $\beta$ -structure rich misfolded form of the amyloid forming protein. It is apparent that the pathogenicity of the protein deposition diseases may be primarily related to the structural nature of the aggregate rather than to the specific sequences of proteins from which they arise (Bucciantini *et al.*, 2002). It is known that a variety of amyloid forming proteins can form ion channels in lipid bilayers; these includes A $\beta$  peptide (Lin *et al.*, 2001), an isoform variant of SAA (Hirakura *et al.*, 2002),  $\beta_2$ -microglobulin (Hirakura and Kagan 2001), poly-L-glutamine (Hirakura *et al.*, 2000a), SOD (Chung *et al.*, 2003), amylin and prion protein (Hirakura *et al.*, 2000b). These observations have led to the channel hypothesis, which states that amyloidogenic proteins insert into cell membranes to form ion channels that allow uncontrollable movements of ions (e.g. Ca<sup>2+</sup> influx), resulting in cell death. However, other studies indicate that amyloid may exert cytotoxicity by other mechanisms, such as via metal dependant induction of oxidative stress (Huang *et al.*, 1999) or by effects on specific (pre-existing) cell membrane Ca<sup>2+</sup> channels (Silei *et al.*, 1999). Amyloid species have also been shown to have the ability to activate microglial cells in the brain triggering acute inflammation resulting in neuron death (Jekabsone *et al.*, 2006). Moreover, it is entirely possible that amyloid forming proteins and their aggregates might exert toxicity via multiple routes.

## 1.4 Cellular defences against misfolded/unfolded proteins

Given that protein aggregates are toxic, it is not surprising that mechanisms have evolved to enable cells to respond to the effects of a diverse range of insults on protein

structure (Sherman and Goldberg 2001). Both prokaryotic and eukaryotic cells utilise a post-translational quality control system, consisting of molecular chaperones and energy-dependent proteases, that are designed to repair or remove damaged proteins. Consequently, damaged proteins have three possible fates: rescue by chaperones, destruction by energy-dependent proteases, or aggregation.

#### 1.4.1 Rescue by chaperones

Chaperones are a diverse group of proteins that selectively recognise and bind exposed hydrophobic surfaces of non-native proteins in a non-covalent but stable interaction. The several families of molecular chaperones, defined by size, cellular compartment and function (Table 1.2), work together to prevent protein aggregation and facilitate the correct folding of non-native proteins (Slavotinek and Biesecker 2001). All intracellular chaperones fall under two broad, functionally related categories: *folding helper* and *holding type* chaperones (Bross *et al.*, 1999).

**Table 1.2 Major classes of intracellular chaperone proteins**

Please see print copy for Table 1.2



*Folding helper* type chaperones (Hsp90, Hsp70, Hsp60) facilitate the correct folding of non-native proteins through regulated binding and release. Release of polypeptides is driven by ATP-dependent conformational changes. This permits misfolded proteins to escape their kinetically trapped non-functional conformation and reinitiates the folding process (Slavotinek and Biesecker 2001). *Holding type* chaperones (small Hsps,  $\alpha$ -crystallin) work independently of ATP and offer hydrophobic surfaces for reversible binding of unfolded polypeptides. Their role is thought to be to protect partially folded/misfolded proteins from aggregation until *folding helper* chaperones are available for the energy dependant refolding of the non-native protein bound by the *holding type* chaperone (Bross *et al.*, 1999).

#### 1.4.2 Destruction by energy dependent proteases

Protein degradation within a eukaryotic cell can be mediated by two main structures, the proteasome and the lysosome (Ward 2002). Lysosomes are membrane bound organelles that reside in the cytoplasm of all eukaryotic cells and contain many hydrolytic enzymes including proteases. Classically, lysosomes have been considered non-specific systems for protein degradation. In contrast, recent evidence suggests that up to 30% of cytosolic proteins may contain a short targeting signal (KFERQ) that enables specific transport of chaperone (Hsc73) associated protein directly into lysosomes (chaperone-mediated autophagy) (Cuervo and Dice 1998).

The majority of non-lysosomal protein degradation is performed by the proteasome, a barrel-shaped protein complex that selectively degrades damaged proteins that have been tagged with ubiquitin (see Figure 1.3). No energy is required for the degradation of protein by the proteasome, however the protein destined for proteolysis must be unfolded by an specific energy-dependant cofactor (Bross *et al.*, 1999).

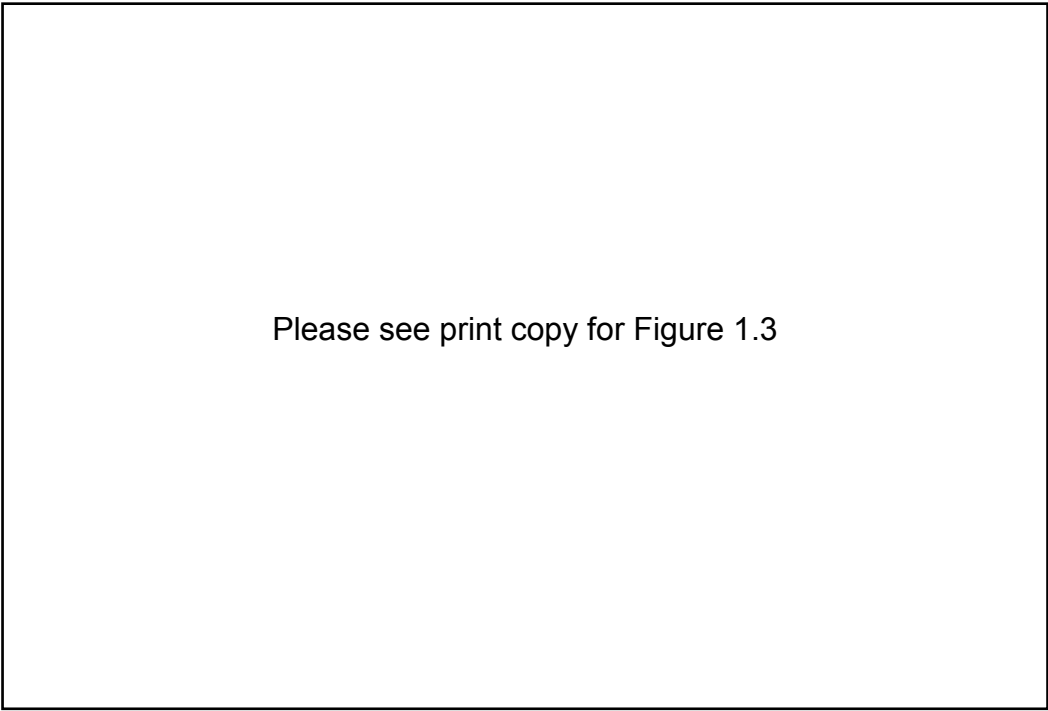
It was previously thought that chaperones promote degradation of damaged proteins by improving their solubility and minimising aggregation, rather than interacting directly with the protease “arm” of the quality control system (Wickner *et al.*, 1999). In contrast, recent data suggests that a non-native protein recognised by chaperones can be targeted for ubiquitination or direct binding to the proteasome via the action of cofactors CHIP

and BAG-1. These cofactors contain a chaperone binding motif or “adapter” which bind to a specific site on the C-terminus of Hsp70 and Hsp90 (two of the most common chaperones in the cytoplasm). The adapter contains a tandem arrangement of three degenerate 34 amino acid repeats (tetratricopeptide repeats, TPRs) adjacent to a highly charged  $\alpha$ -helix (Hohfeld *et al.*, 2001). Binding of cofactors CHIP and BAG-1 inhibits the refolding activity of chaperones (Hsp70 & Hsp90) and promotes either ubiquitination or direct loading of non-native protein onto the proteasome, respectively (Alberti *et al.*, 2002). Chaperone cofactors with TPRs have also been found which promote refolding (Hip and Hop). Thus, chaperones may act as non-native protein scavengers and binding of cofactors may determine if the protein is to be refolded (when bound to Hip or Hop) or degraded (when bound to CHIP or BAG-1) (Alberti *et al.*, 2002).

#### 1.4.3 Protein aggregation

Classically, it was thought that quality control mechanisms were directed towards preventing large scale protein aggregation (called inclusion bodies inside cells), which was thought to be an unfavourable outcome for the cell (Wickner *et al.*, 1999; Sherman and Goldberg 2001). However, recent discoveries have changed the way that certain protein aggregates are viewed: 1) early soluble oligomeric aggregates appear to be the most toxic species (Bucciantini *et al.*, 2002), 2) aggregated proteins are specifically delivered into inclusion bodies in mammalian cells by dynein-dependent retrograde transport on microtubules (Kopito 2000), and 3) *in vitro* experiments show that inclusion bodies seem to be cytoprotective (Tanaka *et al.*, 2004). It now seems that what was thought to be a random process is actually an effort by the cell to compartmentalise the dangerous aggregates away from vital organelles and cellular machinery. These actively created inclusion bodies are called aggresomes and seem to be created when other pathways of protein quality control are overwhelmed, such as in times of stress. Interestingly, in addition to the major aggregated species, aggresomes (transport dependent inclusions) are enriched in molecular chaperones such as Hsp70 (Kopito 2000). The role played by chaperones in these structures is unknown. The association of chaperones with aggresomes may reflect their failed attempt to stabilize or refold the aggregated protein. Alternatively, it is possible that they may somehow facilitate

formation of the aggresome. Coincidentally, reports have linked dynein-dependent transport with a group of chaperone cofactors termed immunophilins. Immunophilins contain a chaperone-binding domain with TPRs similar to the cofactors CHIP and BAG-1 described above. Recent experiments show that Hsp90 can promote the dynein-dependant transport of substrate through the association of the immunophilin FKBP52 (Harrell *et al.*, 2002). This suggests chaperone-mediated transport may be important for disposal of misfolded proteins via aggresome formation when the capacity of the proteasome is reached. Protection for the cell could occur in two distinct ways: 1) the aggregates are compartmentalised (in a intermediate filament (vimentin) cage) which would reduce the interference with cellular function, and 2) the cell may use the aggregate centralisation to facilitate its disposal by an autophagic route (Kopito 2000).



Please see print copy for Figure 1.3

**Figure 1.3 A chaperone based protein quality control model.** Native proteins when exposed to stress may adopt non-native structures. These non-native structures are recognised by chaperones (red discs). Binding of co-chaperones targets non-native proteins to a range of different fates. Chaperone-non-native protein complexes can be degraded (direct loading onto the proteasome, indirect loading onto the proteasome, and chaperone-mediated autophagy), refolded or transported to aggresomes. Similar mechanisms are present in other compartments such as the ER. Reproduced from Yerbury *et al.*. (2005b).

## 1.5 Chaperone-based model for protein quality control.

A model of protein quality control was proposed by Hohfeld *et al.* (2001) which described chaperones employed as non-native protein selectors, with the choice between folding and degradation made by the regulation of chaperone cofactors. In this model the level of pro-folding and pro-degradation cofactors would determine the average time a chaperone would spend on any one pathway and decide the overall fate of non-native proteins. In this chapter the model is extended to include the fact that non-native proteins can be directed to lysosomes for degradation by chaperones (Figure 1.3). This chaperone-based model for protein quality control does not attempt to imply that recognition by chaperones is the only pathway for protein handling, but rather that it is a major pathway in a complex quality control system.

## 1.6 Extracellular vs intracellular environment

So far the discussion of protein quality control has been restricted to cytosolic proteins and mechanisms. However, there is on average 15 L of fluid in the extracellular space in a 70 kg human including 5 L of blood (Guyton 1976). Thus, vast quantities of protein reside extracellularly in the human body. The extracellular fluids can be broken down into several fluids with similar but not identical compositions; interstitial fluid, plasma, cerebrospinal fluid and intraocular fluid. The extracellular space has a lower overall protein concentration (6% in plasma and 2% in interstitial fluid, compared to 30% for intracellular fluids), but has a higher calcium concentration and is more oxidising than the intracellular environment (Sitia and Braakman 2003). The composition of plasma, interstitial fluid and cytosol is depicted in Figure 1.4.

Please see print copy for Figure 1.4

**Figure 1.4. The major constituents of extracellular and intracellular fluids.** The quantities of the substances are represented here as milliequivalents or millimoles per litre. However, in terms of mass, the proteins and non-electrolytes actually comprise about 90% of the dissolved constituents of plasma, 60% of those in the interstitial fluid and about 97% of those in the intracellular fluid (Guyton 1976).

#### 1.6.1 Protein quality control in the extracellular space

The environmental conditions in the extracellular space are more oxidizing than the intracellular environment (Sitia and Braakman 2003). In addition, unlike intracellular proteins, extracellular proteins are constantly exposed to shear stress (e.g. the pumping of plasma through the body) which is known to induce protein unfolding and aggregation (Ker and Chen 1998). Thus it is likely that protein quality control mechanisms, similar to those operating in the intracellular space, are active in extracellular environment. Currently it is largely unknown what quality control mechanisms operate in the extracellular spaces. Since the intracellular protein quality control system is relatively well characterised it makes sense to start by evaluating the likelihood of similar mechanisms in the extracellular space. Intracellular *folding helper* type chaperones have been discovered at low levels in human plasma (~ nM) and associated with cell surfaces (generally cancer cells). Numerous roles have been postulated for the appearance of intracellular chaperones in the extracellular space, such as signalling (Ranford and Henderson 2002), cancer cell invasiveness (Eustace *et al.*, 2004) and immune presentation (Becker *et al.*, 2002). However, the extracellular environment does not permit the major ATP dependant intracellular chaperones (such as

Hsp70 and Hsp90) to act as chaperones outside cells. Since folding helper chaperones require bound ATP to recognise non-native proteins the lack of ATP in the extracellular space (more than  $10^5$  times less abundant than inside cells (Gribble *et al.*, 2000; Farias *et al.*, 2005)) would prohibit the formation of complexes between chaperone and substrate. It is a possibility that the levels of intracellular chaperone in human plasma could be explained by their release from necrotic cells or during viral cell lysis (Becker *et al.*, 2002). However, recent research indicates that at least some of the HSP70 in extracellular spaces has been secreted through non-classical pathways (Mambula and Calderwood 2006). It is possible that there are *folding helper* chaperones specific to the extracellular space. A recent study has shown that one secreted protein, serum amyloid P component (SAP), has some refolding activity *in vitro* (Coker *et al.*, 2000). However, a 10 fold molar excess of SAP was required to effect protein refolding, thus casting doubt on the physiological relevance of the finding. Therefore, the available evidence suggests that folding helper chaperones contribute very little to posttranslational quality control of protein folding in the extracellular space.

The main ingredients of the energy dependant cytoplasmic proteolysis system, ubiquitin and the proteasome, are both found in human plasma ( $\sim 8$  and  $2 \mu\text{g/mL}$ , respectively (Okada *et al.*, 1993)), however the levels of ATP in plasma are reported to be more than  $10^5$  times lower than that inside cells (see above). Thus, current evidence does not support a role for the proteasome in the degradation of extracellular proteins. It is possible that another protease system could do a similar job in the extracellular space. However, general purpose proteolytic enzymes, such as those found in lysosomes, are lacking in the extracellular space (Bouma 1982). There are, nevertheless, several protease families that play specific roles in enzyme activation (plasminogen activator), the coagulation system (thrombin), and tissue remodelling (metaloproteinases). Other enzymes can be released under certain conditions, such as the release of cathepsins (normally lysosomal proteases) from activated macrophages during attempted degradation of large invading cells (Siao and Tsirka 2002). Thus the available results suggest that there are no proteases consistently present in the extracellular space that play a regular role in the degradation of extracellular proteins. This is consistent with

data that demonstrates that extracellular proteins are degraded mostly in the liver and the reticuloendothelial system (Bouma 1982).

The clearance of proteins from the extracellular space for degradation can follow three possible pathways: glomerular filtration, fluid phase endocytosis and adsorptive endocytosis. Data from the 1960s and 1970s indicated that plasma proteins were catabolized in a reaction that followed first order kinetics, moreover, only small and/or positively charged proteins undergo glomerular filtration (~ 0.2% of total protein), and only a handful of *known* proteins use the adsorptive endocytosis route (Bouma 1982). Thus, in the early 1980s it was commonly accepted that the bulk of protein degradation proceeded through fluid phase endocytosis; a totally random process. However, it was found that individual plasma proteins, like intracellular proteins, were continually being degraded and replaced at characteristic rates (Dice & Goldberg 1976) strongly suggesting a non-random process. This prompted the development of a model in which plasma proteins “age” with increased exposure to so called “disturbing factors” and undergo structural changes resulting in an altered protein structure or ‘substate’ recognised by catabolic cells (Margineanu and Ghetie 1983).

## 1.7 An ‘aging’ model

Margineanu and Ghetie (1981) found that a decade of research seemed to run counter to the view that extracellular protein was randomly catabolized. In the 1970’s it had been shown that *in vivo* plasma protein catabolic rates depended on molecular integrity; several independent experiments demonstrated that denatured proteins were catabolised more rapidly from plasma than their native counterparts (Ghetie & Buzila 1972; Gregoriadis 1975; Knowles et al, 1975; Wallerik 1973). This prompted them to construct a new model of protein catabolism. The model was a simple two-step process that focussed on the idea that *in vivo* degradation of extracellular proteins was a selective process: 1) first a protein would undergo molecular modification leading to a form recognisable by catabolic cells, and then 2) this latter form would be internalised and degraded. The model was shown to fit the kinetic data available at the time (Margineanu and Ghetie 1981) and made the following assumptions:

- 1) That there are normal (N) and altered (A) substates of which only the altered substates are recognised and necessarily removed from the system.
- 2) Each protein species is characterised by an initial distribution of substates. This contains both A and N substates, although the abundance of the A substate is practically negligible.
- 3) The aging of plasma proteins is considered to be the result of random and constant action of some microenvironmental factors, acting as disturbing factors (DF). The DF act by varying in time the distribution of the substates as follows: each protein interaction with DFs leads to an increase in the probability for this protein to reach the A substate.

However, little was known about the process or processes leading to the formation of the A substate, and even less was known about the mechanism of recognition by catabolic cells. Since this model was first proposed, there has been much information accumulated in the field of protein folding. It is likely that the N substate represents a protein's native state while the A state is a destabilised conformation. The hypothesised disturbing factors that bring about the A substate are likely to be the commonly known stresses that induce unfolding such as heat, oxidative stress and changes in pH.

The question still remains how are destabilised proteins recognised in the extracellular environment? One promising lead was the discovery that the secreted protein clusterin, present in extracellular fluids at high levels, can act as a molecular chaperone.

## 1.8 Clusterin, the first described extracellular chaperone

Clusterin is a highly conserved secreted glycoprotein found in human plasma, CSF and seminal fluid at concentrations of approximately 100, 2, and 1000 µg/ml (Fritz *et al.*, 1983; Murphy *et al.*, 1988; Choi-Miura *et al.*, 1992), respectively. Clusterin is produced by many different cell types and under certain stress conditions can transit from the cell secretory system to the cytosol, although the precise reason(s) for this remain to be determined (Nizard *et al.*, 2007). The clusterin promoter contains a conserved 14 bp element which is recognized by the transcriptional regulator heat shock factor 1 (HSF1)



(Michel *et al.*, 1997). This places clusterin amongst other intracellular molecular chaperones and heat shock proteins that are also regulated by HSF1 (Michel *et al.*, 1997). Clusterin, in aqueous solution at physiological pH, exists in a range of oligomeric forms, while mildly acidic pH promotes the partial dissociation of oligomers into individual  $\alpha$ - $\beta$  heterodimers (Hochgrebe *et al.*, 2000). Currently, structural characterisation of clusterin is largely limited to predictions based on sequence analyses. These predictions suggest that clusterin has significant contiguous regions of disordered (possibly molten globule-like) conformation that separate other regions of well-defined secondary structure, such as amphipathic  $\alpha$ -helical regions and coiled-coil  $\alpha$ -helices (Dunker *et al.*, 2001; Dunker *et al.*, 2002). As a result, clusterin has been categorized as an intrinsically disordered protein (Dunker *et al.*, 2001; Dunker *et al.*, 2002).

A number of studies have demonstrated that clusterin has a small heat shock protein-like chaperone activity with a remarkable ability to suppress the amorphous aggregation of many different proteins. Clusterin potently inhibits stress-induced protein aggregation in an ATP-independent fashion by forming stable, soluble, high molecular weight complexes with non-native proteins due to its affinity for exposed regions of hydrophobicity (Humphreys *et al.*, 1999; Poon *et al.*, 2000; Yerbury *et al.*, 2007). Clusterin appears to lack the ability to independently refold heat-stressed, non-native enzymes but, similar to the small heat shock proteins, is able to hold heat-inactivated enzymes in a state from which subsequent ATP-dependent refolding by Hsc70 can occur (Poon *et al.*, 2000). Given that there is very little ATP or refolding chaperones in the extracellular space, the physiological significance of this remains uncertain. However, it is likely that clusterin acts to prevent aggregation *in vivo* since immunoaffinity depletion of clusterin from human plasma renders proteins in this fluid more susceptible to aggregation and precipitation (Poon *et al.*, 2002a).

Clusterin has long been known to interact with the cell surface receptor megalin (LRP2) and to complex with A $\beta$  to mediate its uptake by megalin and subsequent degradation (Hammad *et al.*, 1997). It also interacts with other members of the low density lipoprotein (LDL) receptor family - it binds to chicken LR8 and an LDLR-related

protein (Mahon *et al.*, 1999), and uptake of clusterin-leptin complexes by apoER2 and VLDLR has been proposed to facilitate leptin clearance (Bajari *et al.*, 2003). Furthermore, clusterin and LRP1/megalin have been implicated in the clearance of cellular debris by non-professional phagocytes (Bartl *et al.*, 2001). It is likely that by complexing with misfolded extracellular proteins, and keeping them in solution, clusterin delivers non-native proteins to specific receptors for cellular uptake and degradation.

## 1.9 The aims of the current research

The discovery that clusterin has a potent chaperone action poses the question: is there a family of proteins secreted into the extracellular space that protect extracellular proteins from aggregation and facilitate their disposal? Genuine extracellular chaperones must be able to exert their chaperone activity in the extracellular environment. Therefore, they must be robust, abundant and have the ability to work without ATP. It is likely then that any newly discovered extracellular chaperones will be secreted proteins present in the extracellular space at relatively high concentrations. Any such proteins are likely to be a part of an extracellular quality control system that protects the human body from disease. An overall goal of this thesis was to identify novel extracellular chaperone proteins and characterise their ability to inhibit the aggregation of proteins. Specific experimental aims included:

- (i) Discover and functionally characterise normally secreted proteins that have a chaperone action.
- (ii) Determine the effect of extracellular chaperones on amyloid formation by model peptides/proteins and those associated with human disease.
- (iii) Determine the mechanism by which extracellular chaperones exert their effect on amyloid formation.

## Chapter 2

# The Acute Phase Protein Haptoglobin is a Mammalian Extracellular Chaperone with an Action Similar to Clusterin

## Preface

An examination of the literature indicated that haptoglobin, an abundant normally secreted protein, may have a chaperone activity similar to the small heat shock proteins. This chapter summarises work directed towards completing a more thorough examination of the chaperone-like activity of haptoglobin. Part of this work was completed in collaboration with Dr Easterbrook-Smith's laboratory at the University of Sydney. Specifically, circular dichroism spectroscopy and bis-ANS fluorescence measurements shown were performed at the University of Sydney using haptoglobin provided by the author of this thesis.

Publication:

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## 2.1 Abstract

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

## 2.2 Introduction

Haptoglobin (Hp) is an acidic glycoprotein present in most body fluids of humans and other mammals. Individual humans (but not other mammals) express one of three major phenotypic forms of Hp, designated Hp1-1, Hp2-1 and Hp2-2. These phenotypes occur as a consequence of two alleles (HP 1 and HP 2); a crossing-over event is thought to have generated the variant alleles by transposing a large part of the sequence encoding the  $\alpha$  chain (Bowman and Kurosky 1982). In its simplest form, Hp1-1 is a tetramer comprised of two (light)  $\alpha^1$  and two (heavy)  $\beta$  chains linked by disulfide bonds; Hp2-1 and Hp2-2 are disulfide-linked polymerised forms of higher molecular mass. Hp2-2 lacks  $\alpha^1$  chains but contains higher mass  $\alpha^2$  chains instead; the most common phenotype, Hp2-1, contains both  $\alpha^1$  and  $\alpha^2$  chains and is thought to be comprised of a series of polymeric forms of the formula  $(\alpha^1\beta)_2(\alpha^2\beta)_n$  where  $n = 0, 1, 2, 3 \dots$  (Dobryszczycka 1997). The levels of Hp in human plasma are increased up to 8-fold during inflammation, various infections, trauma, tissue damage and in association with neoplasia, leading to Hp being designated as an "acute phase protein" (Bowman and Kurosky 1982; Dobryszczycka 1997). It has been reported that some animals (e.g. cattle) do not constitutively synthesize Hp but only do so in response to stresses such as inflammation (Heegaard *et al.*, 2000).

The best known ligand of Hp is hemoglobin (Hb), which it binds to with extremely high affinity ( $K_D \sim 10^{-15}$  M; (Bowman and Kurosky 1982)). Previously, it was widely believed that a primary biological function of Hp was to clear the body of vascular Hb released from damaged red blood cells. However, recent work has shown in a mouse model that the absence of Hp expression had no significant effect on the clearance of Hb following experimentally induced severe hemolysis; the Hp knock-out mice showed substantially higher mortality under these conditions, which appeared to result from greater oxidative stress (Lim *et al.*, 1998). Formation of the Hp-Hb complex inhibits Hb-mediated generation of lipid peroxides and hydroxyl radical, which is thought to occur in areas of inflammation (Dobryszczycka 1997). Thus, the available evidence indicates that Hp is likely to exert an important anti-inflammatory action *in vivo* by inhibiting oxidative damage mediated by free Hb (Lim 2001). A variety of other

putative biological functions have also been ascribed to Hp. It has been implicated in immune regulation (Louagie *et al.*, 1993), shown to inhibit cathepsin B activity (Snellman and Sylven 1967) and to have pro-angiogenic effects (Cid *et al.*, 1993). Binding of Hp to human neutrophils has been reported to inhibit respiratory burst activity (Oh *et al.*, 1990). In addition, neutrophils have been shown to take up exogenous Hp and store it within cytoplasmic granules - they subsequently secrete it into the local extracellular environment in response to a variety of pro-inflammatory stimuli (e.g. yeast, TNF $\alpha$ , or the chemotactic peptide fMLP (Wagner *et al.*, 1996; Berkova *et al.*, 1999)). Thus, although the functions of Hp are not yet fully understood, the available evidence indicates that it is likely to play an important role in suppressing inflammatory responses.

We have described and characterised the chaperone action of clusterin, a widely distributed and highly conserved glycoprotein found at high levels in human blood (Humphreys *et al.*, 1999; Poon *et al.*, 2000; Poon *et al.*, 2002a; Poon *et al.*, 2002b). Some recent work suggested that haptoglobin (Hp) might also have chaperone activity, which if true, would make it one of a very few known mammalian chaperones present in human body fluids at substantial levels. Both Hp and clusterin are: (i) comprised of disulfide-linked  $\alpha$  and  $\beta$  chains, (ii) heavily glycosylated (Hp and clusterin are 20% and 30% sugar by mass, respectively (Bowman and Kurosky 1982; Kapron *et al.*, 1997)), (iii) constitutively present at high levels in human plasma (Hp at 0.3-1.9 mg/ml (Bowman and Kurosky 1982), clusterin at 60-140  $\mu$ g/ml (Morrissey *et al.*, 2001)), and (iv) expressed at higher levels during a variety of stresses and disease states (Rosenberg and Silkensen 1995; Dobryszcka 1997). Given these similarities, the recent suggestion that Hp might be a chaperone appeared worth closer examination. Previous studies of the putative chaperone action of Hp were restricted to only two stressed proteins and did not demonstrate that, under the conditions tested, the effects measured were specific to Hp (Pavlicek and Ettrich 1998; Pavlicek and Ettrich 1999). The primary aims of this study were to establish whether Hp is a genuine extracellular chaperone and, if so, to compare its mechanisms of action with those of clusterin. To determine whether Hp had a genuine chaperone activity, we assayed the aggregation of a variety of proteins subjected to stresses, including unfractionated proteins in human serum, and tested the

effects of Hp and control proteins in these systems. We also investigated whether Hp, like clusterin and the small heat shock proteins (sHSPs), binds preferentially to stressed proteins to form solubilized high molecular weight complexes. In addition, we tested whether Hp had any ability to inhibit heat-induced loss of enzyme activity or to refold denatured enzymes and whether ATP might be required for either of these processes. Lastly, since it is known that clusterin is activated by mildly acidic pH (Poon *et al.*, 2002a), we tested the effects of pH on the structure and chaperone action of Hp. This report establishes that Hp exerts a genuine chaperone action, provides insights into the mechanism of this action, and identifies similarities and differences between it and that of clusterin and the sHSPs.

## 2.3 Materials and methods

### 2.3.1 Materials

Human serum was obtained from Wollongong Hospital (Wollongong, NSW, Australia) and stored frozen at  $-20^{\circ}\text{C}$  until use. CNBr-activated Sepharose, human hemoglobin, ovotransferrin, lysozyme (from chicken egg white), catalase (from bovine liver), citrate synthase (from porcine heart), alcohol dehydrogenase (from bakers yeast), and superoxide dismutase (SOD; from bovine erythrocytes) were purchased from Sigma (MO, USA). Complete<sup>R</sup> protease inhibitor cocktail was obtained from Roche (Sydney, Australia). Glutathione-S-transferase (GST) from *Schistosoma japonicum* was prepared by thrombin cleavage of recombinant Jun leucine zipper-GST fusion protein and purified by GSH-agarose affinity chromatography as described in (Heuer *et al.*, 1996). Ovotransferrin and GST were biotinylated using NHS-LC-biotin (Pierce, Sydney) following the manufacturer's instructions. Streptavidin-agarose was purchased from Oncogene (Merck, Sydney). Rabbit polyclonal anti-hemoglobin antibody (IgG fraction) was obtained from Dako (Sydney, Australia). The 3A8 murine hybridoma which secretes a monoclonal antibody reactive with human haptoglobin was obtained as a kind gift from Dr Ludwig Wagner (University of Vienna, Austria). A plasmid encoding mutant (chaperone-inactive) A30P  $\alpha$ -synuclein was a gift from Dr Robert Cappai (Department of Pathology, University of Melbourne, Melbourne, Australia). A30P  $\alpha$ -synuclein protein was expressed in *E. coli* and purified by acid precipitation as described in (Souza *et al.*, 2000). 4,4'-bis(1-anilinonaphthalene-8-sulfonate) (bisANS) was obtained from Molecular Probes (Eugene, OR, USA). Human clusterin was purified by immunoaffinity chromatography from human serum as previously described (Wilson and Easterbrook-Smith 1992).

### 2.3.2 Purification of Hp

Hp was purified from human serum using a well-established hemoglobin affinity chromatography method; this method yields native Hp which retains the ability to bind to hemoglobin with high affinity (Javid and Liang 1973). Briefly, human serum (80 ml) was diluted 3 parts to 1 with 10 mM Tris-HCl, pH 7 (TB) containing 5 M NaCl and



then mixed with Hb-Sepharose (5 ml, containing about 50 mg of bound Hb) on an end-over-end stirrer for 1 h at 4 °C. Human hemoglobin (Hb) was bound to CNBr-activated Sepharose using standard methods. The Hb-Sepharose was then washed with TB and unwanted Hb-binding proteins eluted with TB containing 1.6 M guanidine-HCl. Hp was eluted using 10 mM sodium acetate, pH 5, containing 3.5 M guanidine-HCl. The eluate was dialysed extensively against TB and any Hp-Hb complexes subsequently removed by immunoaffinity chromatography using anti-Hb antibody-Sepharose. Hp concentration was determined by absorbance at 280 nm using the molar extinction coefficient  $5.1 \times 10^4$  (corresponding to a 50 kDa  $\alpha\beta$  dimer) (El-Ghmati *et al.*, 2002).

### 2.3.3 Protein precipitation assays

#### 2.3.3.1 Heat stress

Citrate synthase (CS; 3  $\mu$ M) with or without Hp2-1 in CS buffer (14 mM  $\text{Na}_2\text{HPO}_4$ , 3.2 mM Tris, 26 mM NaCl, 0.6 mM EDTA, pH 7.4), was heated in the wells of a 96-well microplate (100  $\mu$ l/well) at 43 °C and protein precipitation, measured as turbidity ( $A^{360}$ ), quantified using a Spectramax 250 plate reader (Molecular Devices, CA, USA). GST (7.8  $\mu$ M), with or without Hp2-1, in phosphate buffer (50 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4), was heated at 55 °C for 20 min in an automated seven-chambered diode array spectrophotometer (Hewlett-Packard GMBH, Germany) and  $A^{360}$  measured as a function of time. Similarly, ovotransferrin (13  $\mu$ M), with or without Hp2-1, in phosphate buffer, was heated to 60 °C for 35 min and protein precipitation measured as above. In other experiments, ovotransferrin (3.3  $\mu$ M, 0.25 mg/ml) with or without Hp1-1, Hp2-1 or Hp2-2 (all at 40  $\mu$ g/ml), also in phosphate buffer, was heated to 60 °C for 30 min and  $A^{360}$  measured as a function of time; in all subsequent experiments, the Hp2-1 phenotype was used.

#### 2.3.3.2 Oxidative stress

Lysozyme (70  $\mu$ M), with or without Hp2-1, was incubated in the wells of a 96-well microplate (100  $\mu$ l/well) for 18 h at room temperature in oxidative stress buffer (1 mM  $\text{H}_2\text{O}_2$ , 2 mM EDTA, 2 mM Na ascorbate, 2 mM  $\text{FeCl}_3$ ), which produces hydroxyl radicals (Wang and Spector 1994); changes in  $A^{360}$  were measured in a Spectramax 250

microplate reader. To confirm that the effects of Hp were specific, in some experiments, superoxide dismutase (SOD) or the chaperone-inactive A30P mutant of  $\alpha$ -synuclein (Souza *et al.*, 2000) (Souza *et al.*, 2000) were used as control proteins. These were added to the following final concentrations for each of the indicated substrates: 6.7  $\mu$ M (CS), 17  $\mu$ M (GST), 8.3  $\mu$ M (ovotransferrin) and 33  $\mu$ M (lysozyme), with other conditions as specified above.

#### *2.3.3.3 The effects of pH on the ability of Hp to inhibit heat-induced precipitation of proteins*

CS (0.3 mg/ml) with or without Hp2-1 (0.15 mg/ml) in CS buffer (adjusted to pH 6.0-7.5), was heated at 43 °C and precipitation measured using a Spectramax 250 microplate reader. In other experiments, ovotransferrin (1 mg/ml) with or without Hp2-1 (0.25 mg/ml) in phosphate buffer (adjusted to pH 6.0-8.0), was heated to 60 °C in either quartz cuvettes or a 384-well microplate and precipitation measured using a diode array spectrophotometer (as described above) and a FluoStar microplate reader (BMG Labtech, Melbourne, Australia), respectively.

#### *2.3.3.4 Precipitation of proteins in whole human serum*

To selectively deplete human serum of Hp, an immunoaffinity column bearing the monoclonal anti-Hp antibody 3A8 was used. 3A8 was purified from tissue culture supernatant using protein G chromatography and then coupled to CNBr-activated Sepharose using standard methods. Unfractionated normal human serum (NHS; Hp2-1 phenotype, 5 ml, supplemented with Complete<sup>R</sup> protease inhibitor cocktail) was passed repeatedly over a 3 ml packed volume column of Sepharose CL 4B (control) or 3A8-Sepharose (to remove Hp). To minimize dilution, on the first pass, in each case the first 1.5 ml eluted was discarded; this procedure resulted in negligible dilution of the total protein concentration in the serum fractions collected. The yield of Hp from this procedure was about 2.0 mg/ml of serum processed. The 3A8 column was subsequently washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and bound Hp eluted with 2 M guanidine hydrochloride in PBS. Eluted Hp was dialysed extensively against PBS. There was no difference in chaperone activity between Hp purified by Hb-Sepharose versus 3A8-

Sepharose chromatography (data not shown). To confirm depletion of Hp from serum, 10 µl aliquots of sera were separated under non-reducing conditions on a 7.5% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Sartorius, Melbourne, Australia) which was subsequently blocked using 1% (w/v) heat-denatured casein in PBS (HDC/PBS). The blocked membrane was probed using tissue culture supernatant containing 3A8 antibody followed by a 1 in 2000 dilution of sheep-anti-mouse Ig-horseradish peroxidase conjugate (Silenus, Melbourne, Australia) in HDC/PBS. Bound secondary antibody was detected using an enhanced chemiluminescence kit (Pierce, IL, USA). Aliquots (50 µl) of control NHS or haptoglobin-depleted serum (HDS), prepared from the same batch of serum, were diluted 1 in 2 with PBS, supplemented with a final concentration of 7.5 mM sodium azide, and incubated in Eppendorf tubes at 43 °C for 48 h. Precipitate was recovered from the samples by filtering them using 0.45 µm ULTRAFREE centrifugal filtration units (Millipore, Sydney). Each filter was washed with 3 x 500 µl aliquots of PBS before solubilising the filtered and washed precipitate with 4 M guanidine hydrochloride in PBS (heated at 60 °C for 2 h). Protein content was determined using a bicinchoninic acid microprotein assay (Smith *et al.*, 1985).

#### 2.3.4 Detection of high molecular weight complexes formed between Hp and stressed proteins

##### 2.3.4.1 HPLC

Size exclusion chromatography (SEC) was used as a first step to investigate the formation of high molecular weight complexes between Hp and stressed proteins. Ovotransferrin (0.5 mg/ml), Hp2-1 (0.25 mg/ml) and mixtures of both Hp2-1 and ovotransferrin (at the same final concentrations) in phosphate buffer, were incubated for 1 h at either 37 °C or 60 °C. Using the same buffer, GST (0.2 mg/ml), Hp2-1 (0.5 mg/ml) and mixtures of GST and Hp2-1 (at the same final concentrations) were incubated at 37 °C or 57 °C for 40 min. All samples were centrifuged at 10,000 x g for 5 min immediately before analysis. A Biosep-SEC-S4000 column (Phenomenex, CA, USA) was equilibrated with 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, and a 100 µl sample volume loaded onto the column using a Shimadzu VP series HPLC system (Shimadzu, Kyoto,

Japan). Chromatography was performed at room temperature with a flow rate of 0.5 ml/min.

#### *2.3.4.2 Affinity adsorption*

Purified Hp2-1 (0.5 mg/ml) or mixtures of Hp2-1 (0.5 mg/ml) and either biotinylated ovotransferrin (1 mg/ml) or GST (0.2 mg/ml) (in a total volume of 60 µl of PBS) were heated at 60 °C for 1 h (Hp, Hp + GST) or 2 h (Hp + ovotransferrin), or incubated at room temperature for the same periods of time (controls). All samples were then centrifuged (5 min at 10,000 g) to remove insoluble material and shaken for 1 h at room temperature with streptavidin-agarose (50 µl packed volume). The streptavidin-agarose was washed by centrifugation three times with PBS and then boiled in SDS-PAGE sample buffer for several minutes to elute bound protein which was subsequently analysed by SDS-PAGE under non-reducing conditions.

#### *2.3.5 Testing the effects of Hp on heat-induced loss and subsequent recovery of enzyme activity*

Catalase and GST were heated in the presence or absence of Hp2-1 and the remaining enzyme activity assayed. Catalase (150 µg/ml in 44 mM Na<sub>2</sub>HPO<sub>4</sub>, 21 mM NaCl, 50 mM KCl, 5 mM MgCl<sub>2</sub>, pH 7.4) was incubated for 15 min at either 37 °C (unstressed control) or 55 °C (heat stressed), in the presence or absence of 300 µg/ml Hp2-1 (which is sufficient to inhibit most precipitation of catalase under these conditions). Immediately after incubation, samples were diluted 1:1 in refolding buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM KCl, 5 mM MgCl<sub>2</sub>, pH 7.4). To assay catalase activity, 50 µl samples of the above solutions were taken at intervals and added to 0.12% (v/v) H<sub>2</sub>O<sub>2</sub> in 50 mM Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.4; the consumption of H<sub>2</sub>O<sub>2</sub> was measured as a decrease in absorbance at 210 nm. Similarly, GST (200 µg/ml in 40 mM Na<sub>2</sub>HPO<sub>4</sub>, 35 mM NaCl, 50 mM KCl, 5 mM MgCl<sub>2</sub>, pH 7.4) was incubated for 5 minutes at either 37 °C or 57 °C in the presence or absence of 500 µg/ml Hp2-1 (which is sufficient to inhibit most GST precipitation under these conditions). The samples were then immediately diluted 1:1 in refolding buffer. To assay GST activity, 200 µl samples were taken at intervals and added to 800 µl of 2 mM glutathione, 2 mM 1-chloro-2,4-dinitrobenzene, 50 mM

Na<sub>2</sub>HPO<sub>4</sub> pH 7.4, and the production of 1-S-glutathionyl-2,4-dinitrobenzene measured as an increase in absorbance at 340 nm. For both catalase and GST, in some experiments, 2 mM ATP was included in the buffer during the initial incubation and also in the refolding buffer.

### 2.3.6 ATPase assays

Production of ADP from ATP was measured using an enzyme-coupled assay in which ADP production is linked to oxidation of NADH. The ATPase reaction buffer contained 2 mM Hepes, pH 8.0, 10 mM MgCl<sub>2</sub>, 100 mM KCl, 10 µM EDTA, 170 µM ATP, 840 µM phosphoenol pyruvate, 105 µM NADH, 37.4 units/ml of lactate dehydrogenase and 31.5 units/ml of pyruvate kinase (Lilley and Portis 1997). The reaction buffer (0.8 ml) was held in a quartz cuvette, maintained at 37 °C, and NADH oxidation monitored as a decrease in absorbance at 340 nm, measured as a function of time after (i) no additions, or (ii) the addition of a final concentration of 20 µg/ml Hp2-1. The validity of this assay was confirmed by showing that (i) addition of exogenous ADP (21 nmol) or (ii) generation of ADP from ATP through phosphorylation of creatine (1.1 µmol added) catalysed by creatine phosphokinase (0.87 units added) both led to NADH oxidation (Figure 2.6C). In order to test the possibility that Hp might exhibit ATPase activity only when complexed with stressed proteins, a 20 µl aliquot of a mixture of Hp2-1 (0.8 mg/ml) and ovotransferrin (1.0 mg/ml) that had been heated at 60 °C for 30 min was added to the ATPase reaction buffer above and the assay performed as described.

### 2.3.7 Dynamic light scattering

Samples of 0.1mg/mL Hp2-1 in phosphate buffer (adjusted to pH 6.0-7.5) were analysed by dynamic light scattering using a Zetasizer Nano ZS and Dispersion Technology software v3.00 (Malvern Instruments Ltd., U.K.). Solutions were passed through a 0.2 µm pore size filter immediately before analysis. For the purpose of these analyses, Hp was assigned a typical protein refractive index of 1.45 (Kohl *et al.*, 1994).

### 2.3.8 Circular dichroism spectroscopy

A Jasco J-720 spectropolarimeter, linked to a Neslab RTE-111 cooling system, was used to acquire circular dichroism (CD) data. Far-UV (190–260 nm) CD studies were performed using a 1 mm path length cell with Hp2-1 at 200 µg/ml in 5 mM sodium phosphate, pH 7.5 or 5 mM MES, pH 6.0 at 20 °C. Spectra were acquired at 20 millidegree sensitivity with a step resolution of 0.5 nm and a bandwidth of 1 nm. Estimates of the percentages of  $\alpha$ -helical,  $\beta$ -sheet,  $\beta$ -turn and unordered secondary structure were made by deconvolution of CD spectral data using the programs CDSSTR (Johnson 1999) and CONTIN-LL (Provencher and Glochner 1981).

### 2.3.9 bis-ANS fluorescence measurements

The fluorescence of bisANS bound to Hp was measured using an Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, VIC, Australia). Samples of Hp2-1 (100 µg/ml in either 50 mM MES, 50 mM NaCl, pH 6.0 or 50 mM phosphate, 50 mM NaCl, pH 7.5) were maintained at 20 °C. bisANS was added to give various concentrations up to 16 µM and after each addition the fluorescence of the samples at 500 nm was measured with an excitation wavelength of 385 nm. The data shown have been corrected for the fluorescence of control samples containing bisANS only. Equation (1), which describes the binding of bisANS to a single class of binding sites on Hp, was fitted to the data by non-linear regression analysis using SigmaPlot v8.02 (SPSS, Chicago, IL, USA).

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

In this equation, F, F<sub>max</sub> and K<sub>d</sub> are the observed fluorescence, the fluorescence at saturating concentrations of bisANS and the apparent dissociation constant, respectively.

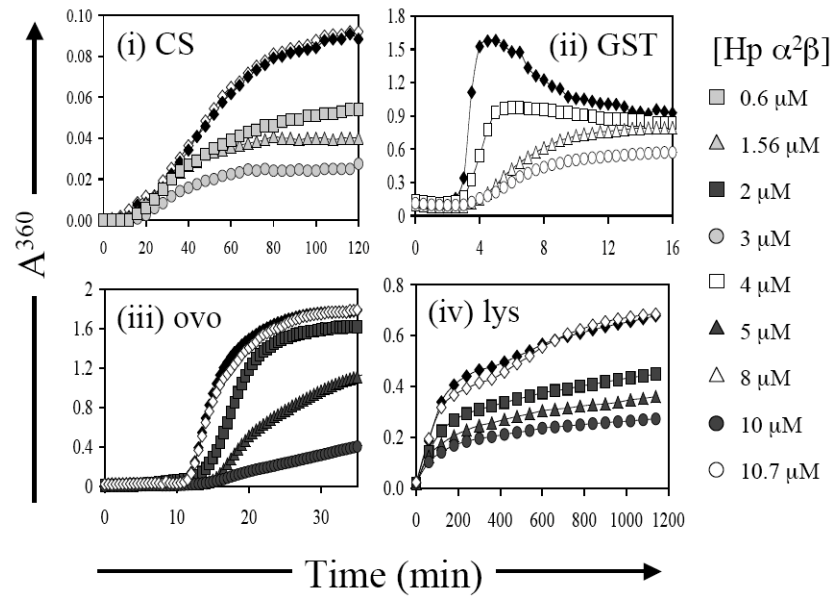
## 2.4 Results

### 2.4.1 Hp protects proteins from stress-induced precipitation

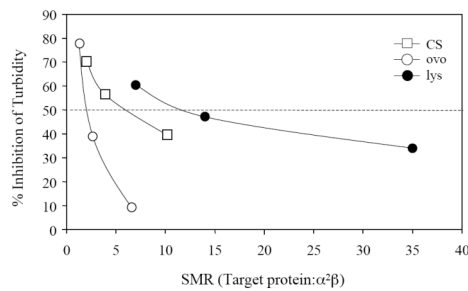
Supra-physiological temperatures (and other stresses) will induce proteins to unfold and subsequently aggregate. For a given heat stress, different proteins will unfold at different rates, depending on their individual structural stability. The available evidence indicates that although different proteins may require a greater or lesser heat stress to unfold at comparable (experimentally convenient) rates, the pathways of unfolding remain the same regardless of the temperature (Day *et al.*, 2002). We tested the ability of Hp to inhibit the aggregation of several different proteins induced by heating to 43-60 °C, and also one protein induced to aggregate by oxidative stress. Like clusterin (Poon *et al.*, 2002a), Hp is very structurally stable; it does not precipitate when heated for extended periods at 60 °C (data not shown). Furthermore, even after prolonged heating at 70 °C, there is only a small change in its circular dichroism spectrum and it actually has a slightly enhanced ability to inhibit protein precipitation (Ettrich *et al.*, 2002).

Heating citrate synthase at 43 °C resulted in a gradual precipitation of protein, shown by an increase in absorbance at 360 nm ( $A^{360}$ ), reaching a maximum after about 120 min (Figure 2.1A(i)). GST at 55 °C produced extensive protein precipitation within 5-10 min (Figure 2.1A(ii)). In the absence of Hp (trace represented by solid diamonds), the reduction in  $A^{360}$  after about 5 min corresponded to the formation of large macroscopically visible aggregates, with a concomitant “clearing” of finer microaggregates from suspension. Heating ovotransferrin at 60 °C resulted in extensive protein precipitation within 30 min (Figure 2.1A(iii)). Oxidative stress produced a slow precipitation of lysozyme, which approached a maximum over a period of 10-20 h (Figure 2.1A(iv)); when Hp2-1 was incubated alone, a low level of turbidity (less than 15% of that detected for lysozyme alone) was detected after 20 h of oxidative stress (data not shown). Hp2-1 gave dose-dependent inhibition of heat-induced precipitation of GST, ovotransferrin and citrate synthase (Figure 2.1A(i)-(iii)) and oxidative stress-induced precipitation of lysozyme (Figure 2.1A(iv)). In each case, further increases in

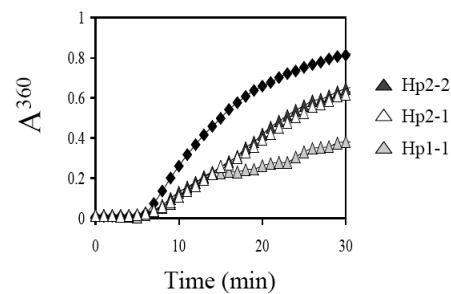
A



B



C



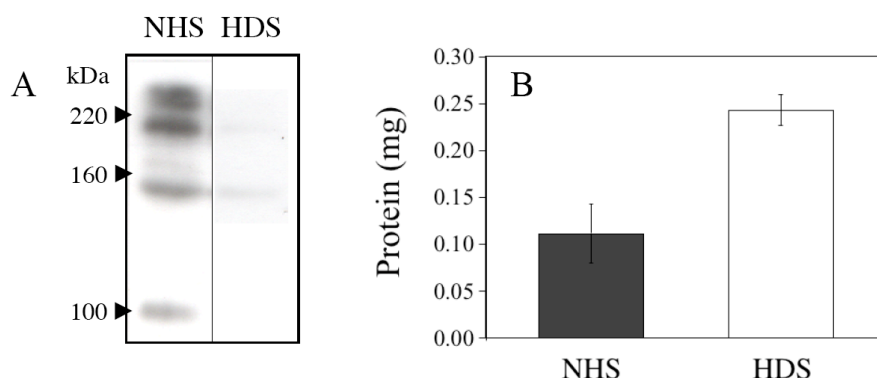
**Figure 2.1 Inhibition of stress-induced protein precipitation by Hp.** (A) Turbidity (measured as  $A_{360}$ ) as a function of time for (i) citrate synthase (CS; 3  $\mu M$ ) and Hp2-1 heated at 43 °C, (ii) GST (7.8  $\mu M$ ) and Hp2-1 heated at 55 °C, (iii) ovotransferrin (ovo; 13  $\mu M$ ) and Hp2-1 heated at 60 °C, and (iv) lysozyme (lys; 70  $\mu M$ ) and Hp2-1 exposed to oxidative stress as described in Materials and Methods. The key indicates the molar concentrations of Hp2-1 "subunit" (assumed to be comprised of  $\alpha^2\beta$  with a mass of 50 kDa) used. On panels (i), (iii) & (iv), the empty symbols represent the result in the presence of SOD (see Materials and Methods for details). (B) The percentage inhibition by Hp2-1 of the turbidity of solutions of citrate synthase (CS) and ovotransferrin (ovo) induced by heat, and lysozyme (lys) induced by oxidative stress, plotted as a function of the SMR of target protein:Hp  $\alpha^2\beta$  "subunit". The lines drawn simply indicate the trends in the data and have no theoretical significance. (C) Turbidity (measured as  $A_{360}$ ) as a function of time for ovo (0.25 mg/ml, 3.3  $\mu M$ ) and 40  $\mu g/ml$  of either Hp1-1, Hp2-1 or Hp2-2 (see key), heated at 60 °C. Owing to the variable structures of Hp2-1 and Hp2-2 (Dobryszczycka 1997), it is not possible to meaningfully compare their respective molar concentrations. The data points shown are individual measurements and in each case are representative of at least three independent experiments.



the ratio of Hp to substrate protein further reduced the extent of substrate protein precipitation (data not shown). These effects were specific to Hp2-1 because SOD and the A30P mutant of  $\alpha$ -synuclein did not prevent precipitation of any of the target proteins under these conditions (data for SOD shown in Figure 2.1A(i), (iii) & (iv)), compare traces represented by empty versus solid diamonds; data for SOD/GST, and for the effects of A30P on all proteins tested, are not shown).

Since many chaperones exist in solution as aggregates of an ill-defined number of monomers, a convention that has been adopted when dealing with the interactions between chaperones and other proteins is to define stoichiometry in relation to the individual subunits of the chaperone and the protein with which it interacts (the subunit molar ratio, SMR). In the case of human Hp, this is complicated because it occurs in three different phenotypes comprised of different and variable assemblies of  $\alpha$  and  $\beta$  chains (Bowman and Kurosky 1982; Dobryszczyka 1997). Most experiments were done with Hp2-1; in these cases, for simplicity, when molar ratios were required for calculations of SMR, we based our calculations on the assumption that each Hp “subunit” is comprised of  $\alpha^2\beta$  (with a mass of about 50 kDa, inclusive of glycosylation (Bowman and Kurosky 1982)). The potency of the inhibition of target protein precipitation by Hp2-1 varied, depending on the target protein. This is shown in Figure 2.1B, in which the percent inhibition of turbidity ( $A^{360}$ ) by Hp2-1 at the end of each of the time-courses shown in Figure 2.1A is plotted as a function of the subunit molar ratio (SMR) of target protein to Hp  $\alpha^2\beta$  dimers; data for GST are not plotted because the formation of macroscopic aggregates in the control makes calculation of percent inhibition problematic. It is apparent that the inhibition was most potent for lysozyme, with 50% inhibition being achieved at a lysozyme: $\alpha^2\beta$  ratio of  $\sim 12:1$ . To achieve comparable inhibition of heat-induced turbidity for CS and ovotransferrin, the data suggest that target protein: $\alpha^2\beta$  ratios of  $\sim 6:1$  and  $\sim 2:1$ , respectively, were required. Although all of the phenotypic forms of Hp were capable of inhibiting stress-induced protein precipitation, they differed in their potency. When tested at equal mass ratios (0.25 mg/ml ovotransferrin and 40  $\mu$ g/ml of Hp1-1, Hp2-1 or Hp2-2), Hp1-1 inhibited heat-induced turbidity for ovotransferrin at 30 min by about 50%, while the corresponding inhibition by both Hp2-1 and Hp2-2 was about 20% (Figure 2.1C).

Immunoaffinity chromatography successfully depleted nearly all Hp from human serum (Figure 2.2A). If Hp acts as a chaperone *in vivo*, then its selective depletion from whole human serum should enhance stress-induced precipitation of proteins in serum. This was found to be the case. Under the conditions tested, from 50  $\mu$ l aliquots of sera, about 0.1 mg of protein precipitated from NHS but about 0.25 mg of protein precipitated from HDS under the same conditions (Figure 2.2B). This difference was reproducible and statistically significant ( $p = 0.02$ , Student's t-test). Collectively, the results indicate that the chaperone action of Hp can protect both purified proteins and unfractionated proteins in whole human serum from stress-induced precipitation.



**Figure 2.2 Endogenous Hp inhibits heat-induced precipitation of proteins in human serum.** (A) Immunoblot showing the depletion of Hp2-1 from human serum by immunoaffinity chromatography. Aliquots (10  $\mu$ l) of normal human serum (NHS) and Hp-depleted serum (HDS) were analysed using 3A8 anti-Hp antibody (see Materials and Methods for more details). The position of molecular mass standards are indicated by arrow heads at the left of the image. (B) Histogram showing the amount of protein precipitated from 50  $\mu$ l aliquots of NHS and HDS heated at 43  $^{\circ}$ C for 48 h. Data points shown are means of triplicate measurements, error bars represent standard errors of the means. The results shown are representative of two independent experiments.

#### 2.4.2 Hp interacts with stressed proteins to form HMW complexes

Chaperones such as clusterin and the sHSPs are known to form high molecular weight (HMW) complexes with partly unfolded proteins under stress conditions (Carver *et al.*, 1995; Humphreys *et al.*, 1999). We used size exclusion chromatography as a first step

to investigate whether Hp forms complexes with stressed proteins; ovotransferrin and GST were used as “target” proteins in these assays. The variably sized absorbance peaks migrating at a position corresponding to a species of molecular weight less than 15 kDa (indicated by the dashed arrows on Figure 2.3A) represent azide present in some of the samples analysed or minor “buffer fronts” in other samples. The range of the molecular mass standards available limit what can be deduced from the SEC profiles for Hp alone, however, it is clear that Hp migrated under these conditions as a broad, asymmetric peak centred at an elution time of about 380 s, with more than half the material eluting before this time and a small fraction eluting at or near the exclusion limit of the column (indicated by the arrow labelled 2000 kDa). Thus, most of the Hp2-1 species migrated at a rate consistent with a mass in the range 220-2000 kDa, with a small fraction migrating as species with a mass of 2000 kDa or greater; this is consistent with it being comprised of a series of variably glycosylated disulfide-linked polymers spanning a wide range of masses. The elution profiles of untreated versus heat-treated Hp were very similar (Figure 2.3A), indicating that there was no major change in the aggregation state of Hp in solution in response to heat. In the case of ovotransferrin, when the protein was heated in the absence of Hp, most but not all of it precipitated and was removed by centrifugation before loading onto the column; the remaining ovotransferrin migrated with the same mobility as unheated ovotransferrin (Figure 2.3A(i)).

When GST was heated in the absence of Hp, essentially all of it precipitated and it was therefore not detected by SEC (Figure 2.3A(ii), 4th trace from top). When unheated mixtures of Hp and either ovotransferrin or GST were analysed by SEC, the resulting absorbance profiles were consistent with the proteins migrating independently of one another (Figure 2.3A). However, when stressed mixtures were analysed, in each case a HMW fraction eluted either at or near the exclusion limit of the column (Figure 2.3A, indicated by the horizontal bars). When analysed by SEC, in comparison to heated mixtures of Hp and GST, there appeared to be more species migrating at 300-350 s in heated mixtures of Hp and ovo, suggesting that the average size of complexes formed between Hp and ovotransferrin was less than those formed between Hp and GST. SDS-PAGE analysis of HMW fractions obtained by SEC of mixtures of Hp and

Please see print copy for Figure 2.3

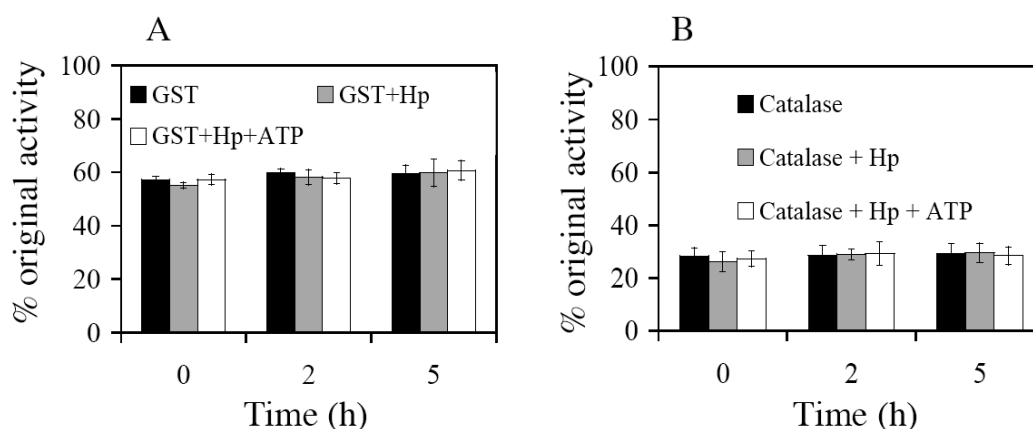
**Figure 2.3 *Hp forms HMW complexes with stressed proteins.*** (A) Results of SEC analyses of (i) Hp2-1 and ovotransferrin, or (ii) Hp2-1 and GST following incubation alone or as mixtures at either 37 °C or 57/60 °C (indicated in the key to the right of the traces; see Materials and Methods for other details). The positions of molecular mass standards are indicated by arrows at the top of the figure). (B) Image of sections of a Coomassie blue stained SDS-PAGE gel (electrophoresed under non-reducing conditions), showing proteins affinity adsorbed by streptavidin-agarose from mixtures that had been heated at 60 °C or incubated at room temperature (RT), containing Hp2-1 alone or Hp2-1 and either biotinylated ovotransferrin (ovo) or biotinylated GST. In each case, the results shown are representative of at least two independent experiments. The identity of the bands labelled at the right of the figure was established by comparison with molecular mass standards (not shown) and the known masses of Hp2-1 molecular species (Pastewka *et al.*, 1975).

ovotransferrin or GST indicated that they only contained both Hp and the respective target protein following heat stress (data not shown). Similar results were obtained from analyses of heat-stressed CS and oxidatively stressed lysozyme (data not shown). This suggested that Hp was forming soluble, HMW complexes with stressed proteins. This interpretation was confirmed by using streptavidin-agarose to affinity adsorb proteins from unheated and heated solutions of Hp and mixtures of Hp and biotinylated ovotransferrin/GST. The adsorbed material was subsequently analysed by SDS-PAGE. In fractions prepared from unstressed mixtures, only the biotinylated target protein was detected (Figure 2.3B). Furthermore, after being heated when in solution alone, Hp did not bind to the streptavidin-agarose beads (Figure 2.3B). However, fractions prepared

from stressed mixtures of Hp and target protein contained Hp (Figure 2.3B). This demonstrates that Hp formed complexes with the target proteins only after heating. Similar results were obtained for affinity adsorption analyses of mixtures of Hp and CS (exposed to heat-stress) or lysozyme (exposed to oxidative stress) (data not shown). Collectively, the above results establish that during experimental stresses, but not otherwise, Hp bound to stressed proteins to form soluble HMW complexes.

### 2.4.3 Hp does not protect enzymes from heat-induced loss of activity, or independently promote protein refolding, and lacks ATPase activity

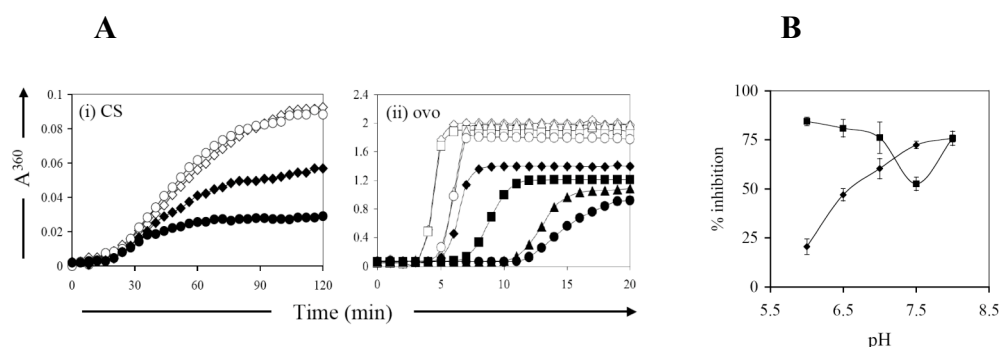
To investigate whether Hp is capable of protecting enzymes from stress-induced loss of function, and whether ATP has an effect on any such ability, we tested the enzyme activity of catalase and GST before and after exposure to heat stress in the presence or absence of Hp and ATP. The presence of Hp2-1 (at sufficient concentrations to provide protection against precipitation) had no effect on the loss of catalase or GST activity in either case, regardless of the presence of ATP (Figures 2.4A & B, time 0). Furthermore, when acting alone, and regardless of the presence of ATP, Hp2-1 was unable to promote the recovery of catalase or GST activity following heat stress (Figures 2.4A & B, times 2 & 5 h). Lastly, purified Hp2-1 had no detectable ATPase activity either when tested alone or following heat-induced association with ovotransferrin (data not shown).



**Figure 2.4** *Hp does not protect enzymes from heat-induced loss of activity or enhance their subsequent recovery of activity.* Bar graphs showing the level of (A) GST and (B) catalase activity as a function of time after heat stress. Additions present during and after heat stress are indicated in the respective keys. The data shown are means of triplicate measurements and the error bars represent standard errors of the means. The data shown is representative of 3 independent experiments.

#### 2.4.4 Effects of pH on the chaperone action and structure of Hp

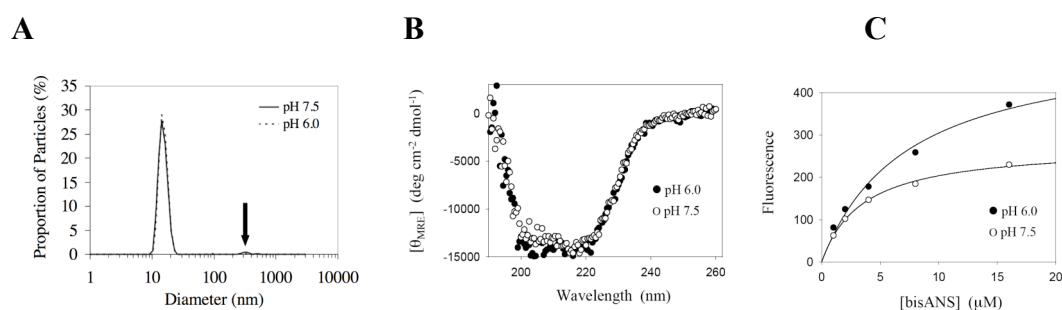
We previously showed that mildly acidic pH (i) increased the efficiency with which clusterin inhibited the precipitation of stressed proteins (Poon *et al.*, 2002a), (ii) induced dissociation of clusterin oligomers in solution into the heterodimeric form (Poon *et al.*, 2002a), and (iii) increased the exposure of regions of hydrophobicity on the molecule (Hochgrebe *et al.*, 2000). Given the functional parallels between clusterin and Hp, we investigated the effects of pH on the chaperone action and structure of Hp. In assays of heat-induced protein precipitation, in the absence of Hp, changes in pH between 6.0 and 7.5 had little effect on the time course or extent of precipitation of CS (Figure 2.5A(i)). In the case of ovotransferrin, the extent of precipitation was similar at all pH values but proceeded at a slightly faster rate at pH 6.0-6.5 than at pH 7.0-7.5 (Figure 2.5A(ii)); this may indicate that changes in the ionization of ovotransferrin side chains induced by mildly acidic pH promote more rapid intermolecular interactions when the protein partly unfolds in response to heat. In contrast, in the presence of Hp, the extent of protein precipitation was significantly greater at lower pH. Under the conditions tested, the ability of Hp to inhibit heat-induced turbidity in solutions of CS and ovotransferrin was greatest at pH 7.5 (approximately 70% and 64% inhibition, respectively) and was significantly less at pH 6.0 (approximately 40% and 26% inhibition, respectively) (Figure 2.5A). In the case of ovotransferrin, the rate of precipitation in the presence of Hp (like those in its absence) was faster at pH 6.0-6.5 than at pH 7.0-7.5. Collectively the results show that, compared with pH 7.5, at mildly acidic pH, Hp is less efficient at inhibiting the precipitation of stressed proteins from solution. Using ovotransferrin as a substrate we also examined the relative efficiency of the chaperone actions of Hp and clusterin over the pH range 6.0-8.0. The ability of Hp to inhibit heat-induced turbidity in solutions of ovotransferrin increased nearly linearly over the pH range 6.0-7.5 and remained at a similar level over the range pH 7.5-8.0 (Figure 2.5B). In contrast, the ability of clusterin to inhibit heat-induced turbidity of solutions of ovotransferrin was greatest at pH 6.0 and decreased with increasing pH, particularly between pH 7.0-7.5. Surprisingly, under these conditions, the chaperone efficiency of clusterin increased again at pH 8.0 to approach near-maximum levels (Figure 2.5B).



**Figure 2.5 Effects of pH on the chaperone action of Hp.** (A) (i) Turbidity of solutions of CS alone (0.3 mg/ml) (empty symbols) or CS (0.3 mg/ml) and Hp2-1 (0.15 mg/ml) (solid symbols) at pH 6.0 (diamonds) and 7.5 (circles), as a function of time of heating at 43 °C. (ii) Turbidity of solutions of ovotransferrin (ovo) alone (1 mg/ml) (empty symbols) or ovotransferrin (1 mg/ml) and Hp2-1 (0.25 mg/ml) (solid symbols) at pH 6.0 (diamonds), 6.5 (squares), 7.0 (triangles), or 7.5 (circles), as a function of time of heating (in water-jacketed quartz cuvettes) at 60 °C. Data points shown are individual measurements and in each case are representative of at least 3 independent experiments. (B) Plot showing the % inhibition of the heat-induced turbidity of solutions of ovotransferrin (1 mg/ml) by Hp2-1 (0.25 mg/ml; diamonds) and clusterin (40 µg/ml; squares), as a function of pH over the range 6.0-8.0 (these experiments were done using 384-well microplates; see Materials and Methods). The percentages are calculated from the differences in  $A^{360}$  at the end of a 2 h time course. Data points are means of 3 replicates and the error bars represent standard errors of the means; the results shown are representative of 3 independent experiments.

The results indicated that the efficiency of the chaperone action of Hp was strongly affected by changes in pH over the range 6.0-7.5. We then examined the effects of pH over this range on the solution size of Hp (probed by dynamic light scattering), its secondary structure (probed by far-UV CD) and the extent to which it exposed hydrophobicity to solution (probed by bisANS fluorescence). Dynamic light scattering measurements indicated that there was no significant change in the solution size of Hp2-1 at pH 6.0 versus 7.5. At both pH values, about 98% of Hp2-1 species in solution had a diameter in the range 9-25 nm with a mean of 15.1 nm (Figure 2.6A). At pH 7.5 (but not at pH 6.0), a minor additional population of much larger particles (representing

about 1% of all particles in solution) was present; these particles had a diameter in the range of 250-400 nm with a mean diameter of about 317 nm (indicated by the arrow on Figure 2.6A). The far-UV CD spectra of Hp2-1 at pH 6.0 and 7.5 were virtually superimposable; deconvolution of the spectra into their  $\alpha$ -helical,  $\beta$ -sheet,  $\beta$ -turn and unordered components showed that there was no significant differences between these at pH 6 compared to pH 7.5 (Figure 2.6B). In contrast, there were pH-dependent differences in the binding of the fluorescent hydrophobic probe bisANS to Hp; dose-dependent increases in the fluorescence of bisANS bound to Hp2-1 were observed at both pH 6 and pH 7.5 but the fluorescence at saturating levels of bisANS was about two-fold higher at pH 6 than at pH 7.5 (Figure 2.6C). It was assumed that there is a single class of non-cooperative bindings sites for bisANS on Hp and the data were fitted to equation (1) (see Materials and Methods) by non-linear regression; these analyses gave values for Fmax of  $527 \pm 5$  and  $275 \pm 8$  at pH 6.0 and 7.5 respectively, with corresponding values for Kd of  $7.3 \pm 1.6$  and  $3.5 \pm 0.3$   $\mu$ M.



**Figure 2.6 Effects of pH on the structure of Hp.** (A) Histogram plot showing the results of dynamic light scattering analyses of Hp2-1 at pH 6.0 and 7.5. The proportions of particles with different estimated diameters is plotted (see Materials and Methods for further details). (B) CD spectra of Hp2-1 at pH 6.0 (●) and 7.5 (○). The data shown are means of three scans; mean residue ellipticity is plotted, calculated for Hp2-1, assuming a molar extinction coefficient of  $5.1 \times 10^4$  M<sup>-1</sup>cm<sup>-1</sup> (El-Ghmati *et al.*, 2002). (C) Dependence of the fluorescence (in arbitrary units) of Hp2-1:bisANS complexes on bisANS concentration at pH 6.0 (●) and pH 7.5 (○) (measured as described in Materials and Methods). Data shown are representative of two independent experiments.



## 2.5 Discussion

Previous work showed that human Hp inhibited the aggregation of two proteins induced by *in vitro* stresses:  $\gamma$ -crystallin exposed to heat or oxidative stress (Pavlicek and Ettrich 1998), and catalase exposed to heat (Pavlicek and Ettrich 1999). It was also shown that compared with its effects on aggregation induced by heat or oxidative stress, Hp less efficiently inhibited the aggregation of  $\gamma$ -crystallin following its rapid dilution from 8 M urea (Pavlicek and Ettrich 1999). These earlier studies (i) were restricted to two substrate proteins, (ii) did not demonstrate that the effects of Hp were specific, and (iii) did not establish a mechanism for the putative chaperone action of Hp. Results presented here show that Hp inhibited the stress-induced precipitation of a variety of purified proteins. This included the heat-induced precipitation of CS, GST and ovotransferrin, and oxidative stress-induced precipitation of lysozyme; the use of control proteins demonstrated that the protective effects of Hp were specific (Figure 2.1A and data not shown). All three Hp phenotypes inhibited the heat-induced precipitation of ovotransferrin, although at equivalent mass concentrations, Hp1-1 was the most efficient (Figure 2.1C). Furthermore, although we have not identified which specific protein(s) in human serum precipitate in response to mild heating, we showed that immunoaffinity depletion of Hp from serum more than doubled the amount of protein that precipitated in response to the treatment (Figure 2.2B). Collectively, the results indicate that Hp has the ability to protect many different proteins from stress-induced precipitation and its effects in whole human serum suggest that this activity may be relevant *in vivo*.

The SMRs of interaction between the four proteins tested and Hp2-1 (calculated based on a 50 kDa  $\alpha^2\beta$  Hp "subunit") sufficient to inhibit most stress-induced precipitation were 2.0:1 (CS), 0.73:1 (GST), 1.3:1 (ovotransferrin), and 7.0:1 (lysozyme). This indicates that Hp is a more efficient chaperone than the intracellular sHSPs; the available data suggest that sHSPs bind stressed proteins at a SMR of one or more subunits of sHSP to one partially folded protein subunit (i.e. at best, SMR = 1:1 (Farahbakhsh *et al.*, 1995; Lindner *et al.*, 1998)). However, SMR-based comparisons indicate that Hp is a less efficient chaperone than clusterin, which under similar

conditions to those used in the current study only required SMRs of target protein:clusterin of 12.5:1 (Humphreys *et al.*, 1999) and 21:1 (Poon *et al.*, 2000) to inhibit most of the heat-induced precipitation of GST and ovotransferrin, respectively (these ratios are calculated assuming a clusterin "subunit" is comprised of the 80 kDa  $\alpha\beta$  heterodimer).

Size exclusion and affinity adsorption analyses indicated that Hp2-1 formed soluble, HMW complexes with stressed proteins (Figure 2.3 and data not shown). Clusterin and the sHSPs form similar HMW complexes with stressed proteins (Carver *et al.*, 1995; Humphreys *et al.*, 1999). Many other chaperones, but not clusterin (Poon *et al.*, 2000), have an ATP-dependent ability to refold unfolded proteins following stresses. A BLAST analysis of the NCBI non-redundant protein sequence database showed that there is no sequence similarity between Hp and any known ATPases or other proteins with nucleotide binding domains. However, to eliminate the possibility that Hp might exert refolding activity by utilizing a novel ATP binding domain, we tested whether Hp could protect enzymes from heat-induced loss of activity or promote post-stress recovery of enzyme activity, in either the presence or absence of ATP. Using GST and catalase as targets, our results demonstrate that, when acting alone, Hp has no effect on the heat-induced loss of enzyme activity or on their ability to recover activity after heat stress, regardless of the presence or absence of ATP (Figure 2.4). We also demonstrated that Hp lacks detectable ATPase activity (data not shown). These results indicate that Hp lacks the ability to independently refold heat-stressed enzymes and that ATP does not play a direct role in its chaperone action. We have not excluded the possibility that, like the sHSPs, Hp may hold stressed proteins in a refolding-competent state, from which the native conformations may be recovered by interaction with ATP-dependent chaperones *in vivo*. However, the only known chaperones with established refolding activity present in human body fluids (e.g. Hsp70) occur at very low (ng/ml) levels (Wright *et al.*, 2000). Thus even if Hp could stabilise stressed proteins in a refolding-competent state, the physiological relevance of this in extracellular body fluids would be questionable.

Clusterin is the first identified chaperone activated by mildly acidic pH; it exists in solution as heterogeneous aggregates and is induced to de-oligomerize at mildly acidic pH, which may expose more chaperone binding sites to solution (Poon *et al.*, 2002a). In solution, both Hp2-1 and Hp2-2 are known to consist of variably sized oligomers made up of different numbers of disulfide-linked  $\alpha\beta$  units (Dobryszczyka 1997); the quaternary structure of Hp in aqueous solution is unknown. In contrast with clusterin, but like the sHSP  $\alpha$ -crystallin (Poon *et al.*, 2002a), the efficiency of the chaperone action of Hp2-1 rapidly decreases as the pH is reduced from 7.5 to 6.0 (Figure 2.5). Furthermore, at least when acting on heat-stressed ovotransferrin, the chaperone action of Hp2-1 is near-maximum efficiency at pH 7.5 and remains high at pH 8.0 (Figure 2.5B); even at pH 9.0, Hp2-1 maintains this high level of chaperone efficiency (data not shown).

The difference between clusterin and Hp with respect to the effects of pH on their chaperone actions suggests that their mechanisms may be quite distinct. We examined the mass of Hp2-1 in solution using SEC and dynamic light scattering (DLS). HPLC SEC analyses of Hp2-1 at pH 7.4 indicated that it migrated as a broad asymmetrical peak, consistent with species in the range 220-2000 kDa (Figure 2.3A). DLS measurements indicated that over the range 6.0 to 7.5, pH had little effect on the solution size of Hp2-1; at either pH the vast majority of Hp species were distributed normally about a mean diameter of approximately 15 nm (Figure 2.6A). At pH 7.5 only, about 1% of particles had an estimated diameter of more than 300 nm (which would correspond to oligomers containing up to hundreds of  $\alpha\beta$  units). These very large oligomers were not detected at pH 6.0, suggesting that they were not stabilized by disulfide bonds and resulted from pH-sensitive non-covalent interactions. However, given that they comprise such a small proportion of the Hp2-1 species in solution, it appears very unlikely that the effects of pH on their formation is an important mechanism affecting the chaperone action of Hp. Thus, although changes in oligomerization state have been implicated in the effects of pH on the chaperone activity of clusterin (Poon *et al.*, 2002a), there is no evidence that a similar mechanism operates in the case of Hp. Therefore, it follows that pH primarily affects the chaperone action of Hp by altering some aspect(s) of its secondary and/or tertiary structure.

Far-UV CD analyses of Hp2-1 indicated that varying pH over the range 6.0-7.5 had no significant effect on the predicted contents of  $\alpha$ -helical,  $\beta$ -sheet, and  $\beta$ -turn regions (Figure 2.6B), suggesting that the secondary structure of Hp does not undergo any gross changes over this pH range. However, an inherent limitation of CD measurements is that they do not identify specific locations of structural changes in a molecule, they only provide overall structural information. The results of experiments measuring the fluorescence of bisANS bound to Hp2-1 indicate that the protein undergoes a pH-dependent structural change (not detected by far-UV CD analyses). As a consequence of these changes, bisANS fluoresced more intensely when bound to Hp at pH 6 compared to pH 7.5, however, Hp had a higher affinity for bisANS at pH 7.5 than at pH 6 (Figure 2.6C). This latter observation may provide an explanation for our finding that Hp is more efficient at inhibiting protein precipitation at pH 7.5 compared to pH 6.0; the higher affinity hydrophobic binding sites exposed at pH 7.5 may be important in the interactions of Hp with stressed proteins (which presumably involves its binding to exposed hydrophobic surfaces on these proteins). The pH-induced changes in bisANS binding detected appear unrelated to the isoelectric point of the protein, which is 3.9-4.2 (Bowman and Kurosky 1982).

Although we have not measured the dissociation constants, the demonstration of stable complexes formed between Hp and stressed proteins (Figure 2.3) suggests that Hp binds to stressed proteins with high affinity. Therefore, collectively, the available data suggests that, *in vitro*, Hp potently inhibits stress-induced protein aggregation by binding (via an ATP-independent mechanism) to partly unfolded proteins to form stable HMW solubilized complexes but does not itself effect protein refolding. In these respects, the chaperone action of Hp is like that of clusterin and the sHSPs. However, these three types of chaperones clearly differ with respect to the molar efficiencies with which they inhibit protein aggregation (see above) and the effects of pH and temperature on their actions. Current understanding indicates that increased temperatures induce de-oligomerization of sHSPs (which like clusterin aggregate in solution) to yield a chaperone-active species in which the hydrophobic surfaces previously located at the interfaces between the subunits of the aggregate are now

available to bind to target proteins (Haslbeck *et al.*, 1999; van Montfort *et al.*, 2001; Gu *et al.*, 2002). An activation mechanism analogous to that proposed for the sHSPs has been suggested for clusterin, in which reduced pH (rather than increased temperature) induces enhanced chaperone action (Poon *et al.*, 2002a). Mildly acidic pH has no effect on the size of most Hp species in solution and decreases the chaperone efficiency of both the sHSP  $\alpha$ -crystallin (Poon *et al.*, 2002a) and Hp (Figure 2.5B). Therefore, at least over the range pH 6.0-7.5, the chaperone action of Hp shows a similar pattern of dependence on pH as the sHSP  $\alpha$ -crystallin. The effects of temperature on the solution size and chaperone efficiency of Hp have yet to be examined in detail.

In healthy individuals, the proportion of Hp in circulation complexed with Hb is very low because Hp-Hb complexes are rapidly cleared and degraded by liver-resident macrophages (Higa *et al.*, 1981). Hp probably has discrete chaperone and Hb-binding sites because complexation with Hb has only a limited effect on the ability of Hp to inhibit the heat-induced aggregation of catalase (when compared with uncomplexed Hp, the Hp-Hb complex is about 25% less effective (Ettrich *et al.*, 2002)). Hp inhibits the pro-inflammatory oxidative effects of free Hb by binding to it with very high affinity and mediates its uptake via the CD163 macrophage cell surface receptor (Kristiansen *et al.*, 2001). Our results raise the possibility that another, complementary, mechanism by which Hp may exert an anti-inflammatory action *in vivo* is via it inhibiting the inappropriate self-association of "damaged" (misfolded) extracellular proteins. Misfolded proteins have been implicated as underlying inflammatory events in atherosclerosis (Ursini *et al.*, 2002) and Alzheimer's disease (Casserly and Topol 2004). It is interesting to speculate that, like its role in directing the uptake of Hb via CD163, Hp may also facilitate the cellular uptake and degradation of misfolded proteins to which it complexes.

Last we propose that Hp and clusterin are the first clearly identified members of a small family of known extracellular mammalian chaperones, present in human plasma at levels of about 100  $\mu$ g/mL or greater, which comprise a critical defence mechanism against the clinically dangerous consequences of inappropriate protein aggregation in extracellular body spaces. Other chaperones normally found inside cells, such as Hsp70,

are also found in human plasma, but their low concentrations in this fluid (in the ng/mL range) mean that they are unlikely to substantively inhibit precipitation of plasma proteins; their release from damaged cells is likely to at least partly explain their occurrence in extracellular fluids (Wright *et al.*, 2000).

## Chapter 3

# Protease Activation of $\alpha_2$ -Macroglobulin Modulates a Chaperone-Like Action with Broad Specificity

## Preface

$\alpha_2$ -macroglobulin presented itself as a candidate for investigation after being linked to disposal of Alzheimer's disease associated A $\beta$  peptide and being compared to intracellular chaperones because of its ability to promote presentation of antigen on class I MHC molecules. Preliminary experiments suggested that it did indeed possess a small heat shock protein-like chaperone action with broad substrate specificity. At this point a fellow graduate student at the University of Wollongong, Katie French, was recruited onto the project. As a result, the work described in this chapter includes data collected by both myself and Katie French.

Publication:

French K<sup>1</sup>, **Yerbury JJ**<sup>1</sup>, Wilson MR. (2008) Protease activation of  $\alpha_2$ -macroglobulin modulates a chaperone-like action with broad specificity. *Biochemistry*. 47, 1176-1185.

<sup>1</sup> These authors contributed equally to this work

### 3.1 Abstract

$\alpha_2$ -Macroglobulin ( $\alpha_2$ M) is a major human blood glycoprotein best known for its ability to inhibit a broad spectrum of proteases by a unique trapping method. This action induces an “activated” conformation of  $\alpha_2$ M with an exposed binding site for the low density lipoprotein receptor, facilitating clearance of  $\alpha_2$ M-protease complexes from the body. This report establishes that protease activation also modulates a potent chaperone-like action of  $\alpha_2$ M which has broad specificity for proteins partly unfolded as a result of heat or oxidative stress. Protease-mediated activation of  $\alpha_2$ M abolishes its chaperone-like activity. However, native  $\alpha_2$ M is able to form soluble complexes with stressed proteins and then subsequently become activated by interacting with a protease, providing a potential mechanism for the *in vivo* clearance of  $\alpha_2$ M/stressed protein/protease complexes. We propose that  $\alpha_2$ M is a newly discovered and unique member of a small group of abundant extracellular proteins with chaperone properties that patrol extracellular spaces for unfolded/misfolded proteins and facilitate their disposal.



### 3.2 Introduction

$\alpha_2$ -Macroglobulin ( $\alpha_2$ M) is a major human blood glycoprotein assembled from four identical 180 kDa subunits into a 720 kDa tetramer. The 180 kDa subunits are disulphide bonded to form dimers, which non-covalently interact to yield the final tetrameric quaternary structure (Jensen and Sottrup-Jensen 1986).  $\alpha_2$ M is well known for its ability to inhibit a broad spectrum of proteases, which it accomplishes using a unique trapping method. When exposed to a protease,  $\alpha_2$ M undergoes limited proteolysis at its bait region leading to a large conformational change, physically trapping the protease within a steric “cage” (Sottrup-Jensen 1989). The trapped protease forms a covalent linkage with  $\alpha_2$ M by reacting with an intramolecular thiol ester bond to yield “activated”  $\alpha_2$ M ( $\alpha_2$ M\*), which exposes a receptor recognition site for low density lipoprotein receptor related protein (LRP) (Sottrup-Jensen 1989). By directly interacting with the thiol ester bond, small nucleophiles such as methylamine can also activate  $\alpha_2$ M (Imber and Pizzo 1981).

Although human  $\alpha_2$ M is best known for its protease inhibitor function, it has also been shown to bind to and promote clearance of other endogenous and exogenous molecules, consistent with a broader protective function.  $\alpha_2$ M is known to bind to cytokines and growth factors (without converting to  $\alpha_2$ M\*), including transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), interleukin 8 (IL-8), platelet derived growth factor-BB (PDGF-BB), nerve growth factor- $\beta$  (NGF- $\beta$ ), and vascular endothelial growth factor (VEGF) (reviewed in refs (LaMarre *et al.*, 1991; Feige *et al.*, 1996)). The affinity of  $\alpha_2$ M for most cytokines is higher in the activated state, and while in this state  $\alpha_2$ M can deliver them via receptor mediated endocytosis to lysosomes for degradation (Crookston *et al.*, 1994). In addition,  $\alpha_2$ M has been shown to bind to the pathogen *Trypanosoma cruzi* and promote its phagocytosis (Araujo-Jorge *et al.*, 1990).  $\alpha_2$ M is also known to bind to endogenous disease-associated proteins, including the A $\beta$  peptide associated with Alzheimer’s disease (Narita *et al.*, 1997; Mettenburg *et al.*, 2002),  $\beta_2$ -microglobulin which forms insoluble deposits in dialysis related amyloidosis (Motomiya *et al.*, 2003) and prion protein associated with plaques in Creutzfeldt-Jakob disease (Adler and Kryukov 2007). Interestingly,  $\alpha_2$ M has been

shown to suppress the aggregation of A $\beta$  and in association with the  $\alpha_2$ M receptor, LRP, protect cells from its toxicity (Du *et al.*, 1997; Narita *et al.*, 1997; Hughes *et al.*, 1998; Fabrizi *et al.*, 2001). Previous work has indicated that  $\alpha_2$ M complexed to either protein or peptide ligands is immunogenic (Chu and Pizzo 1993; Binder *et al.*, 2001; Binder *et al.*, 2002; Binder 2004).  $\alpha_2$ M bound peptides are internalised by LRP and fragments of the peptide are subsequently re-presented on the cell surface. This response is identical to the one elicited by peptides chaperoned by intracellular heat shock proteins (Srivastava 2002). Collectively, the abilities of  $\alpha_2$ M to bind many diverse ligands (Crookston *et al.*, 1994), inhibit A $\beta$  aggregation, and influence the immune response to peptides, prompted us to examine whether  $\alpha_2$ M might be a novel member of a small group of abundant extracellular proteins with chaperone properties ("extracellular chaperones"; ECs) that have been proposed as major elements of a quality control system for the folding state of proteins in extracellular body fluids (Yerbury *et al.*, 2005b). There are a number of similarities between  $\alpha_2$ M and the previously identified ECs clusterin (Wilson and Easterbrook-Smith 2000) and haptoglobin (Yerbury *et al.*, 2005a).  $\alpha_2$ M, clusterin and haptoglobin are all secreted glycoproteins with distant evolutionary relationships to complement (Bowman and Kurosky 1982; Kirszbaum *et al.*, 1989; Dodds and Law 1998). In addition, all three are: (i) structurally comprised of disulfide linked subunits (Bowman and Kurosky 1982; Jensen and Sottrup-Jensen 1986; Wilson and Easterbrook-Smith 2000), (ii) abundant in human plasma ( $\alpha_2$ M 2 - 4 mg/ml (Sottrup-Jensen 1989), clusterin 50-370  $\mu$ g/ml (O'Bryan *et al.*, 1990) and haptoglobin 0.3-1.9 mg/ml (Bowman and Kurosky 1982), (iii) mediate ligand degradation by receptor mediated endocytosis (Ashcom *et al.*, 1990; Hammad *et al.*, 1997; Kristiansen *et al.*, 2001), and (iv) are known to co-localise with Ab deposits in Alzheimer's disease (Powers *et al.*, 1981; Calero *et al.*, 2000; Fabrizi *et al.*, 2001).

We hypothesized that, like clusterin and haptoglobin,  $\alpha_2$ M might have the ability to bind to a wide variety of partly unfolded stressed proteins to prevent their aggregation and keep them soluble. Results presented here establish that  $\alpha_2$ M does indeed have such an activity and that this activity is modulated by protease activation. A model is also presented to describe the potential physiological significance of these findings.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Bovine serum albumin (BSA), citrate synthase (CS, porcine), 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bisANS), lysozyme (Lys, chicken), creatine phosphokinase (CPK, rabbit), superoxide dismutase (SOD, bovine), ovotransferrin (Ovo, chicken), trypsin (type 1, bovine), soybean trypsin inhibitor (type 1), N-alpha-benzoyl-DL-arginine-p-nitroaniline (BAPNA), phenylmethylsulfonylfluoride (PMSF), propidium iodide (PI) and methylamine hydrochloride were purchased from Sigma (MO, USA). Triton X-100 (TX-100) was from Ajax Chemicals (Sydney, Australia). Glutathione-S-transferase (GST) from *Schistosoma japonicum* was prepared as previously described (Heuer *et al.*, 1996). A plasmid encoding a GST-RAP fusion protein (RAP is an inhibitor of ligand binding to low density lipoprotein family receptors) was obtained as a kind gift from Dr Y. Li (Washington University School of Medicine, MO, USA) and purified in the same way as GST. CS, CPK and Lys were biotinylated using NHS-LC-biotin (Pierce, Sydney, Australia) as per the manufacturer's instructions. Streptavidin-agarose was purchased from Calbiochem (Sydney, Australia). Streptavidin-Alexa 488 was from Invitrogen (Sydney). Rabbit polyclonal anti- $\alpha_2$ M and anti-DNP antibodies (IgG fractions) were obtained from Dako Cytomation (CA, USA); rabbit-anti-trypsin antibody (IgG fraction) was from Abcam (Cambridge, UK). Horseradish peroxidase and fluorescein conjugates of sheep-anti-rabbit IgG antibody (SaRIgG-HRP and SaRIgG-FITC, respectively) were from Chemicon (Melbourne, Australia).

#### 3.3.2 Purification and in vitro activation of $\alpha_2$ M

$\alpha_2$ M was purified from heparinized human plasma using a  $\text{Zn}^{2+}$  HiTrap<sup>TM</sup> chelate-affinity column (GE Healthcare) followed by size exclusion chromatography (SEC) as previously described (Imber and Pizzo 1981). The concentration of  $\alpha_2$ M was determined by absorbance at 280 nm (extinction coefficient for a 1% solution = 8.93 (Barrett 1981)). Native  $\alpha_2$ M was converted to activated  $\alpha_2$ M ( $\alpha_2$ M\*) by incubation with either methylamine or trypsin (Bonner *et al.*, 1992). Native  $\alpha_2$ M (4.85  $\mu$ M) was incubated overnight at room temperature (RT) with 0.15 M methylamine HCl in 0.5 M

Tris, pH 8.2, and then dialysed against phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4).  $\alpha_2\text{M}$ -trypsin complexes were formed by incubating  $\alpha_2\text{M}$  (4.85  $\mu\text{M}$ ) in 25 mM Tris, pH 8.0, with a 6-fold molar excess of trypsin at 37 °C for 2 h; unbound trypsin was inactivated with excess soybean trypsin inhibitor.  $\alpha_2\text{M}$ -trypsin complexes were purified by SEC using a Biosep<sup>®</sup>-SEC-S4000 column equilibrated in PBS. Successful activation of  $\alpha_2\text{M}$  was confirmed by native PAGE and trypsin binding assay (performed as described in (Van Leuven *et al.*, 1981) and (Bonner *et al.*, 1992), respectively);  $\alpha_2\text{M}^*$  species were stored in PBS, pH 7.4 at -20 °C.

### 3.3.3 Protein precipitation assays

Individual solutions of  $\alpha_2\text{M}$  or  $\alpha_2\text{M}^*$  (0.3-7  $\mu\text{M}$ ) and CS (6  $\mu\text{M}$ ) in 50 mM Tris, 2 mM EDTA, pH 8, or mixtures of CS and  $\alpha_2\text{M}$  or  $\alpha_2\text{M}^*$  (at the same final concentrations) were heated at 43 °C for 4 h in a 384 well plate and precipitation measured as turbidity (absorbance at 360 nm,  $A^{360}$ ). Absorbance readings were acquired every 4 min using a FLUOstar plate reader (BMG Labtech, Germany). In similar experiments, individual solutions of CPK (25  $\mu\text{M}$ ),  $\alpha_2\text{M}$  or  $\alpha_2\text{M}^*$  (0.3-3.5  $\mu\text{M}$ ), or mixtures of CPK and  $\alpha_2\text{M}$  or  $\alpha_2\text{M}^*$  (at the same final concentrations) in PBS were heated at 43 °C for 3 h and precipitation measured as described above. In related experiments,  $\alpha_2\text{M}$  was pre-incubated for 1 h at RT with a 2:1 molar ratio of bisANS: $\alpha_2\text{M}$  before being added to CS and CPK aggregation reactions (like those just described) at final  $\alpha_2\text{M}$  concentrations of 3.5  $\mu\text{M}$  and 0.7  $\mu\text{M}$ , respectively. In control experiments 7  $\mu\text{M}$  bisANS alone was added to aggregation reactions. In addition, individual solutions in PBS of GST (8  $\mu\text{M}$ ) and Ovo (12.5  $\mu\text{M}$ ) were incubated with or without  $\alpha_2\text{M}$  (0.7-7  $\mu\text{M}$ ) at 60 °C and the precipitation measured as above. Lastly, individual solutions of Lys (70  $\mu\text{M}$ ),  $\alpha$ -syn (70  $\mu\text{M}$ ), and  $\alpha_2\text{M}$  or trypsin-activated  $\alpha_2\text{M}^*$  (at 0.7-7  $\mu\text{M}$ ), or mixtures of Lys or  $\alpha$ -syn and  $\alpha_2\text{M}$  or  $\alpha_2\text{M}^*$  (at the same final concentrations) were incubated in wells of a 96-well plate (100  $\mu\text{l}$ /well) at 37 °C in oxidative stress buffer (OSB; 100  $\mu\text{M}$   $\text{CuSO}_4$ , 4 mM  $\text{H}_2\text{O}_2$  in PBS); changes in  $A^{360}$  were measured in a SpectraMax Plus<sup>384</sup> microplate reader (Molecular Devices, USA). As controls, the effects of SOD and BSA on protein precipitation induced by oxidative and heat stress were tested in similar assays.

### 3.3.4 Precipitation of proteins in whole human serum

$\alpha_2$ M was selectively depleted from normal human serum (NHS) using a  $\text{Zn}^{2+}$  HiTrap™ chelate-affinity column (GE Healthcare). Approximately 2.0-2.5 mg of  $\alpha_2$ M and about 0.5 mg of contaminant proteins were recovered per ml of serum; the latter were separated from  $\alpha_2$ M by Superose 6 SEC, concentrated by ultrafiltration, and then added back to the  $\alpha_2$ M depleted serum ( $\alpha_2$ MDS) to reconstitute them to approximately their original concentrations. A sample of double depleted serum (DDS) was also prepared by immunoaffinity depletion of clusterin from  $\alpha_2$ MDS, as previously described (Poon *et al.*, 2000); depletion of  $\alpha_2$ M and clusterin from sera was confirmed by immunoblotting (See Section 3.3.4.1). To allow for small dilution effects during the depletion steps, the  $A^{280}$  of  $\alpha_2$ MDS, DDS and a "matched" sample of NHS (the same batch of serum from which the depleted sera had been prepared) were determined and used to "normalize" the total protein concentration of each sample; the maximum dilution factor was less than 7% and adjustments to the total protein concentration were made by adding PBS. Aliquots (100  $\mu\text{l}$ ) of NHS,  $\alpha_2$ MDS and DDS were diluted 1:4 with PBS (supplemented with 7.5 mM  $\text{NaN}_3$ , 1 mM PMSF) and incubated at 37 °C for 7 days. Precipitated proteins were recovered using 0.45  $\mu\text{m}$  ULTRAFREE centrifugal filtration units (Millipore, Sydney) which were washed with PBS before the precipitate was solubilised with 2 M guanidine hydrochloride in PBS for 2 h at 60 °C and quantified using a bicinchoninic acid microprotein assay (Smith *et al.*, 1985).

#### 3.3.4.1 Confirming depletion of $\alpha_2$ M and clusterin from human serum

In order to confirm the depletion of  $\alpha_2$ M and clusterin from human serum, samples were subjected to reducing 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Sartorius, Melbourne, Australia). Membranes were blocked overnight with 1% heat-denatured casein (HDC) in PBS, washed with PBS and subsequently incubated with either polyclonal rabbit anti- $\alpha_2$ M (DakoCytomation, Denmark; diluted 1:500 in 1% HDC in PBS) or anti-clusterin G7 antibody (diluted 1:500 in 1% HDC in PBS; (26)) for 2 h at room temperature. Unbound antibody was removed by washing with PBS, and secondary antibody conjugated with HRP (diluted 1:500 in 1% HDC in PBS) added to each membrane and incubated for 2 h at room temperature. Following washes with PBS and 0.1% Triton X-100 in PBS, bound

antibody was detected using an enhanced chemiluminescence kit (Pierce, IL, USA).

### 3.3.5 Detection and purification of $\alpha_2$ M/stressed protein complexes

#### 3.3.5.1 Immunoprecipitations

The following solutions in PBS were incubated for 3 h at either 43 °C or RT:  $\alpha_2$ M (3.5  $\mu$ M), biotinylated CS (CS-b; 6  $\mu$ M), or mixtures of  $\alpha_2$ M and CS-b at the same final concentrations;  $\alpha_2$ M (0.7  $\mu$ M), CPK-b (25  $\mu$ M), or mixtures of  $\alpha_2$ M and CPK-b at the same final concentrations. Similarly,  $\alpha_2$ M (3.5  $\mu$ M), Lys-b (70  $\mu$ M), or mixtures of  $\alpha_2$ M and Lys-b at the same final concentrations were incubated in OSB for 13 h at 37 °C. All samples were then centrifuged (5 min at 10000 g) to remove insoluble material and incubated with shaking for 1 h at RT with streptavidin-agarose (50  $\mu$ l packed volume; Calbiochem, USA). After washing with 0.1% Triton X-100 in PBS, the agarose beads were boiled in SDS-PAGE sample buffer and the eluted proteins analysed by SDS-PAGE under reducing conditions.

#### 3.3.5.2 Native Gel Electrophoresis

CS (6 mM) and  $\alpha_2$ M (3.5 mM), or mixtures of CS and  $\alpha_2$ M at same final concentrations were incubated at 43 °C or room temperature for 4 h and then centrifuged at 13000 x g for 1 minute to remove any precipitated protein. CPK (25 mM) and  $\alpha_2$ M (3.5 mM), or mixtures of CPK and  $\alpha_2$ M at same final concentrations were also prepared as described for CS. Lys (70 mM) and  $\alpha_2$ M (3.5 mM) or mixtures of  $\alpha_2$ M and lys were incubated at 37 °C in oxidative stress buffer overnight. Samples (30  $\mu$ g total protein) were electrophoresed on a 1% native agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 5 mM EDTA, pH 7.5). The gel was run for approximately 2.5 hours at a constant 60 V in TAE buffer, pH 7.5. The gel was then washed with milli-Q water and stained using Imperial<sup>TM</sup> protein stain (Pierce, USA).

#### 3.3.5.3 Purification of complexes

$\alpha_2$ M/CS and  $\alpha_2$ M/CPK complexes were purified by anion exchange chromatography. Mixtures of CS-b (6  $\mu$ M) and  $\alpha_2$ M (3.5  $\mu$ M) or CPK-b (25  $\mu$ M) and  $\alpha_2$ M (0.7  $\mu$ M) were incubated at 43 °C for 3 h, centrifuged at 10000 g for 5 min and dialysed against 20

mM TAPS buffer, pH 9, overnight at 4°C. The samples were then applied to a 1 ml HiTrap Q fast flow Sepharose column (GE Healthcare, Australia) equilibrated with 20 mM TAPS, pH 9, and eluted with a linear gradient of 0-1 M NaCl in the same buffer. The presence of complex in fractions shown by SDS-PAGE to contain both  $\alpha_2$ M and the substrate protein was verified by immunoprecipitation, as described above. Complexes were stored at -20 °C.

### 3.3.6 Preparation of $\alpha_2$ M/stressed protein/trypsin complexes

Using identical conditions to those described above, heated mixtures of  $\alpha_2$ M and either CS-b or CPK-b were subsequently incubated with 1.4  $\mu$ M trypsin for 10 min before adding 2.8  $\mu$ M soybean trypsin inhibitor. These mixtures were then subjected to immunoprecipitation analysis using streptavidin-agarose (as described above). To prepare purified  $\alpha_2$ M/stressed protein/trypsin complexes for use in cell surface binding experiments, ion-exchange-purified  $\alpha_2$ M/CS-b or  $\alpha_2$ M/CPK-b complexes (0.75 mg/ml in PBS) were incubated with a 3-fold molar excess of trypsin for 2 h at 37 °C. Any unbound trypsin was inactivated with excess soybean trypsin inhibitor and subsequently removed by Superose-6 SEC. SDS-PAGE analyses indicated that the stressed protein complexed with  $\alpha_2$ M was not significantly degraded during incubation of  $\alpha_2$ M/stressed protein complexes with trypsin (data not shown).

### 3.3.7 Cell culture and flow cytometry

The JEG-3 (human adenocarcinoma) cell line expressing LRP was obtained from ATCC (VA, USA) and cultured in Dulbecco's Modified Eagle Medium: F-12 (DMEM: F-12) (Invitrogen, USA) supplemented with 5% (v/v) foetal calf serum (FCS; Thermotrace, Australia), incubated at 37 °C and 5% (v/v) CO<sub>2</sub>. Adherent cells were detached using 5 mM EDTA in PBS and then washed by centrifugation at 300 g for 10 min in Hank's binding buffer (HBB; 0.14 M NaCl, 5 mM KCl, 6 mM glucose, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM HEPES, 0.1% (w/v) BSA, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, pH 7.4). Cells were incubated for 30 min on ice with either  $\alpha_2$ M, trypsin-activated  $\alpha_2$ M\*, or trypsin-activated  $\alpha_2$ M/(biotinylated)stressed protein complexes, all at 200  $\mu$ g/ml in HBB. To detect bound  $\alpha_2$ M or  $\alpha_2$ M\*, the cells were subsequently incubated with rabbit anti- $\alpha_2$ M or (control) anti-DNP antibodies (diluted 1:500), and

finally with SaRIgG-FITC (diluted 1:50). To detect bound complexes incorporating biotinylated stressed proteins, cells were incubated with streptavidin-Alexa 488 (5  $\mu$ g/ml). To confirm the specificity of binding, similar experiments were undertaken in which cells were first pre-incubated with either an inhibitory rabbit polyclonal anti-LRP antibody (200  $\mu$ g/ml; kindly donated by S. K. Moestrup, University of Aarhus, Denmark) or GST-RAP (100  $\mu$ g/ml). Immediately before analysis using an LSR II flow cytometer (Becton Dickinson, Sydney), dead cell nuclei were stained using 1  $\mu$ g/ml PI. Excitation was at 488 nm and fluorescence emissions were collected at  $515 \pm 10$  nm (FITC) and  $695 \pm 20$  nm (PI). Electronic gating was used to exclude dead cells from the analyses. Data was collected using FACS Diva software (v4.0; Becton Dickinson) and analysed using FloJo v6.4.1 (Treestar Inc., USA). The significance of differences in binding were assessed using the Student's t-test.

### 3.3.8 Enzyme activity assays

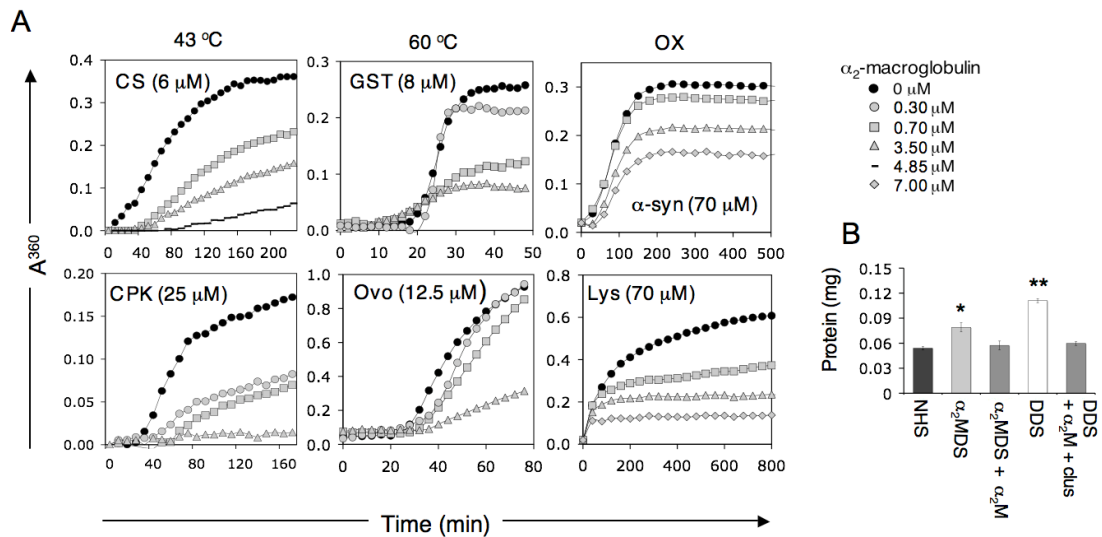
GST (8  $\mu$ M) in PBS was incubated for 30 min at either 37 °C or 60 °C in the presence or absence of 4.85  $\mu$ M  $\alpha_2$ M (which is sufficient to inhibit most precipitation of CS under these conditions). Immediately after heating, the samples were diluted 1:1 in GST refolding buffer (50 mM  $\text{Na}_2\text{HPO}_4$ , 50 mM KCl, 5 mM  $\text{MgCl}_2$ , pH 7.4,  $\pm$  2 mM ATP). To assay GST activity, 50  $\mu$ l samples were added to 200  $\mu$ l of 2 mM glutathione, 2 mM 1-chloro-2,4-dinitrobenzene, 50 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4, and the production of 1-S-glutathionyl-2,4-dinitrobenzene measured as an increase in  $A^{340}$ . Similarly, 6  $\mu$ M CS in PBS was heated for 45 min at room temperature or 43 °C in the presence or absence of 4.85  $\mu$ M  $\alpha_2$ M (which is sufficient to inhibit most precipitation of GST under these conditions). Immediately after heating, the samples were diluted 1:4 in CS refolding buffer (100 mM Tris-HCl, 35 mM NaCl, 50 mM KCl, 5 mM  $\text{MgCl}_2$ , pH 7.4,  $\pm$  2 mM ATP). To assay CS activity, 10  $\mu$ l samples were added to 240  $\mu$ l of 100 mM Tris-HCl, 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.094 mM acetyl coenzyme A, 0.046 mM oxalacetate, pH 8, and the production of 2-nitro-5-thiobenzoate measured as an increase in  $A^{412}$ . For both enzymes, heating was performed in a Thermo Finemixer SH2000-DX (Finemould Precision Ind. Co., USA), without shaking, and absorbance measurements made using a SpectraMax Plus<sup>384</sup> microplate reader.



## 3.4 Results

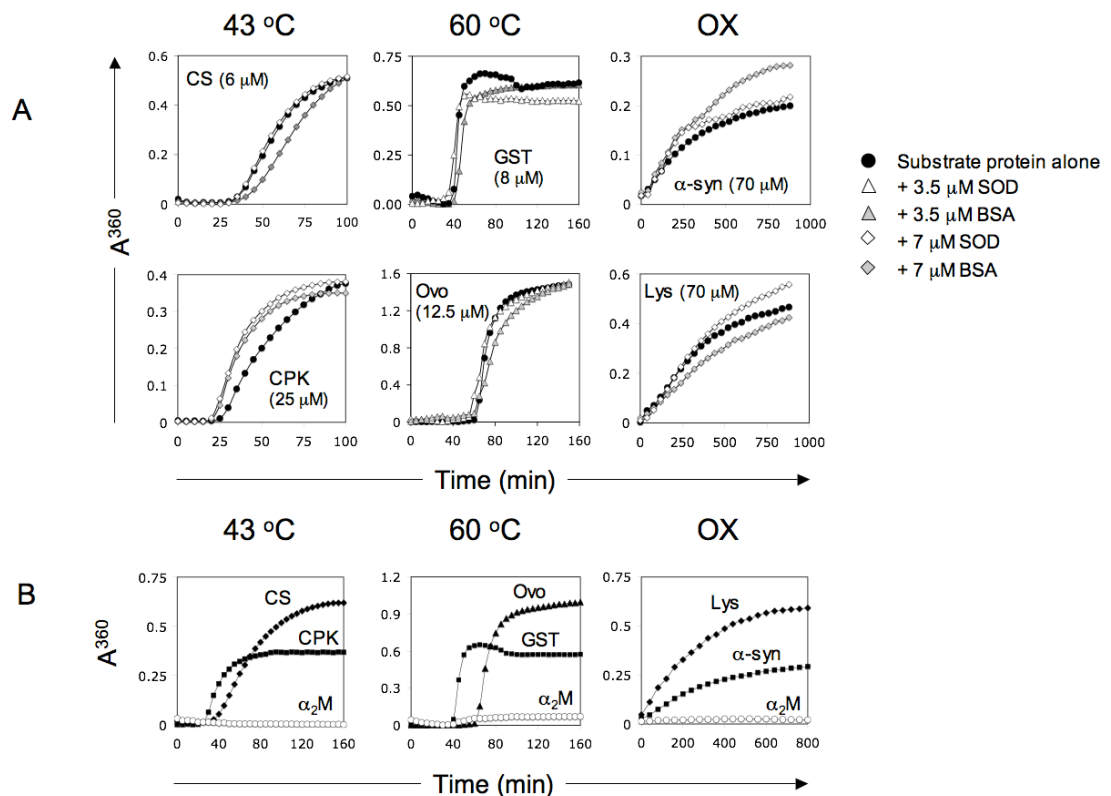
### 3.4.1 $\alpha_2$ M protects proteins from heat and oxidative stress-induced precipitation

Heating of citrate synthase (CS) or creatine phosphokinase (CPK) at 43 °C and ovotransferrin (Ovo) or glutathione-S-transferase (GST) at 60 °C resulted in their gradual precipitation over 50-240 min (Figure 3.1A). Oxidative stress induced slow



**Figure 3.1  $\alpha_2$ M inhibits stress-induced protein precipitation.** (A) CS (6  $\mu$ M) and CPK (25  $\mu$ M) were incubated at 43 °C, Ovo (12.5  $\mu$ M) and GST (8  $\mu$ M) were incubated at 60 °C, and  $\alpha$ -syn (70  $\mu$ M) and Lys (70  $\mu$ M) were subjected to oxidative stress in the presence of various concentrations of  $\alpha_2$ M (indicated in the key). The turbidity associated with protein precipitation was detected as an increase in absorbance ( $A_{360}$ ). In all cases, the control proteins SOD and BSA had negligible effects on protein precipitation (see Figure 3.2). Data points shown are individual measurements and are representative of at least three independent experiments. (B) Histogram showing the total protein precipitated from 100  $\mu$ l aliquots of normal human serum (NHS),  $\alpha_2$ M-depleted serum ( $\alpha_2$ MDS), serum depleted of both  $\alpha_2$ M and clusterin (DDS), and depleted sera supplemented with purified  $\alpha_2$ M and clusterin, diluted 1 in 4 in PBS and incubated at 37 °C for 7 days. Data points represent the means ( $n=3$ )  $\pm$  standard errors (SE). Asterixes denote significant differences when compared to NHS ( $p < 0.05$ , Student's t-test).

precipitation of lysozyme (Lys) over 800 min and a more rapid precipitation of  $\alpha$ -synuclein ( $\alpha$ -syn) over 200 min (Figure 3.1 A). In all cases, the addition of  $\alpha_2$ M produced a dose dependant inhibition of stress-induced precipitation. Under the conditions tested there was no significant aggregation measured for  $\alpha_2$ M alone (Figure



**Figure 3.2 Protein precipitation controls.** (A) Control proteins BSA and SOD have only minor effects on the precipitation of CS (6  $\mu$ M) and CPK (25  $\mu$ M) incubated at 43 °C, Ovo (12.5  $\mu$ M) and GST (8  $\mu$ M) incubated at 60 °C, and  $\alpha$ -syn (70  $\mu$ M) and Lys (70  $\mu$ M) subjected to oxidative stress (OX) (treatments indicated above the plots). (B)  $\alpha_2$ M alone did not undergo significant precipitation under any of the stress conditions tested. The turbidity associated with protein precipitation was detected as an increase in absorbance ( $A^{360}$ ). Data points shown are individual measurements and are representative of two independent experiments.

3.2). Effects corresponding to concentrations of  $\alpha_2$ M up to 7  $\mu$ M are shown; under the conditions tested, higher concentrations of  $\alpha_2$ M gave still greater inhibition of protein aggregation (data not shown). In all cases the addition of control proteins superoxide dismutase (SOD) or bovine serum albumin (BSA) had no significant effect on protein precipitation (Figure 3.2). When aliquots of normal human serum (NHS),  $\alpha_2$ M-depleted serum ( $\alpha_2$ MDS) and serum depleted of both  $\alpha_2$ MDS and clusterin (DDS) prepared from

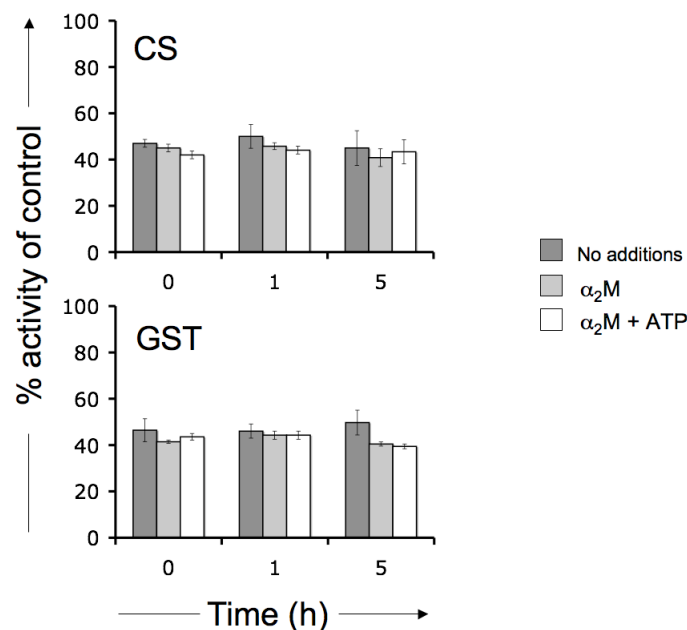
the same original batch of serum (diluted 1 in 4 in PBS) were incubated at 37 °C for 7 days, the  $\alpha_2$ MDS contained significantly more aggregated protein than NHS but less than that in DDS (Figure 3.1B). Adding purified  $\alpha_2$ M or clusterin back to the depleted sera returned the level of protein aggregation back to that of the corresponding undepleted samples (Figure 3.1B). Similar results were obtained (albeit with greater amounts of protein precipitated) when the same sera were heated at 43 °C for 72 h (data not shown). Although we have not yet identified the serum protein(s) protected by  $\alpha_2$ M or clusterin, collectively the results indicate that like clusterin and haptoglobin (Poon *et al.*, 2000; Yerbury *et al.*, 2005a),  $\alpha_2$ M specifically inhibits the stress-induced aggregation and precipitation of a broad range of purified proteins and unfractionated proteins in whole human serum.



**Figure 3.3 Depletion of  $\alpha_2$ M and clusterin from human serum.** Immunoblot showing the depletion of  $\alpha_2$ M from normal human serum (NHS) to yield  $\alpha_2$ M-depleted serum ( $\alpha_2$ MDS) and the subsequent removal of clusterin from  $\alpha_2$ MDS to give double depleted serum (DDS).

### 3.4.2 $\alpha_2$ M does not protect enzymes from heat-induced loss of activity or independently promote protein refolding

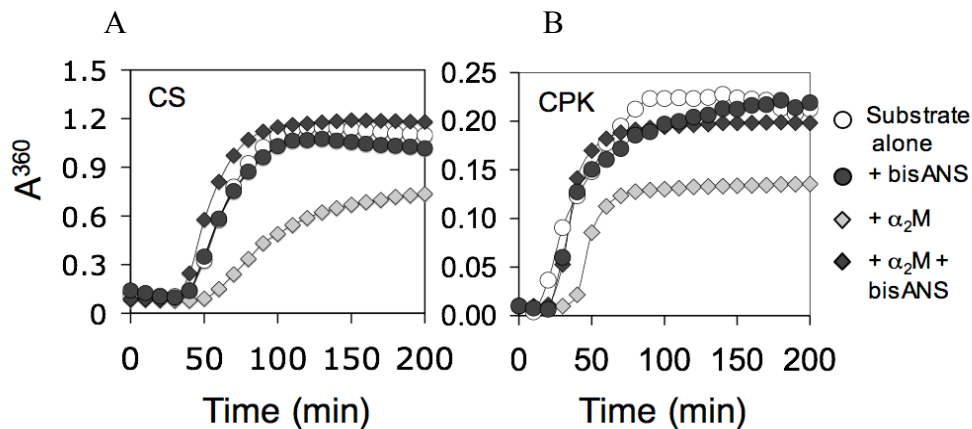
To test whether  $\alpha_2$ M had any inherent refolding activity, we analysed the enzyme activity of CS and GST before and after heat stress in the presence and absence of  $\alpha_2$ M and ATP. At concentrations sufficient to suppress most protein precipitation, and regardless of the presence or absence of ATP,  $\alpha_2$ M had no significant effect on the loss of activity of either CS or GST and did not promote the recovery of enzyme activity when measured at up to 5 h after heat stress (Figure 3.4).



**Figure 3.4**  $\alpha_2$ M does not protect enzymes from heat stress-induced loss of activity or independently promote recovery of activity. Histograms showing the level of CS and GST enzyme activity as a function of time after heat stress (43 °C and 60 °C, respectively). Additional components of the stressed mixtures are shown in respective keys. The data shown are means of triplicate measurements and the error bars indicate SE of the means. For each enzyme, none of the means are significantly different (Student's t-test,  $p > 0.05$ ). The data is representative of 2 independent experiments.

### 3.4.3 The chaperone-like action of $\alpha_2$ M is inhibited by bisANS

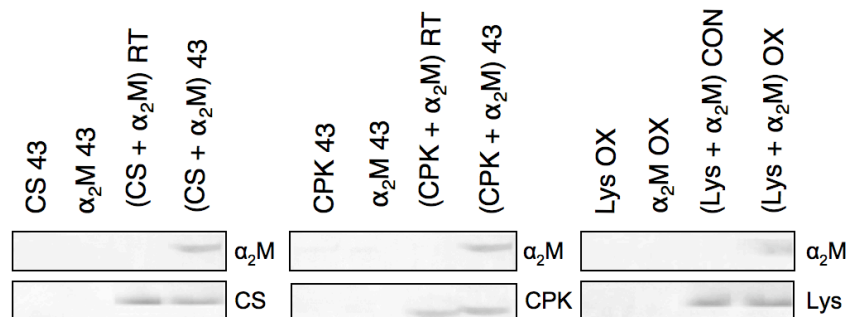
Under the conditions used, the aggregation of CS and CPK was little affected by the hydrophobic probe bisANS (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid) but was partially inhibited by  $\alpha_2$ M (Figure 3.5). Under these same conditions, pre-incubating  $\alpha_2$ M with bisANS abolished its ability to inhibit the aggregation of CS and CPK (Figure 3.5). A trypsin binding assay and native PAGE analysis confirmed that  $\alpha_2$ M was not activated by bisANS under these conditions (data not shown).



**Figure 3.5.** *The chaperone action of  $\alpha_2$ M depends on hydrophobic interactions.* Time-dependent changes in turbidity (measured as  $A^{360}$ ) of heat-stressed CS (6  $\mu$ M) and CPK (25  $\mu$ M), either alone or in the presence of  $\alpha_2$ M (at 3.5  $\mu$ M or 0.7  $\mu$ M, respectively) or bisANS (7  $\mu$ M), or  $\alpha_2$ M (at the same final concentrations) preincubated with bisANS (see *Materials and Methods*). The data points shown are individual measurements. The results shown are representative of at least 3 independent experiments.

#### 3.4.4 $\alpha_2$ M forms stable complexes with stressed proteins

We investigated whether  $\alpha_2$ M, like clusterin (Humphreys *et al.*, 1999) and haptoglobin (Yerbury *et al.*, 2005a), forms stable complexes with stressed proteins. We used native gel electrophoresis to analyse stressed and non-stressed mixtures of CS, CPK and Lys, with and without added  $\alpha_2$ M. In all cases tested, following heat or oxidative stress but not otherwise, samples containing both  $\alpha_2$ M and substrate produced a band of unique electrophoretic mobility suggesting the formation of a complex (data not shown). This interpretation was confirmed by using streptavidin-agarose to affinity adsorb biotinylated (-b) proteins from solutions containing  $\alpha_2$ M, or  $\alpha_2$ M together with one of CS-b, CPK-b or Lys-b. The proteins in these solutions had been either untreated or exposed to stresses previously described. Adsorbed proteins were eluted and analysed by SDS-PAGE. Whether untreated, heated or exposed to oxidizing conditions,  $\alpha_2$ M did not bind to the streptavidin-agarose beads (Figure 3.6).

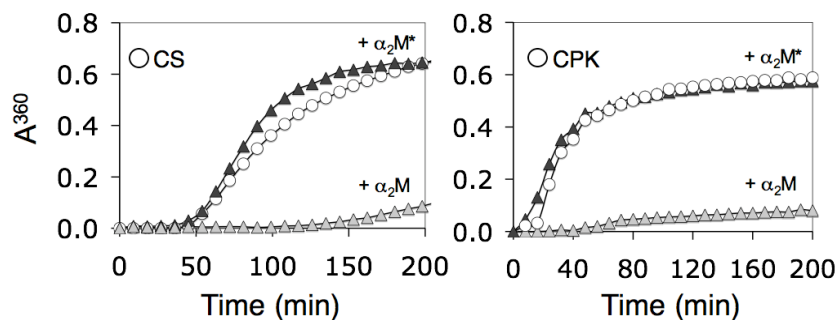


**Figure 3.6  $\alpha_2$ M forms stable complexes with stressed proteins.** Image of sections of Coomassie blue stained 10% SDS-PAGE gels (run under reducing conditions) showing proteins affinity adsorbed by streptavidin-agarose in samples (heated to 43°C or incubated at RT, as indicated) containing  $\alpha_2$ M alone, CS-b or CPK-b alone, or mixtures of  $\alpha_2$ M and CS-b or CPK-b.  $\alpha_2$ M alone, Lys-b alone, or mixtures of  $\alpha_2$ M and Lys-b were also analysed under oxidative (OX) and control (CON) conditions. Note that in the absence of  $\alpha_2$ M, under the stress conditions used (43 °C or oxidative conditions) most of the CS-b, CPK-b and Lys-b precipitated from solution and was thus unavailable to bind to streptavidin-agarose. The identity of the bands was established by comparison with molecular mass standards (not shown). The results shown are representative of 2 independent experiments.

In unstressed samples containing only a substrate protein (CS-b, CPK-b or Lys-b) together with  $\alpha_2$ M, only the biotinylated substrate protein was recovered (Figure 3.6). For samples that had been exposed to heat or oxidative stress, and that contained a substrate protein and  $\alpha_2$ M, both proteins were detected in the bead eluate. Little or no detectable substrate protein was recovered from similarly stressed solutions of substrate alone, because in the absence of  $\alpha_2$ M most of the substrate precipitated from solution under these conditions (Figure 3.6). Collectively, these results demonstrate that  $\alpha_2$ M forms stable non-covalent complexes with substrate proteins under conditions of heat and oxidative stress.

### 3.4.5 Pre-activation with trypsin abolishes the chaperone-like action of $\alpha_2$ M

Under the conditions tested,  $\alpha_2$ M potently inhibited the heat-induced precipitation of CS and CPK (Figure 3.7). However, under the same conditions, trypsin-activated  $\alpha_2$ M\* had little or no effect on substrate protein precipitation (Figure 3.7), demonstrating that protease activation of  $\alpha_2$ M effectively abolishes its chaperone-like activity. The kinetics and extent of precipitation of CS and CPK shown in Figure 3.7 differ somewhat from those shown in Figure 3.1A; this resulted from the use of different commercial batches of the two proteins in the different experiments.



**Figure 3.7** *Trypsin activation abolishes the chaperone activity of  $\alpha_2$ M.* Time-dependent changes in turbidity (measured as A<sup>360</sup>) of heat-stressed CS and CPK, either alone or in the presence of  $\alpha_2$ M or trypsin-activated  $\alpha_2$ M\* (indicated on plots). The data points are individual measurements; the results shown are representative of at least 3 independent experiments.

### 3.4.6 $\alpha_2$ M/stressed protein complexes retain protease trapping activity

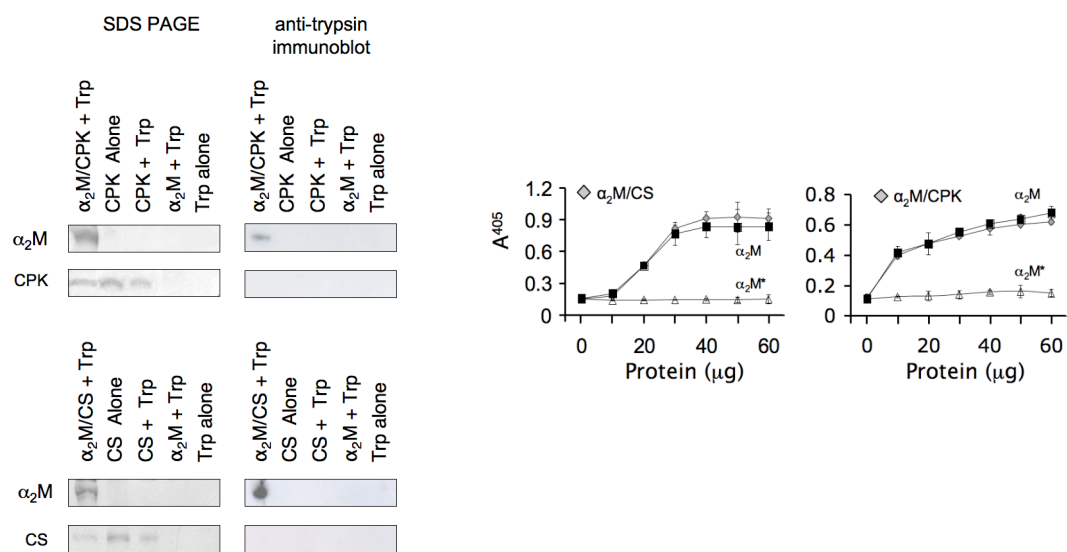
Immunoprecipitation analyses were used to test whether trypsin could interact with  $\alpha_2$ M/stressed protein complexes. This indicated that the only conditions tested under which the immunoprecipitate contained trypsin was when a heated mixture of  $\alpha_2$ M and substrate protein had been subsequently incubated with trypsin (Figure 3.8A). This establishes that under these conditions a complex is formed which contains all three molecular species ( $\alpha_2$ M, heat-stressed protein and trypsin). Under the conditions used (non-reduced 10% SDS PAGE gel), trypsin detected in the immunoblots migrated to essentially the same position as native  $\alpha_2$ M (i.e. above a 250 kDa molecular mass marker and near the top of the gel, where resolution between molecules of different

mass is very limited). This suggests that when trypsin interacts with  $\alpha_2$ M/stressed protein complexes, it undergoes the trapping reaction to become covalently linked with  $\alpha_2$ M. SDS PAGE analyses indicated that under the conditions used, when complexed with  $\alpha_2$ M, CS and CPK were not degraded by trypsin (data not shown). This is consistent with results demonstrating that when complexed with prion protein,  $\alpha_2$ M protected it from degradation by proteinase K ( $\alpha_2$ M does not directly inhibit proteinase K (Adler and Kryukov 2007)). The interaction of  $\alpha_2$ M/stressed protein complexes with a protease was also probed using the classic trypsin trapping assay. Interaction with trypsin converts  $\alpha_2$ M to  $\alpha_2$ M\* and coincidentally traps the protease in a steric “cage” (Feldman *et al.*, 1985). In this situation, trypsin remains able to cleave substrates less than about 10 kDa which are small enough to diffuse into the cage (Ganrot 1966; Barrett and Starkey 1973). In contrast,  $\alpha_2$ M\* is unable to bind trypsin. A molar excess of trypsin was incubated with purified complexes formed between  $\alpha_2$ M and heat-stressed CS or CPK and any unbound trypsin subsequently inactivated using soybean trypsin inhibitor (which is sterically unable to access and inactivate trypsin bound to  $\alpha_2$ M (Ganrot 1966)). Residual trypsin activity, attributable to complexation with  $\alpha_2$ M, was measured using the low molecular weight substrate BAPNA. Purified  $\alpha_2$ M/CS and  $\alpha_2$ M/CPK complexes showed dose-dependent trypsin binding activity which, on a mass basis, was very similar to that measured for native  $\alpha_2$ M (Figure 3.8B). This indicates that  $\alpha_2$ M remains in its native form when complexed with heat-stressed proteins and suggested that the mass of the complexes tested was dominated by  $\alpha_2$ M. Size exclusion chromatography (SEC) analyses supported this interpretation; Superose 6 chromatography was unable to resolve  $\alpha_2$ M/CS and  $\alpha_2$ M/CPK complexes from native  $\alpha_2$ M (data not shown). The resolving power of SEC is limited at these high molecular masses and limited further by the fact that  $\alpha_2$ M migrates as a broad peak. However, it suggests that the complexes consist of a single 720 kDa  $\alpha_2$ M tetramer bound to one or a small number of the much smaller substrate protein molecules.

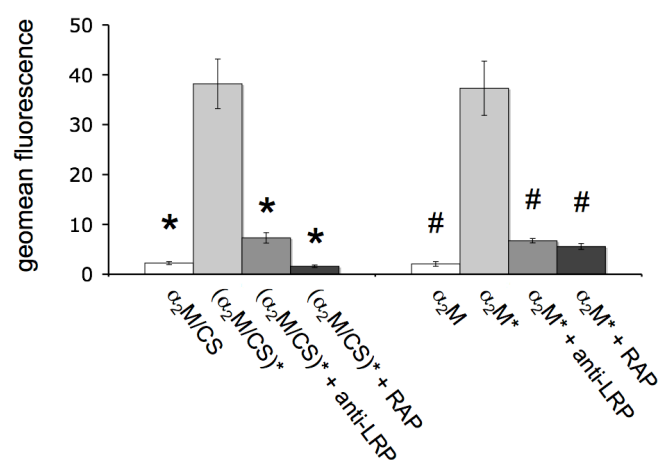


### 3.4.7 $\alpha_2$ M/stressed protein complexes bind to LRP after proteolytic activation

Native  $\alpha_2$ M and  $\alpha_2$ M/(heat-stressed)CS complexes showed little binding to JEG-3 cells; in contrast, following activation with trypsin, both  $\alpha_2$ M\* and trypsin-activated  $\alpha_2$ M/CS complexes (i.e. ( $\alpha_2$ M/CS)\*) showed substantial binding (Figure 3.9). This binding was primarily to the cell surface receptor LRP because it was strongly inhibited by both an anti-LRP antibody and GST-RAP (Figure 3.9). Similar results were obtained using CPK as the heat-stressed substrate protein (data not shown).



**Figure 3.8  $\alpha_2$ M/stressed protein complexes retain the ability to trap trypsin.** (A) Image of sections of non-reduced Coomassie blue stained 10% SDS PAGE gels (left panels) and corresponding immunoblots probed with an anti-trypsin antibody (right panels), showing proteins affinity absorbed by streptavidin-agarose from: samples incubated with trypsin containing CS-b or CPK-b alone, or  $\alpha_2$ M alone (all at room temperature), or mixtures of  $\alpha_2$ M and either CS-b or CPK-b which had been heated at 43 °C before mixing with trypsin (i.e.  $\alpha_2$ M/CS and  $\alpha_2$ M/CPK). On the SDS PAGE gels, the identity of bands was established by comparison with molecular mass standards (not shown); where detected on the immunoblots, trypsin migrated to the same position as  $\alpha_2$ M (left lanes). Results for heated CS or CPK alone, and for unheated mixtures of  $\alpha_2$ M and either CS or CPK are not shown here (see Figure 3.6). The results shown are representative of two independent experiments. (B) Native  $\alpha_2$ M, methylamine activated  $\alpha_2$ M\* and purified  $\alpha_2$ M/CS and  $\alpha_2$ M/CPK complexes (indicated on plots) were assayed for trypsin binding activity. Data points represent means (n=3)  $\pm$  SE; the results shown are representative of 3 independent experiments.



**Figure 3.9 Trypsin activates  $\alpha_2$ M /stressed protein complexes to expose an LRP binding site.**

Histogram plot showing the average geometric mean fluorescence ( $n = 3, \pm$  SE, arbitrary units) for immunochemical detection of the binding to JEG-3 cells of  $\alpha_2$ M, trypsin activated  $\alpha_2$ M\*, native  $\alpha_2$ M/CS complexes and trypsin-activated  $\alpha_2$ M /CS complexes (i.e.  $(\alpha_2$ M/CS)\*). In some cases, cells were pre-incubated with an inhibitory anti-LRP antibody or GST-RAP (indicated below the x-axis). The values shown have been corrected for the fluorescence associated with cells stained with negative control antibody. The results shown are representative of 3 independent experiments. Significant differences ( $p < 0.05$ ) are indicated by \* (vs.  $(\alpha_2$ M/CS)\*, left) and # (vs.  $\alpha_2$ M\*, right).

### 3.5 Discussion

Previous work showed that  $\alpha_2$ M could suppress the formation of A $\beta$  fibrils and reduce their toxicity (Du *et al.*, 1997). In addition,  $\alpha_2$ M was recently compared with intracellular chaperones because of its ability to behave as an immunogen when complexed to peptides (Srivastava 2002). Two key properties of chaperones are selective binding to non-native protein conformations to form stable complexes, and inhibition of the irreversible aggregation of non-native protein conformations (Fink 1999). The possibility that  $\alpha_2$ M might have chaperone properties with broad substrate specificity has not been tested before. The intrinsic stability of globular mammalian proteins means that none of these will normally unfold and aggregate under "physiological conditions" (i.e. neutral pH, 37 °C) at an experimentally convenient rate. Clearly, this is a physiological necessity, otherwise our bodies would routinely be choked with pathological protein aggregates. Protein aggregation *in vitro* requires solution conditions (such as increased temperature or oxidative conditions) such that the native structure is partially or completely disrupted but under which interactions such as hydrogen-bonding are not completely inhibited. Using such conditions, protein aggregates formed *in vitro* are indistinguishable from those aggregates found *in vivo* (Stefani and Dobson 2003). Furthermore, although different proteins may require a greater or lesser heat stress to unfold at comparable (experimentally convenient) rates, the pathways of unfolding remain the same regardless of the temperature (Day *et al.*, 2002).

Our data show for the first time that  $\alpha_2$ M can inhibit the aggregation and precipitation of a broad variety of proteins induced by heat or oxidative stress (Figure 3.1A). During inflammation and carcinoma, the *in vivo* levels of H<sub>2</sub>O<sub>2</sub> and Cu<sup>2+</sup> can approach those used in this study to exert oxidative stress (Abdulla *et al.*, 1979; Weiss 1989); millimolar levels of H<sub>2</sub>O<sub>2</sub> are known to have negligible effects on the structure and function of  $\alpha_2$ M (Wu and Pizzo 1999; Khan and Khan 2004). It was also shown that the selective removal of  $\alpha_2$ M from whole human serum renders proteins in this fluid more susceptible to precipitation, even at 37 °C (Figure 3.1B). Collectively, these results indicate that  $\alpha_2$ M has potent *in vitro* chaperone properties that are likely to be

relevant *in vivo*. Like clusterin (Humphreys *et al.*, 1999),  $\alpha_2$ M can inhibit protein precipitation even when present at sub-stoichiometric levels (Figure 3.1A). This is likely to result from the stabilization of a small fraction of the total pool of substrate molecules which are misfolded and which would otherwise act as aggregation nuclei. The substrate-specific variation in the ratio of  $\alpha_2$ M:substrate required to suppress precipitation is typical of the behaviour seen with other proteins having chaperone properties, including the small heat shock proteins (Carver *et al.*, 2003), clusterin (Humphreys *et al.*, 1999) and haptoglobin (Yerbury *et al.*, 2005a), and probably results from substrate-specific differences in the proportion of misfolded substrate molecules present under set conditions. The demonstration that endogenous  $\alpha_2$ M and clusterin significantly inhibit the spontaneous precipitation of proteins in unfractionated human serum incubated at 37 °C may have important medical implications. Abundant extracellular proteins with this type of chaperone property may act as an important line of defense against inappropriate extracellular protein aggregation which underpins a variety of serious human diseases (Yerbury *et al.*, 2005b). The effects of  $\alpha_2$ M and clusterin on plasma protein precipitation are additive (Figure 3.1B), suggesting that even though they are promiscuous in their interactions with different substrate proteins, they may provide complementarity with respect to the endogenous extracellular proteins they protect.

In the case of two enzymes tested, when acting alone,  $\alpha_2$ M did not protect enzymes from heat-induced loss of activity or promote recovery of this activity following heat stress, regardless of the presence or absence of ATP (Figure 3.4). Moreover, a motifscan ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)) and a BLAST search failed to identify any known ATPase motifs or sequence similarity to any known ATPases, respectively. The demonstration that the hydrophobic probe bisANS suppressed the ability of  $\alpha_2$ M to inhibit heat-induced substrate protein aggregation (Figure 3.5) indicates that it binds to stressed proteins, at least in part, via hydrophobic interactions. Previous work has shown that, overall,  $\alpha_2$ M contains more surface exposed hydrophobicity after it has been activated (Birkenmeier *et al.*, 1989). However, this does not exclude the possibility that specific region(s) of exposed hydrophobicity on  $\alpha_2$ M important in the chaperone-like action are sterically more accessible to stressed

proteins prior to protease activation. It is also possible that there are other unknown determinants required for binding to stressed proteins that are affected by the conformational changes associated with protease activation.

Lastly, we used immunoprecipitation analyses (Figure 3.6) to show that  $\alpha_2$ M forms stable soluble complexes with stressed proteins. Collectively, the above functional characteristics demonstrate that  $\alpha_2$ M possesses similar chaperone properties as the small heat shock proteins (sHSPs), clusterin and haptoglobin (Carver *et al.*, 1995; Yerbury *et al.*, 2005a). Although we have not excluded the possibility that, like some of the sHSPs (Fink 1999),  $\alpha_2$ M may hold stressed proteins in a refolding-competent state, there are no known chaperones with established refolding activity present at significant levels in extracellular body fluids (Yerbury *et al.*, 2005b) and thus any such activity would be of questionable physiological relevance.

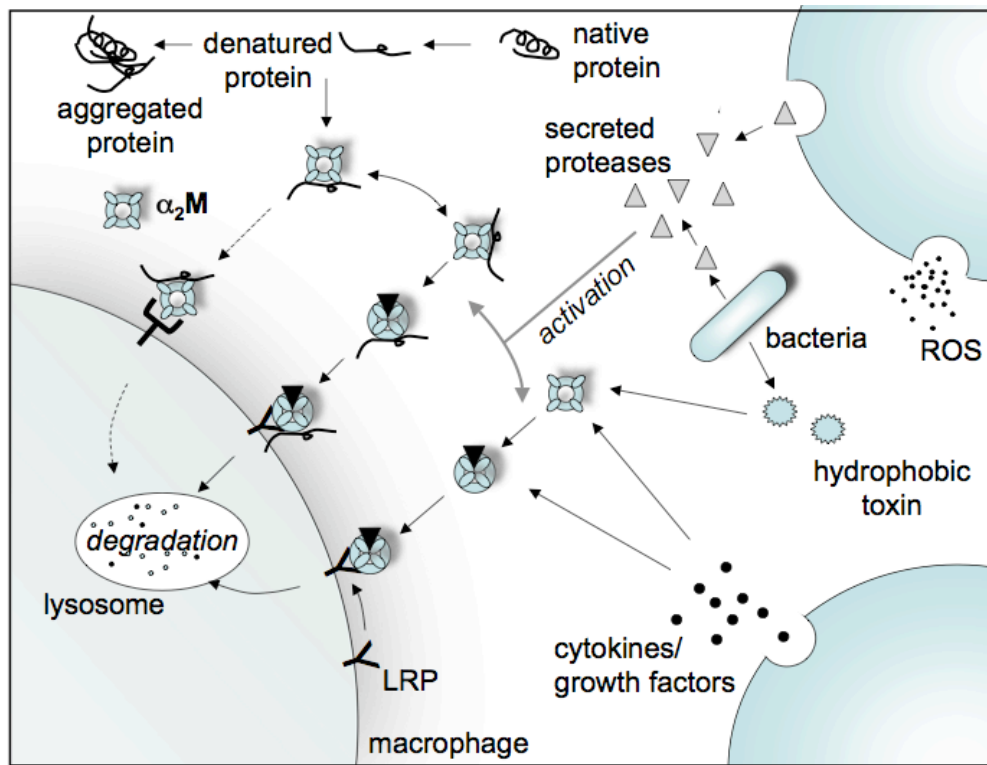
Two major types of binding reactions that have been previously described for  $\alpha_2$ M are the protease trapping and thiol ester/covalent linking reactions, which are involved in  $\alpha_2$ M activation (Barrett 1981).  $\alpha_2$ M also binds to a range of non-protease ligands, independently of its activation, including concanavalin A, phytohemagglutinin, aspartate aminotransferase, myelin basic protein, histone H4, endotoxin (Barrett 1981), A $\beta$ , cytokines and growth factors (Mettenburg *et al.*, 2002).  $\alpha_2$ M is known to have discrete binding sites for A $\beta$ , cytokines and LRP (Mettenburg *et al.*, 2002). The relationship between binding sites on  $\alpha_2$ M for misfolded proteins versus the many other ligands remain to be defined. However, whatever binding mechanism(s) underly the chaperone-like action of  $\alpha_2$ M, they are clearly prevented if the molecule is first activated by undergoing a protease trapping reaction (Figure 3.7).

It had previously been shown that a lysozyme- $\alpha_2$ M-elastase complex could be formed when, using a 100-fold molar excess of lysozyme, all three were incubated together at room temperature (Chu and Pizzo 1993). The association with lysozyme appears to result from it becoming non-specifically trapped within the protease "cage" following elastase-mediated cleavage of the  $\alpha_2$ M bait region, rather than specific binding to  $\alpha_2$ M. Thus, although this previous study demonstrated formation of a trimolecular complex

with  $\alpha_2$ M, it is not directly comparable to the stressed protein- $\alpha_2$ M-trypsin complexes described here, which are formed at low stoichiometries. Data presented here establishes that the protease trapping action of  $\alpha_2$ M prevents it from subsequently exerting a chaperone-like action but that if it first complexes with a stressed protein, it remains able to later trap proteases forming a stressed protein- $\alpha_2$ M-protease complex (Figure 3.8A&B). This establishes  $\alpha_2$ M as the first known mammalian protein with both chaperone-like and protease inhibitor activities. We also demonstrated that following interaction with a protease (trypsin), but not otherwise,  $\alpha_2$ M incorporated into complexes with heat-stressed proteins exposed binding site(s) for LRP, consistent with it adopting an “activated” conformation (Figure 3.9). Conversion to  $\alpha_2$ M\* was also indicated by the demonstration of a covalent association between trypsin and  $\alpha_2$ M in these complexes (Figure 3.8A). It follows that if one important function of  $\alpha_2$ M is to bind to and solubilize extracellular proteins with non-native conformations, and subsequently mediate their clearance by LRP, then interaction with a protease may be one *in vivo* switch to trigger LRP-mediated uptake of  $\alpha_2$ M/stressed protein complexes. At physiological locales such as sites of inflammation, this process would be facilitated by the relative abundance of proteases. Uptake of non-activated  $\alpha_2$ M/stressed protein complexes via other currently unknown cell surface receptors is also possible (Figure 3.10).

The affinity of  $\alpha_2$ M for most cytokines (e.g. TGF $\beta$ ) is higher when it is in the activated state, and while in this state  $\alpha_2$ M delivers the ligands to LRP for uptake and subsequent degradation (Crookston *et al.*, 1994). Thus, the model we propose (Figure 3.10) substantively expands a previous paradigm that  $\alpha_2$ M is a scavenger/disposal vehicle for a variety of extracellular proteins. In addition to  $\alpha_2$ M, mice also express a structurally and functionally closely related protein, murinoglobulin-1. The only study to ablate the expression of both these proteins in mice did not directly examine their role in clearing extracellular misfolded proteins. However, the results of this study implied a general anti-inflammatory action for the proteins, which is consistent with such a role (Umans *et al.*, 1999). We propose that  $\alpha_2$ M is a newly discovered and unique member of a small group of abundant proteins with chaperone properties that patrol extracellular spaces for

unfolded/misfolded proteins and facilitate their disposal. Such an activity would contribute to the important anti-inflammatory actions of  $\alpha_2$ M *in vivo*.



**Figure 3.10 Proposed model for  $\alpha_2$ M function.** At sites of inflammation factors such as elevated temperature, reactive oxygen species (ROS) and lowered pH may cause damage to extracellular proteins inducing them to partially unfold. Bacteria present at the site may secrete pathogenic proteases and toxins, while host immune cells are known to secrete proteases in an attempt to destroy invading pathogens. In addition, a variety of cell types may locally secrete cytokines or growth factors. Native  $\alpha_2$ M may exert a broad anti-inflammatory action by binding to and promoting the clearance of (i) endogenous or exogenous proteases, and (ii) other ligands such as denatured proteins, hydrophobic toxins (such as endotoxin) and cytokines. The  $\alpha_2$ M-mediated clearance of non-protease ligands can occur via LRP following the activation of  $\alpha_2$ M-ligand complexes by interaction with proteases, which are likely to be abundant at sites of inflammation. Clearance of native  $\alpha_2$ M-ligand complexes might also occur via other cell surface receptors, independently of protease activation.

## Chapter 4

# The Extracellular Chaperone Clusterin Influences Amyloid Formation and Toxicity by Interacting with Pre-Fibrillar Structures

## Preface

Much was known about the potent ability of clusterin to chaperone amorphously aggregating proteins. However, there was little information available on the effect of clusterin on amyloid formation. This work set out to systematically examine the effect of clusterin on amyloid formation and toxicity using a large panel of unrelated proteins and peptides. Some of the work, in particular work on cc $\beta$ <sub>w</sub> peptide and calicitonin, was performed by Sarah Meehan and Stephen Poon at the University of Adelaide and the University of Cambridge, respectively.

Publication:

**Yerbury JJ**, Poon S, Meehan S, Thompson B, Kumita JR, Dobson CM, Wilson MR (2007) The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. *FASEB Journal* **21**, 2312-22.



## 4.1 Abstract

Clusterin is an extracellular chaperone present in all disease-associated extracellular amyloid deposits, however, its roles in amyloid formation and protein deposition *in vivo* are poorly understood. The current study initially aimed to characterise the effects of clusterin on amyloid formation *in vitro* by a panel of eight protein substrates. Two of the substrates (Alzheimer's beta peptide and a PI3-SH3 domain) were then used in further experiments to examine the effects of clusterin on amyloid cytotoxicity and to probe the mechanism of clusterin action. We show that clusterin exerts potent effects on amyloid formation, the nature and extent of which vary greatly with the clusterin:substrate ratio, and provide evidence to suggest that these effects are exerted via interactions with pre-fibrillar species that share common structural features. Pro-amyloidogenic effects of clusterin appear to be restricted to conditions in which the substrate protein is present at a very large molar excess; under these same conditions clusterin co-incorporates with substrate protein into insoluble aggregates. However, when clusterin is present at much higher but still sub-stoichiometric levels (e.g. a molar ratio of clusterin:substrate = 1:10), it potently inhibits amyloid formation and provides substantial cytoprotection. These findings suggest that clusterin is an important element in the control of extracellular protein misfolding.

## 4.2 Introduction

Numerous age-related, systemic and neurological disorders are associated with the deposition of highly structured protein aggregates usually known as amyloid or amyloid-like fibrils, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Creutzfeldt-Jakob disease (Chiti and Dobson 2006). In many cases, the deposits formed in these and other protein conformation disorders are located extracellularly where they exert pathogenic effects by organ disruption or by cytotoxicity. Although the processes that control the folding of proteins inside cells are relatively well understood, very little is known of the corresponding extracellular processes. Clusterin is the best characterised abundant extracellular chaperone and has recently been proposed to form part of an extracellular protein quality control system (Yerbury *et al.*, 2005b).

Amyloid fibrils found *in vivo* exhibit common structural features independent of the identity of the parent protein. Intracellular amyloid aggregates are found co-localised with components of the intracellular protein quality control system, including chaperones and ubiquitin (Sherman and Goldberg 2001). In a remarkable parallel observation, all disease-associated insoluble extracellular protein deposits tested, including those characterised as amyloid, co-localize with clusterin (Table 4.1). The roles of clusterin in amyloid formation and protein deposition are, however, poorly understood. Clusterin is a well conserved, secreted glycoprotein found in most extracellular fluids which has a potent ATP-independent chaperone action similar to that of the small heat shock proteins. It inhibits stress-induced amorphous protein aggregation by binding to exposed regions of hydrophobicity on non-native protein conformations, to form high-molecular-weight but still soluble complexes (Humphreys *et al.*, 1999).

**Table 4.1. Protein deposition disorders in which clusterin has been found co-localised with extracellular protein deposits.**

Disease	Main constituent	Reference
Alzheimer's disease	A $\beta$	(Calero <i>et al.</i> , 2000)
Creutzfeldt-Jakob disease	PrP	(Freixes <i>et al.</i> , 2004)
Gerstmann-Straussler-Scheinker disease	PrP	(Chiesa <i>et al.</i> , 1996)
Gelatinous drop-like corneal dystrophy	keratoepithelin	(Nishida <i>et al.</i> , 1999)
Lattice type I corneal dystrophy	M1S1	(Nishida <i>et al.</i> , 1999)
Age-related macular degeneration	drusen	(Sakaguchi <i>et al.</i> , 2002)
Pseudoexfoliation (PEX) syndrome	PEX material	(Zenkel <i>et al.</i> , 2006)
Down's syndrome	A $\beta$	(Kida <i>et al.</i> , 1995)
HCHWA-Dutch type	A $\beta$	(Maat-Schieman <i>et al.</i> , 1996)
Familial British dementia	ABri	(Ghiso <i>et al.</i> , 1995)
Atherosclerosis	LDL/ApoB100	(Witte <i>et al.</i> , 1993)

References refer to studies that have found clusterin co-localised with deposits related to the respective disease.

Limited data from previous studies suggest that clusterin can affect the amyloid-forming process both *in vitro* and *in vivo*. Clusterin has been reported to inhibit *in vitro* amyloid formation by apolipoprotein C-II (apoC-II) (Hatters *et al.*, 2002), the A $\beta$  peptide (Oda *et al.*, 1995; Matsubara *et al.*, 1996; Hughes *et al.*, 1998) and a fragment of the prion protein, PrP<sup>106-126</sup> (McHattie and Edington 1999). Depending on the conditions, however, it has been reported to either promote or suppress the cytotoxicity of A $\beta$  (Oda *et al.*, 1995; Boggs *et al.*, 1996; Matsubara *et al.*, 1996; Hughes *et al.*, 1998). Similarly, work with PDAPP mice (a transgenic mouse model for Alzheimer's disease) in which clusterin expression is ablated has provided results which sometimes appear contradictory. For example, when compared with matched litter mates, ablation of clusterin expression decreased the levels of thioflavin-S staining of material in brain sections and the number of visibly damaged neurons in PDAPP mice. These findings were interpreted as indicating that clusterin expression promoted A $\beta$  amyloid formation

and toxicity (DeMattos *et al.*, 2002). However, in a background of apolipoprotein E negative (apoE<sup>-/-</sup>) PDAPP mice, the ablation of clusterin expression had the opposite effects, promoting the early onset of A $\beta$  deposition and material staining with thioflavin-S (DeMattos *et al.*, 2004). Such evidence shows that the *in vivo* effects of clusterin on amyloid formation are likely to involve multiple interactions and processes making it critical to better understand the nature and mechanism(s) of interactions between clusterin and amyloid-forming proteins through *in vitro* studies. This objective was the global aim of the current study in which the effects of clusterin on amyloid formation by a broad range of unrelated proteins were examined using a variety of complementary approaches. In addition, we selected two protein substrates, one associated with disease (A $\beta$ <sub>1-42</sub>, Alzheimer's disease) and the other not (a PI3-SH3 domain), to extend these investigations to examine the effects of clusterin on amyloid-related toxicity and to better characterise the mechanism(s) by which clusterin affects amyloid formation.

## 4.3 Materials and Methods

### 4.3.1 Materials

Clusterin was purified from human serum obtained from Wollongong Hospital (Wollongong, NSW, Australia) as previously described (Wilson and Easterbrook-Smith 1992). Hexafluoroisopropanol (HFIP), lysozyme (from hen egg white), bovine serum albumin (BSA), and  $\kappa$ -casein (from bovine milk) were purchased from Sigma (MO, USA). A plasmid encoding  $\alpha$ -synuclein was a gift from Dr Robert Cappai (Department of Pathology, University of Melbourne, Melbourne, Australia).  $\alpha$ -Synuclein was expressed in *E. coli* and purified by acid precipitation as described previously (Souza *et al.*, 2000). Glutathione-S-transferase (GST) from *Schistosoma japonicum* was prepared by thrombin cleavage of recombinant Jun leucine zipper-GST fusion protein and purified by GSH-agarose affinity chromatography (Heuer *et al.*, 1996). The short coiled-coil  $\beta$  (cc $\beta$ ) peptide, originally designed *de novo* as a model that transforms from a helical conformation at 20 °C into amyloid fibrils at 37 °C (Kammerer *et al.*, 2004), was modified by adding a tryptophan residue at its N terminus (to produce cc $\beta$ <sub>W</sub>; a kind gift from Dr Cait MacPhee, Department of Physics, University of Edinburgh, U. K.). The cc $\beta$  fibrils described herein were indistinguishable from those described previously. Calcitonin was purchased from both Auspep (Melbourne, Australia) and Southampton Polypeptides Limited (Southampton, UK).  $\beta_2$ -microglobulin was a kind gift from Prof Sheena Radford (University of Leeds, UK). A plasmid encoding the amyloidogenic PI3-SH3 domain of bovine phosphatidylinositol-3'-kinase (hereafter referred to simply as SH3) as a GST fusion protein was a kind gift from Dr Jesus Zurdo (University of Cambridge, UK). SH3 was expressed in *E. coli*, purified and cleaved from GST using a 5 ml GSTTrapFF cartridge (GE Healthcare, Sydney, Australia) following the manufacturer's instructions. A $\beta$ <sub>1-42</sub> was purchased from Biopeptide (San Diego, CA, USA), resuspended in HFIP and divided into aliquots in which the solvent was left to evaporate (the peptide 'film' was frozen at - 80 °C). Monoclonal anti-A $\beta$  antibody WO2 (in culture supernatant) was a kind gift from Dr Kevin Barnham (Department of Pathology, University of Melbourne, Australia).

#### 4.3.2 Fibril formation in vitro

SH3 solutions (250  $\mu$ M, unless otherwise indicated), or mixtures of SH3 and clusterin, in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , and 8 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.5) were heated to 60  $^\circ\text{C}$  whilst shaking at 500 rpm for 72 h in a Thermo Finemixer SH2000-DX (Finemould Precision Ind. Co., USA). Directly before use,  $\text{A}\beta_{1-42}$  was resuspended in buffer (two parts 20 mM NaOH, diluted in 7 parts Milli Q water and 1 part 10X PBS) and centrifuged at 13,000 g for 10 minutes to remove any aggregated material.  $\text{A}\beta_{1-42}$  (10  $\mu$ M, unless otherwise indicated), in the presence or absence of clusterin, was incubated at 37  $^\circ\text{C}$  in oxidizing buffer (OB) which consisted of PBS containing 0.9 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 100  $\mu$ M  $\text{CuCl}_2$ , 600  $\mu$ M glycine, pH 7.5, whilst shaking for 8 h in a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Australia). In similar experiments, the relative ability of clusterin to inhibit fibril formation in reactions that were seeded with pre-formed aggregates of the same protein (sampled at different times during a previous aggregation reaction) was examined. SH3 (500  $\mu$ M) and  $\text{A}\beta_{1-42}$  (10  $\mu$ M) were incubated as described above; samples were then taken from each aggregation reaction at various time points and stored frozen at - 20  $^\circ\text{C}$ . These samples were used to seed fibril formation reactions at a final molar ratio of pre-aggregated substrate molecules to monomers of 1:9, in the presence or absence of clusterin (at molar ratios of clusterin:SH3 = 1:100, clusterin: $\text{A}\beta$  = 1:50). The following proteins were all treated as described below, with and without added clusterin. Samples of  $\alpha$ -synuclein ( $\alpha$ -syn, 70  $\mu$ M), lysozyme (lys, 70  $\mu$ M) and  $\kappa$ -casein ( $\kappa$ -cas, 52  $\mu$ M) in 50 mM  $\text{Na}_2\text{HPO}_4$  buffer, pH 7.4, were shaken at 500 rpm and 57  $^\circ\text{C}$  for 192 h, 172 h and 72 h, respectively, in an IKA Vibrax VXR orbital shaker (IKA, USA).  $\beta_2$ -microglobulin ( $\beta_2\text{m}$ , 85  $\mu$ M) in 25 mM sodium acetate, pH 2.1, was shaken for 336 h at 500 rpm and 37  $^\circ\text{C}$  in a Thermo finemixer SH2000-DX. cc $\beta_w$  (60  $\mu$ M) in 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.8, was shaken for 40 min at 37  $^\circ\text{C}$  in a FLUOstar OPTIMA fluorescence plate reader. Calcitonin (Calc, 150  $\mu$ M) was incubated in 50 mM  $\text{Na}_2\text{HPO}_4$  buffer, pH 7.4, at 37  $^\circ\text{C}$  for 20 h. In other experiments, to confirm that clusterin did not form Thioflavin T reactive aggregates under the conditions used, clusterin (at 1.0-12.5  $\mu$ M) was incubated alone in 50 mM  $\text{Na}_2\text{HPO}_4$  buffer, pH 7.4, for 24 h at 37  $^\circ\text{C}$  and up to 192 h at 60  $^\circ\text{C}$ , or in 25 mM sodium acetate, pH 2.1, for 336 h at 37  $^\circ\text{C}$ .

#### 4.3.3 Thioflavin T fluorescence assays

Thioflavin T (50  $\mu$ M) was either added to aliquots of samples taken at specific time points following the initiation of fibril formation (Iys,  $\kappa$ -cas,  $\alpha$ -syn,  $\beta_2$ m, SH3) or to the reaction mixture at the beginning of the time course (calc, A $\beta$  and cc $\beta_w$ ). Fluorescence was measured on a FLUOstar OPTIMA fluorescence plate reader using excitation and emission windows of 450  $\pm$  10 and 490  $\pm$  10 nm, respectively.

#### 4.3.4 Transmission electron microscopy (TEM)

Formvar and carbon-coated nickel electron microscopy grids were prepared by the addition of 2  $\mu$ l of protein sample at a concentration of 1 mg/ml. After several minutes, the grids were washed with 3  $\times$  10  $\mu$ l H<sub>2</sub>O and negatively stained with 10  $\mu$ l of uranyl acetate (2% (w/v), Agar Scientific, UK). The grids were dried with filter paper between each step. Samples were viewed under 20-125 K magnifications at 120 kV excitation voltages using a Philips CM100 transmission electron microscope, and images were analyzed using the SIS Megaview II Image Capture system (Olympus, Germany).

#### 4.3.5 Cytotoxicity

SH-SY5Y cells (a kind gift from Dr Kevin Barnham, Department of Pathology, University of Melbourne, Australia) were cultured at 37 °C and 5 % (v/v) CO<sub>2</sub> in full medium, which consisted of DMEM:F12 medium containing 2.5 % (v/v) fetal bovine serum (FBS) (both from Trace Biosciences, Melbourne, Australia). Cells suspended in full medium were added to a 96 well plate (100  $\mu$ l/well containing 5,000 cells) and left to attach overnight before washing with DMEM:F12. The cells were then cultured as above for 48 h in FBS-free AIM-V medium (Invitrogen, Melbourne, Australia), with or without additives. Fibril formation by SH3 (250  $\mu$ M) or A $\beta$  (10  $\mu$ M) was initiated as described above; in some reactions, clusterin or a control protein (BSA) was included to give molar ratios of clusterin/BSA:substrate of 1:500 (SH3) or 1:10 (A $\beta$ ). To analyse cytotoxicity, aliquots of SH3 and A $\beta$  reactions (taken at 12 h and 2 h, respectively) were added to cells alone to give final concentrations of 10  $\mu$ M and 1  $\mu$ M, respectively, or in some cases (from reactions lacking clusterin/BSA) supplemented with clusterin or BSA to give clusterin/BSA:substrate = 1:10. In other experiments, clusterin or BSA alone were added to cells to give a final concentration of 1.0  $\mu$ M. Calcein-AM was used to

measure cell viability (Lichtenfels *et al.*, 1994). Calcein-AM (1  $\mu$ M) was added to cells and left to incubate for 30 min before analysing fluorescence using a FLUOstar OPTIMA plate reader and excitation and emission windows of 485  $\pm$  10 nm and 520  $\pm$  10 nm, respectively. The significance of differences in fluorescence was assessed using the Student's t test. Calcein-AM is membrane-permeable and non-fluorescent; it is cleaved by esterases in the cytoplasm of viable cells to release fluorescent, membrane-impermeable calcein which remains trapped inside viable cells. Thus the resulting level of cell-associated calcein fluorescence is proportional to the number of viable cells.

#### 4.3.6 Effects of clusterin on the sedimentation properties of substrate protein aggregates

At the conclusion of *in vitro* fibril formation time courses, samples of SH3 and A $\beta$ , with or without clusterin, were centrifuged for 30 min at 10,000 g. The supernatant was removed and the pellet resuspended and subsequently washed repeatedly in PBS. SH3 samples were then analysed by 15% SDS PAGE and stained with Coomassie blue. A $\beta$  samples were analysed by 15% SDS PAGE and subsequent immunoblotting using anti-A $\beta$  monoclonal antibody. The presence of clusterin in supernatant and pelleted fractions was tested by applying these fractions to nitrocellulose membranes which were subsequently blocked with HDC (1% (w/v) heat denatured casein, 0.04% (w/v) thimerosal, in PBS). The presence of clusterin was detected using a mixture of G7, 41D and 78E monoclonal anti-clusterin antibodies (Lakins *et al.*, 2002). Bound anti-A $\beta$  and anti-clusterin antibodies were detected with HRP-conjugated sheep anti-mouse Ig antibody (Silenus, Melbourne, Australia) followed by enhanced chemiluminescence (ECL) with Supersignal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

#### 4.3.7 Detection of stable clusterin-substrate complexes

Samples of SH3 and A $\beta$ , with or without clusterin, taken from the beginning and end of *in vitro* fibril formation time courses were centrifuged for 30 min at 10,000 g. The supernatants were shaken end-over-end for 1 h at room temperature with anti-clusterin antibody coupled to Sepharose beads (G7-Sepharose; 100  $\mu$ l packed volume) (Humphreys *et al.*, 1999). The G7-Sepharose was washed by centrifugation three times



with PBS and then incubated in 2M guanidine HCl in PBS at pH 7.5 for 15 min to elute bound protein. The mixture was then centrifuged using a 0.45  $\mu$ m Ultrafree-MC centrifugal filter device (Millipore, USA) to separate the beads from the eluted proteins, which were subsequently analysed by SDS-PAGE under non-reducing conditions. To detect A $\beta$ , immunoblotting was performed as described above.

#### 4.3.8 Immuno dot blots

Samples of amyloidogenic proteins were taken at various time points during fibril formation and frozen at - 20 °C until required. Samples (1 mg) were spotted on to nitrocellulose membranes (Pall, FL, USA) and allowed to dry; the membranes were then blocked with HDC. The membranes were incubated for 2 h at 37 °C in PBS containing 10 mg/ml clusterin, or control proteins GST and ovalbumin, before being washed with PBS. Bound clusterin was detected using a mixture of G7, 78E and 41D antibodies. Rabbit anti-GST (Silenus, Melbourne, Australia) and anti-ovalbumin (a gift from S. Easterbrook-Smith, University of Sydney) were used to detect any bound control protein. Bound primary antibodies were detected with sheep anti-mouse Ig-HRP or sheep anti-rabbit Ig-HRP (Silenus, Melbourne, Australia) using ECL as described above.

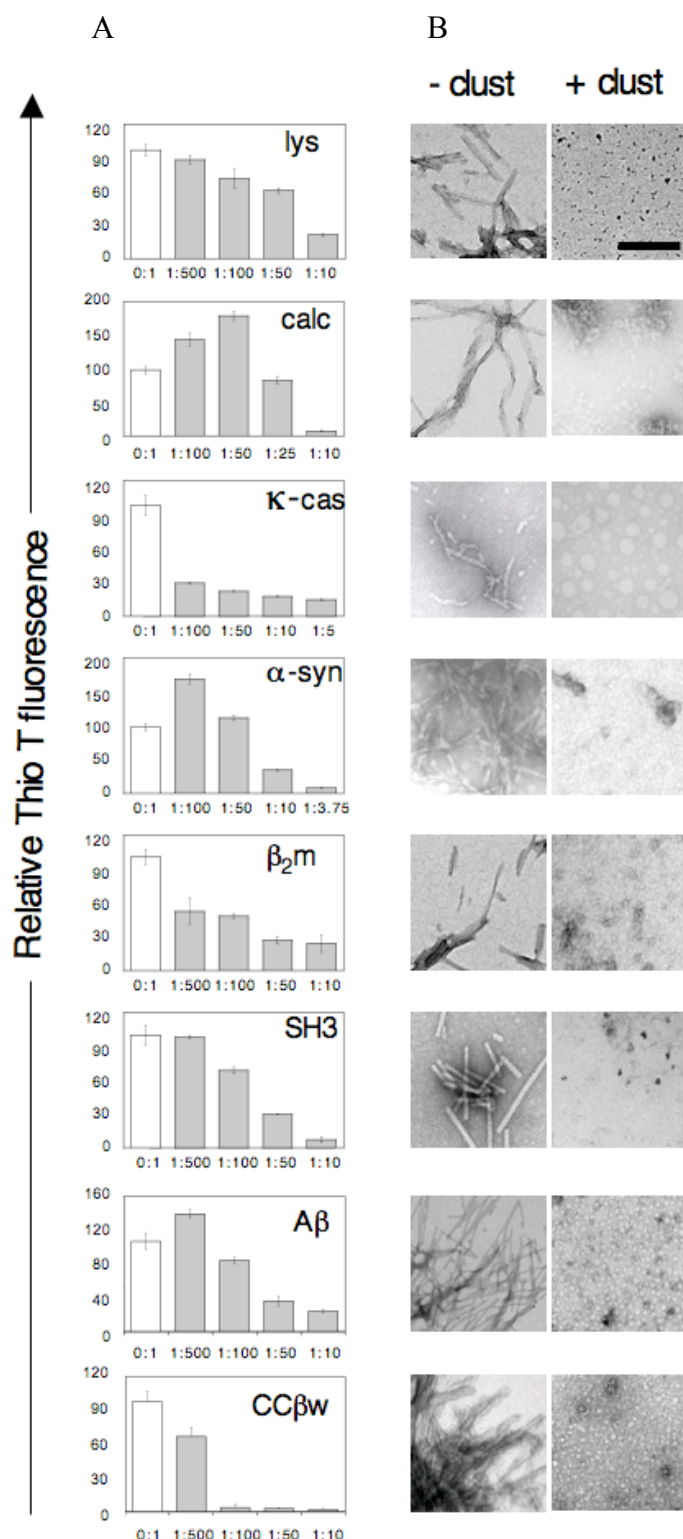
## 4.4 Results

### 4.4.1 Clusterin affects fibril formation in vitro

Under the various conditions used, the aggregates formed from all proteins showed (i) increased thioflavin-T fluorescence (Figure 4.1A), (ii) green birefringence when stained with Congo Red (Figure 4.2), and (iii) fibrillar structures detected by TEM (Figure 4.1B) characteristic of amyloid species. The addition of clusterin to solutions of all proteins tested showed a dose dependent decrease in thioflavin-T fluorescence (Figure 4.1A). However, for calcitonin,  $\alpha$ -synuclein and A $\beta$ , molar clusterin:substrate ratios of 1:50, 1:100 and 1:500, respectively, produced an increase in thioflavin T fluorescence. The minimum molar ratio of clusterin:substrate required to affect the levels of thioflavin T fluorescence differed greatly between the systems tested, which could reflect differences between the substrates and/or the conditions used. A ratio of clusterin:CC $\beta_w$  of only 1:100 was sufficient to reduce thioflavin T fluorescence to almost background levels. Molar ratios of clusterin:substrate needed to inhibit 70-80 % of the thioflavin T fluorescence measured in the absence of clusterin were 1:100 for  $\kappa$ -casein and 1:50 for  $\beta_2$ -microglobulin, SH3 and A $\beta$ . Although under the conditions tested much higher levels of clusterin were needed to similarly inhibit fibril formation of calcitonin, lysozyme and  $\alpha$ -synuclein, even in these cases the effect was markedly sub-stoichiometric (clusterin:substrate  $\sim$  1:10). The presence of a control protein, BSA, had no measurable effect on thioflavin T fluorescence for any of the proteins tested (data not shown). When incubated alone, clusterin did not develop any significant Thioflavin T reactivity under any of the conditions tested (data not shown).

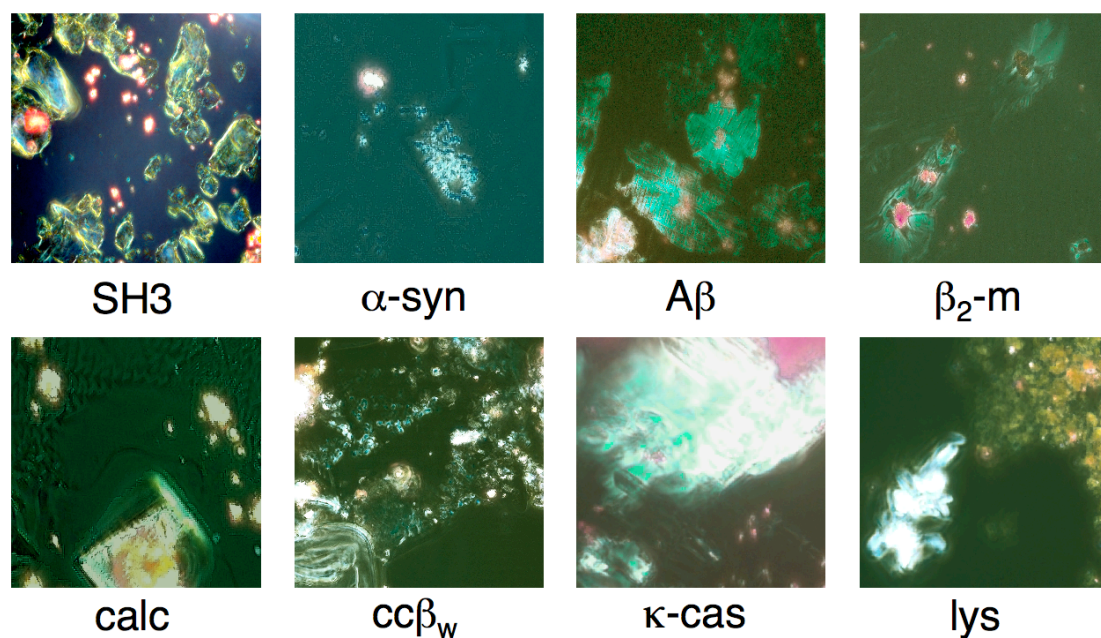
TEM was used to examine the morphology of protein aggregates (at the highest molar ratio of clusterin:substrate shown for each protein in Figure 4.1A). In the absence of clusterin, fibrillar aggregates of dimensions expected for amyloid fibrils in these well characterised systems were observed for all proteins (Figure 4.1B). Under the conditions tested, clusterin inhibited the formation of fibrillar structures in all cases (Figure 4.1B). A variety of structures were observed in reactions containing clusterin including spherical particles of differing diameter (Figure 4.1B;  $\kappa$ -cas, A $\beta$  and CC $\beta_w$ )

and amorphous aggregates (Figure 4.1B; Lys, calc,  $\beta_2$ M and SH3). TEM analysis of samples identified as having increased Thioflavin T fluorescence resulting from a low molar ratio of clusterin:substrate showed them to contain fibrillar aggregates (Figure 4.3).

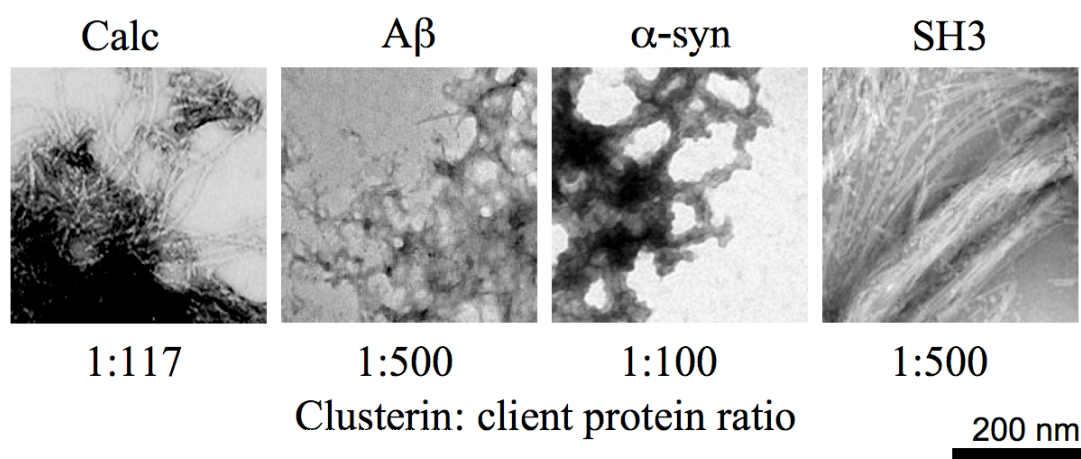


**Figure 4.1 Effect of clusterin on fibril formation.** (A)

Thioflavin T fluorescence (in arbitrary units, AU) of protein species present at the end of aggregation reactions, in the presence (grey bars) or absence (white bars) of various amounts of clusterin (indicated as molar ratios of clusterin:substrate). In each case the data shown are means of triplicates and the error bars are standard errors of the mean (SE). (B) TEM images of final samples taken from aggregation reactions containing either no clusterin (- clust) or the highest molar ratio of clusterin:substrate indicated in (A) (+ clust). In all cases the results shown are representative of two or more individual experiments. The scale bar in the upper right panel applies to all TEM images shown and represents 200 nm.



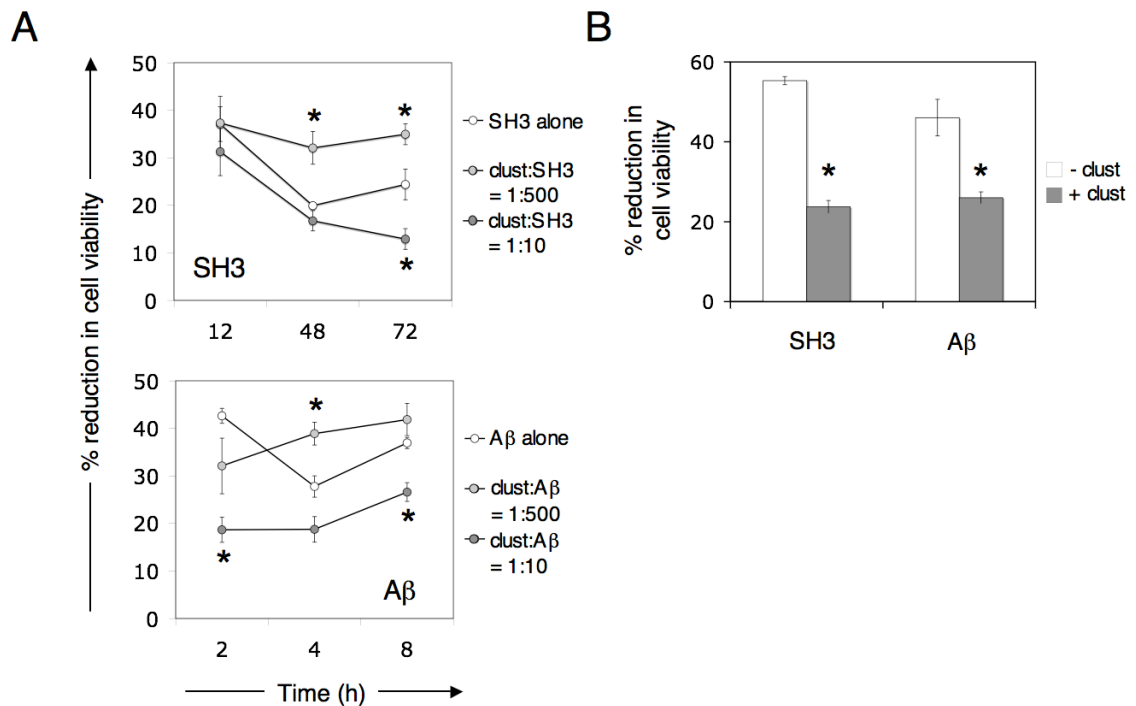
**Figure 4.2** Protein aggregates display green birefringence when stained with Congo red. Light microscopy images of Congo red stained protein aggregates viewed through a cross-polarising filter. Protein aggregates shown were collected at the end of individual time courses in the absence of clusterin.



**Figure 4.3** Effect of low molar ratios of clusterin:substrate on fibril formation. Samples containing low ratios of clusterin:substrate that either promoted an increase in thioflavin T fluorescence or showed no difference to amyloidogenic protein alone (see Figure 4.1) were examined using TEM.

#### 4.4.2 Effects of clusterin on aggregate toxicity

Compared to SH3 alone, at a clusterin:SH3 ratio of 1:10, the toxicity of aggregates generated after 72 h was significantly reduced (Figure 4.4A;  $p < 0.05$ ) but at a clusterin:SH3 ratio of 1:500 the toxicity of aggregates generated after 48 and 72 h was significantly increased (Figure 4.4A;  $p < 0.05$ ). At a clusterin:A $\beta$  1:10 ratio, clusterin significantly suppressed the toxicity of aggregates generated after 2 h and 8 h ( $p < 0.05$ ) but at a clusterin:A $\beta$  1:500 ratio the toxicity of aggregates at 4 h was significantly enhanced ( $p < 0.05$ ). Moreover, when clusterin was added to aggregates of SH3 and A $\beta$  pre-formed in the absence of clusterin, it significantly decreased their cytotoxicity (Figure 4.4B;  $p < 0.001$  and  $p < 0.05$  respectively). Under the conditions tested, the addition of either clusterin or BSA alone to cultures had no significant effect on cell viability (data not shown).



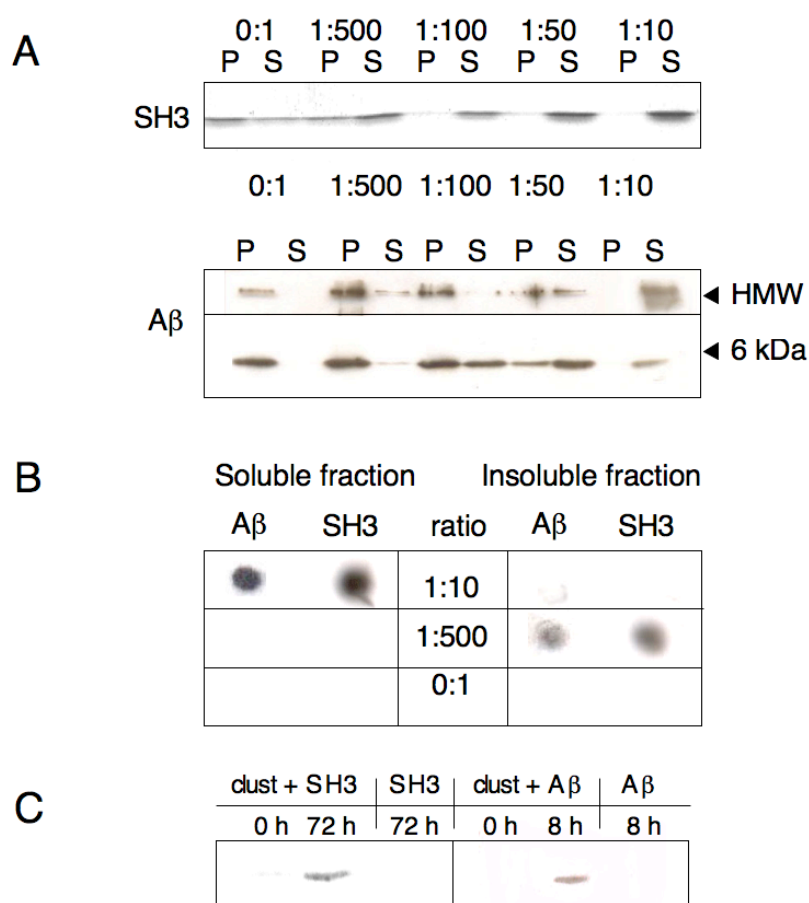
**Figure 4.4 Effects of clusterin on cytotoxicity of SH3 and A $\beta$ .** (A) Reduction in SH-SY5Y cell viability (measured as a decrease in calcein fluorescence; see Section 4.3.5), expressed as a percentage of corresponding values for untreated control cultures, following incubation for 48 h with protein aggregates taken from SH3 and A $\beta$  fibril formation reactions at the times indicated on the x-axis, in the absence or presence of clusterin (see key). Significant differences between the effects of a clusterin-containing sample and the corresponding SH3 or A $\beta$  only sample ( $p < 0.05$ ) are indicated by \*; in each case, the significantly different data point is the one closest to the \*. (B) Changes in the viability of SH-SY5Y cells were

measured as above, 48 h after adding (i) aggregates generated in SH3 or A $\beta$  fibril formation reactions lacking clusterin (- clust), or (ii) the same aggregates added together with clusterin (to give clusterin:SH3 = 1:10 and clusterin:A $\beta$  = 1:10) (+ clust). In (A) and (B), the data points shown represent means of triplicate determinations and the error bars are SE. All results shown are representative of at least two independent experiments.

#### 4.4.3 Clusterin binds amyloid-forming proteins and affects their sedimentation properties

Clusterin, at various ratios relative to the substrate, was added to fibril formation reactions of SH3 and A $\beta$  and at the end of the time course the samples were centrifuged to separate supernatant and pellet fractions. SH3 fractions were analysed by SDS PAGE; in the absence of clusterin most of the SH3 protein was found in the pellet (P) fraction (Figure 4.5A). However, at clusterin:SH3 ratios of 1:100-1:10, almost all the SH3 protein was found in the supernatant (S) fraction. Even at a ratio of clusterin:SH3 of 1:500, most of the SH3 remained in the S fraction (Figure 4.5A). Similarly, immunoblots of A $\beta$  fibril formation reactions showed that the proportion of A $\beta$  in the S fraction increased as the ratio of clusterin:A $\beta$  increased until (at clusterin:A $\beta$  = 1:10) effectively all the A $\beta$  was present in the S fraction (Figure 4.5A). In the absence of clusterin, A $\beta$  was detected as its monomeric form (at about 4.5 kDa) or as SDS-resistant high molecular weight aggregates greater than about 180 kDa in mass (Klug *et al.*, 2003). However, at clusterin:A $\beta$  = 1:10, much of the non-sedimenting A $\beta$  was detected in a broad high molecular weight band, which may represent an SDS-resistant clusterin-A $\beta$  complex (Matsubara *et al.*, 1996). For both SH3 and A $\beta$ , at a clusterin:substrate ratio of 1:10, clusterin was detected only in the S fractions but at a lower ratio (1:500) it was found predominantly in the P fractions (Figure 4.5B). To determine if clusterin was forming stable complexes with the substrate proteins, we immunoadsorbed clusterin (and hence anything to which it was bound) from the initial and the final samples of fibril formation reactions of SH3 and A $\beta$ , and analysed this material by SDS-PAGE and immunoblotting. In fractions prepared from samples of clusterin and substrate protein, prior to incubation, no SH3 or A $\beta$  was detected; similarly, when incubated in the absence of clusterin for the duration of the respective time courses, neither SH3 nor A $\beta$  was bound by immobilised anti-clusterin Ig (Figure 4.5C). However, immunoadsorbed fractions prepared from final samples containing clusterin and substrate protein showed bands

corresponding to SH3 and A $\beta$  (Figure 4.5C). This result demonstrates that clusterin formed stable complexes with the substrate proteins at some point during the time course of the aggregation reactions.



**Figure 4.5 Clusterin reduces the formation of sedimentable aggregates of SH3 and A $\beta$  by forming stable complexes with fibrillogenic intermediates.** (A) Images of Coomassie blue stained SDS-PAGE gel (SH3) and immunoblot (A $\beta$ ) showing supernatant (S) and pellet (P) fractions prepared by centrifugation of final samples taken from fibril formation reactions containing various ratios of clusterin:substrate (indicated above the corresponding lanes). On the A $\beta$  panel, HMW indicates SDS-resistant aggregates migrating at an apparent molecular weight of > 180 kDa; 6 kDa indicates the position of a 6 kDa molecular weight marker. (B) Immuno dot blot detection of clusterin associated with S and P fractions prepared from final samples taken from SH3 and A $\beta$  fibril formation reactions containing various ratios of clusterin:substrate (indicated). (C) Composite image showing proteins that co-precipitate with immunoadsorbed clusterin from initial and final samples of SH3 and A $\beta$  fibril formation reactions (clusterin:SH3 = 1:10, clusterin:A $\beta$  = 1:10). Immunoprecipitations were also performed for final samples of control reactions lacking clusterin. SH3 was detected by Coomassie staining SDS-PAGE gels; A $\beta$  was detected by immunoblotting. In all cases the results shown are representative of two or more individual experiments.

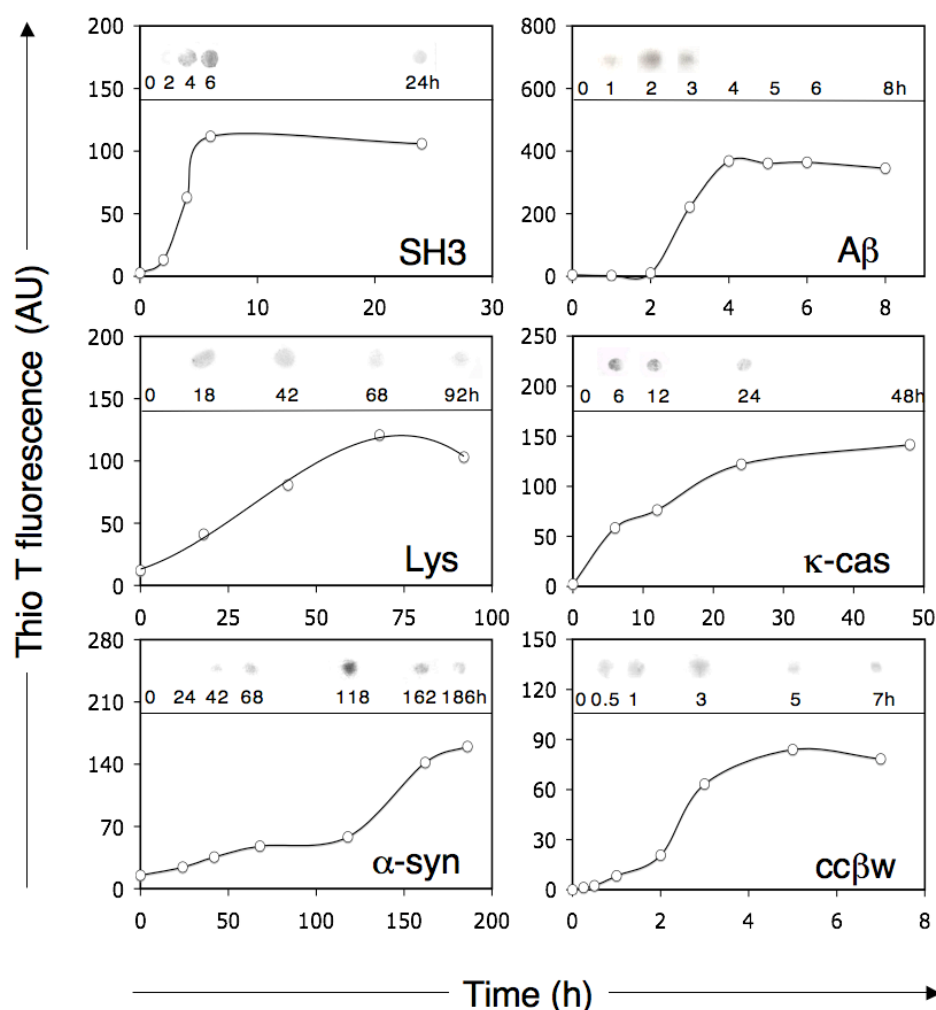
#### 4.4.4 Clusterin binds to intermediate structures on the fibril-forming pathway

Samples taken at different points in time from fibril formation reactions performed (in the absence of clusterin) for the same panel of eight proteins previously examined were spotted on to nitrocellulose membranes; the thioflavin-T reactivity of species present in these reactions was also measured as a function of time (Figure 4.6). When assayed by immuno dot blot, clusterin did not bind to the native proteins present in samples prior to incubation, and bound only weakly or not at all to final samples. Clusterin showed no detectable binding to fibrils after they had been centrifuged and washed (data not shown). In most cases, the strongest binding of clusterin detected was to transient "intermediate" species; in the case of A $\beta$ ,  $\alpha$ -synuclein and cc $\beta$ <sub>w</sub>, maximum binding was detected to species present during the transition between the lag and growth phases (Figure 4.6). For lysozyme and  $\kappa$ -casein, which under these conditions have no detectable lag phase, the strongest binding was to species present early in the growth phase (Figure 4.6). For SH3, the strongest binding was detected at 4-6 h, the initial stage at which Thioflavin T fluorescence reached a maximum value. There was no binding of clusterin detected to species present at any time in  $\beta$ <sub>2</sub>-microglobulin and calcitonin fibril formation reactions (data not shown). In addition, there was no detectable binding of the control proteins GST and ovalbumin to any of the samples of all eight proteins tested (data not shown).

#### 4.4.5 Clusterin affects nucleation and is less effective at suppressing fibril elongation

Increasing the concentration of amyloidogenic proteins will generally favour the self-association of monomers into oligomers able to nucleate the aggregation process. Therefore, in order to test whether clusterin affects nucleation, one clusterin:substrate ratio was selected in each case and the concentration of the substrate protein varied. As expected, as the concentrations of SH3 and A $\beta$  were increased, the level of thioflavin T reactive species increased and the pre-aggregation lag time shortened (Figure 4.7A).





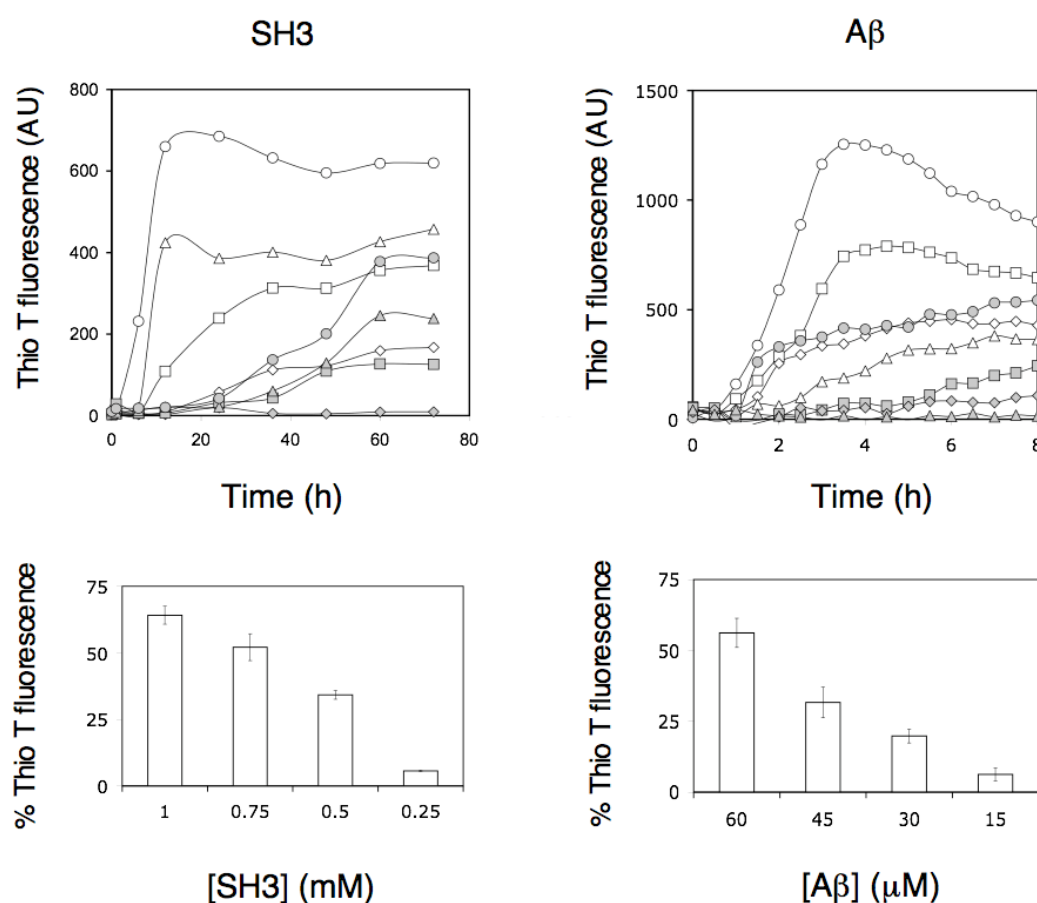
**Figure 4.6 Binding of clusterin to intermediate structures on the fibril forming pathway.** Main panels show the thioflavin-T fluorescence of species present in fibril formation reactions as a function of time. Panel insets show the results of immuno dot blot assays measuring the binding of clusterin to protein species present at different times during fibril formation. In these experiments, the aggregation reactions contained 1 mM SH3 or 10  $\mu$ M A $\beta$ . The results shown are representative of two or more individual experiments.

Clusterin inhibited the development of thioflavin T reactive species in all cases, but as the concentrations of SH3 and A $\beta$  were increased this effect became progressively smaller. For samples containing 0.25 mM SH3, a 1:50 molar ratio of clusterin:SH3 suppressed the thioflavin-T fluorescence measured on the final sample by more than 90 %. However, when SH3 was at a concentration of 1 mM, the same ratio of clusterin:SH3 only decreased thioflavin-T fluorescence by  $\sim$  35% (Figure 4.7B).

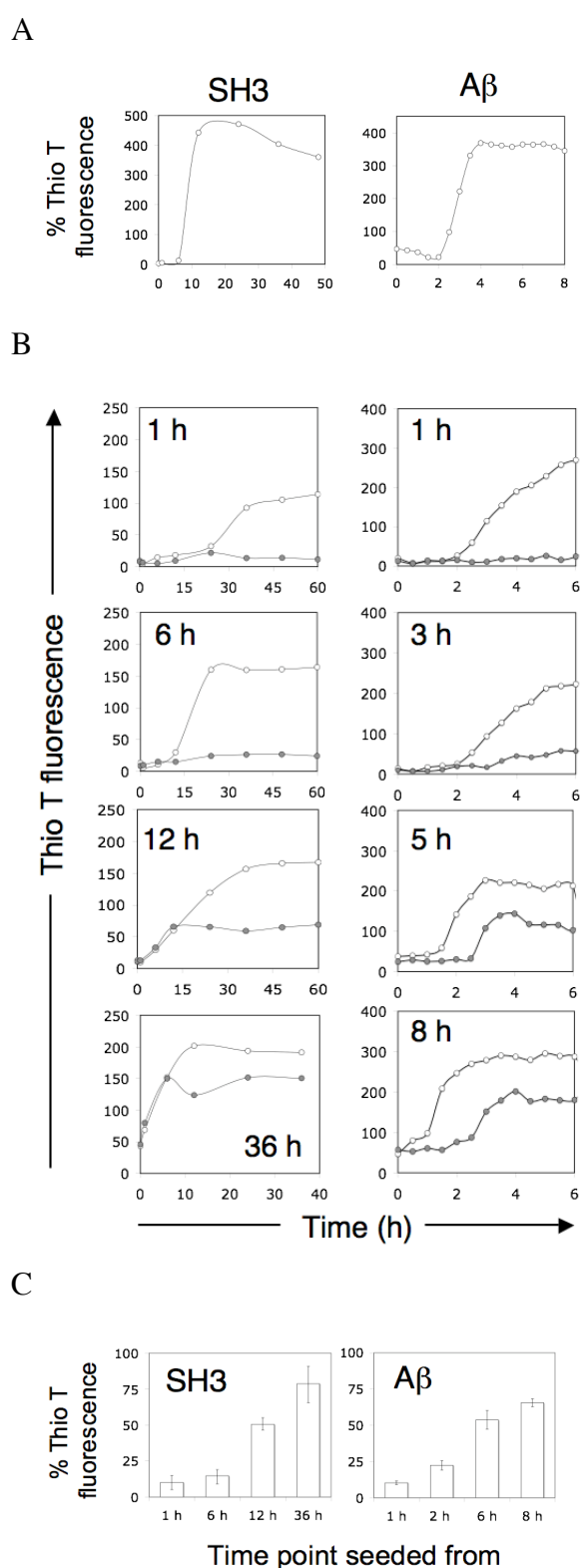
Similarly, a 1:50 molar ratio of clusterin:A $\beta$  suppressed the final thioflavin-T fluorescence by  $\sim 90\%$  for samples containing  $15\ \mu\text{M}$  A $\beta$ , but only by  $\sim 45\%$  when A $\beta$  was at  $60\ \mu\text{M}$  (Figure 4.7B).

As a further means of examining the point at which clusterin exerts its effects on amyloid formation, samples were taken at different times during fibril formation and used to seed amyloid formation in subsequent experiments in the presence or absence of clusterin. Under the conditions used in this study, in the absence of pre-formed amyloid species, SH3 and A $\beta$  exhibited lag phases of  $\sim 6\ \text{h}$  and  $\sim 2\ \text{h}$ , respectively (Figure 4.8A). Note that the kinetics of SH3 aggregation in this reaction (Figure 4.8A) were faster than in most of the subsequent SH3 reactions reported here (Figure 4.8B), as the latter were performed using a lower substrate concentration (see Figure 4.8 legend).

In both the reactions used to generate pre-formed amyloid species, the elongation phase was rapid, after which the level of thioflavin T reactive material remained relatively constant. When samples taken early in these time courses, or from the transition region between lag and growth phases, were used to seed subsequent aggregation reactions, clusterin was able to suppress the generation of thioflavin-T reactive species by up to  $\sim 75 - 90\%$  (Figure 4.8B,C). However, when the samples used to seed the reaction were taken from later points in the initial aggregation time courses, the lag phases of the subsequent aggregation reactions were shortened (Figure 4.8B) and the inhibitory effects of clusterin were significantly less ( $25 - 50\%$  inhibition, Figure 4.7C).



**Figure 4.7 Effect of substrate protein concentration on the relative ability of clusterin to inhibit fibril formation.** (A) Time dependent changes in the thioflavin-T fluorescence of species present in fibril formation reactions containing different concentrations of substrate protein and (where present) a constant clusterin:substrate ratio. Solutions containing SH3 without (empty symbols) or with (solid symbols) clusterin (at clusterin:SH3 = 1:100) were incubated as described in Section 4.3.2 at various concentrations of SH3 (1.0 mM, circles; 0.75 mM, triangles; 0.50 mM, squares; 0.25 mM, diamonds). Similarly, solutions of Aβ without (empty symbols) or with (solid symbols) clusterin (at clusterin:Aβ = 1:50) were incubated at various concentrations of Aβ (15 μM, triangles; 30 μM, diamonds; 45 μM, squares; 60 μM, circles). The data shown here are averages of triplicate measurements and are representative of two individual experiments. (B) Thioflavin T fluorescence of final samples from fibril formation reactions containing clusterin, expressed as a percentage of the respective values for reactions lacking clusterin. In each case, the data shown represent means of triplicate determinations and the error bars are SE.



**Figure 4.8 Effects of clusterin on seeded fibril growth.** (A) Panels showing time dependent changes in the thioflavin-T fluorescence of protein species present in fibril formation reactions in which the concentrations of SH3 and A $\beta$  were 500  $\mu$ M and 10  $\mu$ M, respectively. Aliquots taken at different times from these reactions were used to seed similar reactions (shown in (B)). (B) Thioflavin-T fluorescence as a function of time of protein species in fibril formation reactions that, at zero time, were seeded with pre-formed aggregates taken at the times indicated from the aggregation mixtures represented in (A). These reactions contained 250  $\mu$ M SH3 or 10  $\mu$ M A $\beta$  and were conducted in the absence (empty symbols) or presence of clusterin (solid symbols; clusterin:substrate = 1:100 and 1:50, respectively). Unseeded aggregation reactions had similar timecourses to those of 1 h seeded reactions (data not shown). (C) Thioflavin T fluorescence of final samples taken from fibril formation reactions containing clusterin, expressed as a percentage of the respective values for reactions lacking clusterin (calculated from results such as those shown in (B)). In each case, the data shown represent means of duplicate determinations and the error bars are ranges. The data shown are representative of at least two independent experiments.

## 4.5 Discussion

Previous work indicated that clusterin can inhibit the *in vitro* formation of amyloid aggregates by the prion protein, A $\beta$  and apolipoprotein C-II (Oda *et al.*, 1995; McHattie and Edington 1999; Hatters *et al.*, 2002). It has been shown here that clusterin can potentially inhibit *in vitro* amyloid formation by a broad range of unrelated proteins, suggesting that this activity is not strongly dependent on the identity of the specific polypeptide substrate or whether it is associated with disease (for a summary of results see Tables 4.2 and 4.3). In all cases tested, significantly sub-stoichiometric ratios of clusterin:substrate (from 1:10 to 1:3.5) completely inhibited the formation of fibrils detectable by fluorescence and TEM (Figure 1B). Interestingly, for three of the eight substrate proteins tested (calcitonin,  $\alpha$ -synuclein and A $\beta$ ), when clusterin was present at very low levels relative to the substrate it significantly increased the level of thioflavin-T reactive material formed (Figure 4.1A). One possible explanation is that, at least for calcitonin,  $\alpha$ -synuclein and A $\beta$ , when present at very low levels, clusterin may facilitate amyloid formation by stabilizing the otherwise unstable protein aggregates required to initiate fibril formation.

**Table 4.2. Summary of the effects of clusterin on amyloid formation by eight different proteins.**

Effect	lys	calc	$\kappa$ -cas	$\alpha$ -syn	$\beta_2$ m	SH3	A $\beta$	cc $\beta$ w
Dose dependant decrease in thioT fluorescence	yes	yes	yes	yes	yes	yes	yes	yes
Increased thio T fluorescence at low clusterin:substrate*	no	yes	no	yes	no	no	yes	no
Inhibition of fibril formation at high clusterin:substrate**	yes	yes	yes	yes	yes	yes	yes	yes
Clusterin binding to transient intermediates detected	yes	no	yes	yes	no	yes	yes	yes

\*Low clusterin:substrate ratio varies between substrates but is in the range of (1:50 – 1:500). \*\* High clusterin:substrate ratios are equal to or greater than 1:10.

Given the demonstration that, under differing conditions, clusterin exerts variable effects on amyloid fibril formation, we explored whether this is also true for its effects on amyloid toxicity. Both SH3 and A $\beta$  are known to generate cytotoxic intermediates during amyloidogenesis (Bucciantini *et al.*, 2002; Dahlgren *et al.*, 2002). For both these proteins, at least for some of the time points tested, clusterin enhanced the cytotoxicity of aggregates formed when present during fibril formation at a clusterin:substrate ratio of 1:500, but had the opposite effect when present at the much higher but still substoichiometric ratio of 1:10 (Figure 4.4A). These results clearly indicate that the effects of clusterin on the cytotoxicity of aggregates are complex and depend on both the stage of amyloid formation at which the aggregates are formed and on the clusterin:substrate ratio. Regions of exposed hydrophobicity have been strongly implicated in the cytotoxicity of protein aggregates (Chiti and Dobson 2006). The enhancement of toxicity seen at very low clusterin:substrate ratios may result from there being sufficient clusterin present under these conditions to physically stabilize in solution aggregates bearing multiple hydrophobic surfaces, but insufficient clusterin to bind to and inhibit the cytotoxicity of all hydrophobic surfaces. If this is correct, then it would be expected that at higher clusterin:substrate ratios clusterin would be more effective at masking exposed hydrophobicity on protein aggregates and reducing their cytotoxicity. This was found to be the case, both when clusterin was present during amyloid formation and when it was added to aggregates of SH3 and A $\beta$  pre-formed in its absence (see clusterin:substrate = 1:10 data in Figure 4.4A, and Figure 4.4B). Earlier studies have reported that clusterin enhances (Boggs *et al.*, 1996) or suppresses (Oda *et al.*, 1995) the cytotoxicity of A $\beta$ ; but the conditions used in each of these studies differed greatly, including both the type of peptide (A $\beta$ <sub>1-40</sub> vs A $\beta$ <sub>1-42</sub>), whether or not clusterin was pre-incubated with A $\beta$ , and the effective ratios of clusterin:A $\beta$  tested. The results of the present study show that very different effects can be observed under different experimental conditions, suggesting that the findings of both earlier studies may be broadly correct.

**Table 4.3. Summary of additional results for A $\beta$  and SH3 relating to the effects of clusterin on the toxicity associated with amyloid formation and the mechanism of clusterin action.**

Effect	SH3	A $\beta$
cytoprotection (at high clusterin:substrate)	late*	yes
cytotoxicity (at low clusterin:substrate)	yes	mid*
clusterin retained in soluble fraction	yes	yes
inhibition of seeding by early aggregates	yes	yes
more efficiently inhibits fibril formation at low substrate concentrations	yes	yes

\*Refers to the stage of the time course, late for SH3 is 72 h and mid for A $\beta$  is 4 h.

When increasing amounts of clusterin were added to fibril formation reactions containing either SH3 or A $\beta$ , a progressive increase in the proportion of substrate protein present in the non-sedimenting supernatant (S) fraction at the end of the time course was observed (Figure 4.5A). At a "high" clusterin:substrate ratio (1:10) the substrate remained in the S fraction but at a much lower ratio (1:500) it was incorporated into the sedimenting pellet (P) fraction (Figure 4.5B). We found that clusterin did not detectably bind to washed, preformed fibrils formed from any of the eight substrate proteins tested (data not shown). These results suggest that under amyloidogenic conditions, as is the case under conditions when amorphous aggregates are formed (Humphreys *et al.*, 1999), clusterin can enhance substrate protein solubility even when present at significantly sub-stoichiometric ratios. In the case of proteins forming amorphous aggregates, clusterin appears to exert this effect by promoting the formation of soluble high molecular weight complexes with substrate molecules present in non-native conformations (Humphreys *et al.*, 1999). The presence of clusterin and substrate molecules in supernatant fractions prepared from amyloid-forming reactions in which clusterin had inhibited fibril formation (Figure 4.5B) suggests that similar clusterin-substrate complexes might be forming under these conditions. This was confirmed for both SH3 and A $\beta$  substrates by immunoprecipitation analyses (Figure 4.5C).

Clusterin inhibits the amorphous aggregation of a variety of proteins *in vitro* when present at molar clusterin:substrate ratios of 1:5.5 - 1:0.65 (Humphreys *et al.*, 1999); this action appears to be a consequence of clusterin binding to regions of exposed hydrophobicity on substrate molecules (Poon *et al.*, 2002a). In contrast, data presented here and elsewhere (Hughes *et al.*, 1998; Hatters *et al.*, 2002), show that clusterin can influence amyloid formation at much lower clusterin:substrate ratios (as low as 1:500). The apparent difference in stoichiometry between the effects of clusterin on protein aggregation producing amorphous versus amyloid type aggregates is likely to relate to the low concentration of protein oligomers that nucleate fibril formation thought to be present in amyloid-forming reactions (Harper and Lansbury 1997). The very low clusterin:substrate ratios that affect the amyloid-forming pathway are consistent with clusterin interacting with species present in low abundance such as those that nucleate growth, or their precursors. Immuno dot blot analyses showed that for most proteins tested clusterin bound most strongly to transient protein species that are presumably more abundant during fibril nucleation and/or growth (Figure 4.6). It is not clear why binding to corresponding species present in calcitonin and  $\beta_2m$  fibril formation reactions was not detected, although one possibility is that the interacting species in these cases are at very low levels. In the case of SH3, maximum binding was detected to species present at 4-6 h, corresponding to the initial stage at which maximum Thioflavin T fluorescence was obtained. Electron microscopic examination of these samples indicated that they consisted of a mixture of spherical and short fibrillar aggregates (data not shown). Thus, in this case also, it is feasible that clusterin was binding to transient intermediate species. In the case of SH3 and A $\beta$  it was shown that on a molar basis clusterin suppressed amyloid formation more efficiently at lower substrate concentrations (when self association of substrate molecules with non-native conformations into oligomers is less favoured; Figure 4.7). Similarly, clusterin appears more efficient at inhibiting fibril formation reactions which are seeded with species taken from early time points of preceding reactions, compared with those seeded with species taken from later time points (Figure 4.8). These results suggest that clusterin primarily exerts its effects on amyloid formation by interacting with transient protein species that are most abundant prior to fibril elongation; these interacting species are



probably small oligomers that are either structural precursors to, or fully functional, aggregation nuclei.

Collectively, our results indicate that clusterin, at sub-stoichiometric levels, exerts substantial effects on *in vitro* amyloid formation by a broad variety of substrate proteins, and suggest that these effects result from interactions with pre-fibrillar species at very low abundance. At least *in vitro*, clusterin does not bind detectably to either native substrate proteins or mature amyloid fibrils. The relatively low level of substrate specificity characterising this action suggests that clusterin interacts with species sharing common structural features present on the amyloid forming pathways of many different proteins. We have shown that clusterin forms stable complexes with fibril-forming proteins and reduces their propensity to sediment from solution. Our results, and those from a recent study using human lysozyme as a substrate protein (Kumita *et al.*, 2007), suggest that clusterin does not bind to native or non-native monomers but instead support the hypothesis that clusterin interacts with oligomeric species that may function as nucleation points for fibril formation. Our results clearly show that an important determinant of the nature and extent of the effects of clusterin on both amyloid formation and toxicity is the clusterin:substrate ratio. This conclusion has important potential implications for the likely effects of clusterin on amyloid formation *in vivo*, as clusterin may exert differing effects on the extracellular folding landscape depending on its relative abundance compared to amyloid forming substrate proteins in particular biological contexts. For example, although clusterin is present in human plasma at about 100 µg/ml (Morrissey *et al.*, 2001), in cerebrospinal fluid it is present at only about 2 µg/ml (Calero *et al.*, 2000). It is therefore feasible that in the central nervous system, clusterin might under some conditions enhance amyloid formation and toxicity (DeMattos *et al.*, 2002) and yet elsewhere in the body where it is more abundant (e.g. in plasma), exert the opposite effect. Also, the evidence that clusterin does not bind to mature amyloid fibrils, but at very low clusterin:substrate ratios is incorporated into insoluble amyloid material, provides a tenable explanation for the otherwise curious observation that clusterin is found associated with a variety of disease-related amyloid deposits *in vivo* (Table 4.1). It appears likely that the association of clusterin with these deposits represents its failed attempts to maintain the

solubility of amyloid-forming species under disease-specific conditions of very high molar substrate excess.

In conclusion, the abundant extracellular chaperone clusterin exerts potent effects on the formation of amyloid *in vitro* by a wide range of proteins, including examples known to be associated with disease and others that are not. The nature and extent of these effects show a limited dependence on the identity of the substrate protein but in contrast vary with the clusterin:substrate ratio. Clusterin appears to interact with pre-fibrillar species that share common structural features and depending on the prevailing conditions, can either promote or suppress amyloid formation and toxicity. However, in all cases tested, at molar ratios of clusterin:substrate of 1:10 or greater, clusterin potently inhibited amyloid formation and provided substantial cytoprotection. These findings suggest that clusterin may be an important part of an armoury of mechanisms that defend against the consequences of extracellular protein misfolding. They also raise the possibility that, at least in some circumstances, increasing the levels of clusterin *in vivo* could be a therapeutic tool in the fight against extracellular protein deposition disorders.

Chapter 5

The Extracellular Chaperones  $\alpha_2$ -Macroglobulin and Haptoglobin  
Suppress Amyloid Formation by Interacting with Intermediate  
Species.

**Preface**

The initial experimental chapters of this thesis demonstrate that  $\alpha_2$ -macroglobulin and haptoglobin have the ability to chaperone amorphously aggregating proteins. However, their effects on amyloid formation were almost unknown. This work set out to systematically examine the effect of  $\alpha_2$ -macroglobulin and haptoglobin on amyloid formation using a panel of unrelated proteins and peptides. Some of the work, in particular work on human lysozyme, was performed by collaborators at the University of Cambridge.

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## 5.1 Introduction

The pathology of more than 40 human degenerative diseases is associated with the deposition of fibrillar proteinaceous aggregates known as amyloid. These *protein deposition diseases* affect many of the tissues and organs of the human body and are comprised of various sporadic, familial (e.g. Alzheimer's (AD) and Parkinson's (PD) diseases), and transmissible neurodegenerative disorders (e.g. spongiform encephalopathies). It is thought that proteins aggregate and form deposits when the normally efficient protein quality control machinery is overwhelmed (Muchowski and Wacker 2005). Much current research is focussed on the protein quality control machinery and the role it plays in these disorders. 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 has only recently been proposed that extracellular counterparts to these exist (Yerbury *et al.*, 2005b). One such extracellular chaperone, clusterin, has been shown to potently inhibit amyloid formation when it was present during the early stages of fibrillogenesis (Hughes *et al.*, 1998; Hatters *et al.*, 2002; Kumita *et al.*, 2007; Yerbury *et al.*, 2007). Clusterin appears not to bind to the native form of the substrates tested, nor does it detectably bind to mature fibrils, but appears to bind some species important to the nucleation event (*see section 1.3.3.1 How is amyloid formed*) which results in inhibition of fibril formation (Kumita *et al.*, 2007; Yerbury *et al.*, 2007). Recently, two other secreted glycoproteins, haptoglobin and  $\alpha_2$ -macroglobulin, have been shown to have small heat shock protein-like chaperone activity and to suppress the amorphous aggregation of a range of unrelated proteins (Yerbury *et al.*, 2005a; French *et al.*, 2008). The role of these abundant extracellular chaperones (ECs) in the formation of amyloid deposits *in vivo* is emerging as an exciting field.

$\alpha_2$ -Macroglobulin ( $\alpha_2$ M) is an abundant human blood glycoprotein, comprised of ~10% carbohydrate by mass (Bowman and Kurosky 1982). It is best known for its ability to inhibit a broad range of proteases. Upon interaction with a protease,  $\alpha_2$ M undergoes limited proteolysis at its bait region leading to a major conformational change,

physically trapping the protease within a steric “cage” (Sottrup-Jensen 1989). The trapped protease forms a covalent linkage with  $\alpha_2$ M by reacting with an intramolecular thiol ester bond to yield a conformationally altered form known as “activated” or “fast”  $\alpha_2$ M ( $\alpha_2$ M\*), which exposes a receptor recognition site for low density lipoprotein receptor related protein (LRP) (Sottrup-Jensen 1989). Small nucleophiles such as methylamine can also activate  $\alpha_2$ M by directly interacting with the thiol ester bond (Imber and Pizzo 1981).

Aside from its interactions with proteases,  $\alpha_2$ M binds to a wide range of ligands including those associated with protein deposition disorders.  $\alpha_2$ M binds A $\beta$  peptide (Narita *et al.*, 1997), prion protein (Adler and Kryukov 2007) and  $\beta_2$ -microglobulin (Motomiya *et al.*, 2003), which are associated with Alzheimer’s disease, spongiform encephalopathies and dialysis related amyloidosis, respectively. In addition,  $\alpha_2$ M binds to cytokines and growth factors (Mettenburg *et al.*, 2002), and to a range of hydrophobic molecules including endotoxin, phenyl-Sepharose, and liposomes (Barrett 1981). The binding to hydrophobic molecules does not inhibit the trapping of proteases and is not known to be associated with any conformational changes (Barrett 1981).  $\alpha_2$ M is found associated with amyloid deposits in AD and spongiform encephalopathies (Fabrizi *et al.*, 2001; Adler and Kryukov 2007). Previous work has indicated that  $\alpha_2$ M can inhibit the formation of amyloid fibrils by A $\beta$  peptide (Hughes *et al.*, 1998) and protect cells from A $\beta$  toxicity in an LRP dependant fashion (Du *et al.*, 1997). Whether the anti-amyloidogenic action of  $\alpha_2$ M applies to other amyloid forming proteins has not been tested. It was recently shown that  $\alpha_2$ M has a promiscuous ATP-independent chaperone action similar to that of clusterin (French *et al.*, 2008). It forms stable complexes with misfolded proteins and maintains their solubility but is unable to effect their refolding (French *et al.*, 2008).

Haptoglobin (Hp) is a secreted acidic glycoprotein produced mainly in the liver and found in most body 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" (Bowman and Kurosky 1982; Dobryszczycka 1997). In humans, a crossing-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 ( $\alpha^1\beta$ )<sub>2</sub> structure (~100 kDa). However, in Hp 2-1 and Hp 2-2, an additional cysteine residue in the  $\alpha^2$  chain allows the formation of a series of various sized disulfide-linked  $\alpha\beta$  polymers (~100 to ~500 kDa). Hp is most well known for its high affinity binding to hemoglobin (Hb) ( $K_D \sim 10^{-15}$  M) (Bowman and Kurosky 1982). Formation of the Hp-Hb complex inhibits Hb-mediated generation of lipid peroxides and hydroxyl radical, which is thought to occur in areas of inflammation (Dobryszczycka 1997). When complexed to Hb, Hp is known to be recognized by the cell surface receptors CD163 and Mac-1 and taken up by receptor-mediated endocytosis for degradation (Graversen *et al.*, 2002). Hp has also been implicated in immune regulation (Louagie *et al.*, 1993) and shown to inhibit cathepsin B activity (Snellman and Sylven 1967). Taken together, the available evidence indicates that Hp is likely to play an important role in suppressing inflammatory responses.

Human Hp specifically inhibits the precipitation of a wide variety of proteins induced by a range of stresses (Pavlicek and Ettrich 1999; Yerbury *et al.*, 2005a). Like clusterin, Hp forms stable, 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 increased the amount of protein that precipitated in response to stresses (Yerbury *et al.*, 2005a). 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 $\beta$  amyloid deposits *in vivo* (Powers *et al.*, 1981).

The aims of the current study were to determine if  $\alpha_2$ M and Hp could affect the *in vitro* formation of amyloid by a range of unrelated proteins and, if so, to characterise the mechanism(s) involved. We selected three amyloid forming substrates that are associated with disease (A $\beta_{1-42}$ , calcitonin, and lysozyme associated with Alzheimer's disease, localized amyloidosis and familial systemic amyloidosis respectively) and one other that is not (amyloidogenic peptide cc $\beta_w$ ).

## 5.2 Materials and Methods

### 5.2.1 Materials

$\alpha_2$ M and Hp were purified from human serum obtained from Wollongong Hospital (Wollongong, NSW, Australia) as previously described (Yerbury *et al.*, 2005a; French *et al.*, 2008). Thioflavin T, hexafluoroisopropanol (HFIP),  $\kappa$ -casein, and bovine serum albumin (BSA) were purchased from Sigma (MO, USA).  $\alpha$ -synuclein was expressed in *Escherichia coli* and purified by acid precipitation as described (Souza *et al.*, 2000). Calcitonin and the short coiled-coil  $\beta$  (cc $\beta_w$ ) peptide, which transforms from a helical conformation at 20 °C into amyloid fibrils at 37 °C (Yerbury *et al.*, 2007), were purchased from Auspep (Melbourne, Australia). A $\beta_{1-42}$  was purchased from Biopeptide (San Diego, CA, USA), resuspended in HFIP and divided into aliquots in which the solvent was left to evaporate (the peptide ‘film’ was frozen at - 80 °C). The non-natural variant of human lysozyme, I59T, was expressed and purified as described (Kumita *et al.*, 2006). Monoclonal anti-A $\beta$  antibody WO2 supernatant was a kind gift from Dr Kevin Barnham (Department of Pathology, University of Melbourne, Australia).

### 5.2.2 Fibril formation in vitro

Immediately before use, A $\beta_{1-42}$  was resuspended in buffer (two parts 20 mM NaOH, diluted in 7 parts Milli Q water and 1 part 10X PBS) and to remove any aggregated material was centrifuged at 13,000 g for 10 minutes. A $\beta_{1-42}$  (10  $\mu$ M), in the presence or absence of either Hp or  $\alpha_2$ M, was incubated at 37 °C in oxidizing buffer (OB; PBS containing 0.9 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 100  $\mu$ M CuCl<sub>2</sub>, 600  $\mu$ M glycine, pH 7.5), whilst shaking for 8 h in a 384 well plate using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Australia). cc $\beta_w$  (60  $\mu$ M) in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8, was shaken for 300 min at 37 °C in a 384 well plate using a FLUOstar OPTIMA fluorescence plate reader. Calcitonin (Calc, 150  $\mu$ M) was incubated in 50 mM Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.4, at 37 °C for 300 min. Fibril formation of A $\beta$ , cc $\beta_w$  and calcitonin was measured as *in situ* thioflavin T fluorescence using a FLUOstar OPTIMA with an excitation wavelength of 440 nm (slit-width 10 nm) and an emission scan from 450–600 nm (slit-width 10 nm). The non-natural lysozyme variant, I59T (6.8  $\mu$ M, 0.1 M sodium

citrate buffer, pH 5.0) was incubated at 60 °C with stirring and light-scattering was monitored at 500 nm with slit-widths of 5 nm in a Cary Eclipse spectrofluorimeter (Varian Ltd., Oxford UK). Samples of  $\alpha$ -synuclein ( $\alpha$ -syn, 70  $\mu$ M) and  $\kappa$ -casein ( $\kappa$ -cas, 52  $\mu$ M) in 50 mM Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.4, in the absence or presence of Hp, were shaken in 1.5 ml microfuge tubes using an IKA Vibrax VXR orbital shaker (IKA Works, Inc., Wilmington, NC, USA) at 57 °C for 192 h and 72 h respectively. To confirm that Hp and  $\alpha_2$ M did not form Thioflavin T reactive aggregates under the conditions used, they (at 1.0-12.5  $\mu$ M) were incubated alone in 50 mM Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.4, for 24 h at 37 °C, with or without OB and up to 192 h at 60 °C.

### 5.2.3 Thioflavin T fluorescence assays

Thioflavin T (fresh stock solutions prepared daily in PBS and passed through 0.22  $\mu$ m pore size filters) was added to a final concentration of 50 mM to aliquots of samples taken at specific time points during reaction time courses. Thio-T binding was measured in a Cary Eclipse spectrofluorimeter or FLUOstar OPTIMA with an excitation wavelength of 440 nm (slit-width 10 nm) and an emission scan from 450–600 nm (slit-width 10 nm).

### 5.2.4 Transmission electron microscopy (TEM)

Samples 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 was used to view samples under 20-125 K magnifications at 80 kV excitation voltages. Images were analyzed using the SIS Megaview II Image Capture system (Olympus, Germany).

### 5.2.5 Effects of $\alpha_2$ M and Hp on the sedimentation properties of substrate protein aggregates

At the conclusion of *in vitro* fibril formation time courses, samples of A $\beta$  and calcitonin, with or without  $\alpha_2$ 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 $\beta$  samples were analysed by 15% SDS PAGE and subsequent immunoblotting using WO2 (anti-A $\beta$  monoclonal antibody). Calcitonin samples were



analysed by applying samples to nitrocellulose membrane (Pall, FL, USA) before blocking and probing with anti-calcitonin polyclonal antibodies (Abcam, Sydney, Australia). Bound specific antibodies were detected with either HRP-conjugated sheep anti-mouse Ig or sheep anti-rabbit Ig antibodies (Silenus, Melbourne, Australia) followed by enhanced chemiluminescence (ECL) with Supersignal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

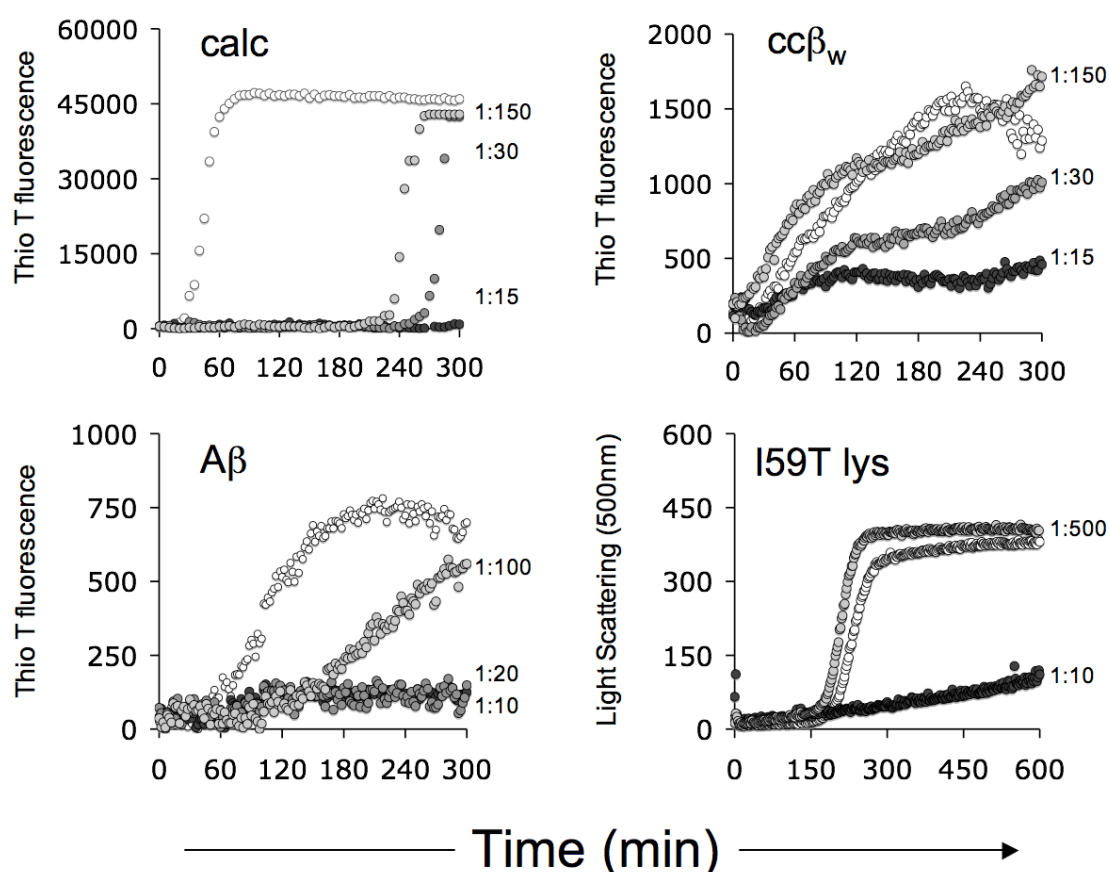
#### 5.2.6 Immuno dot blots

Samples of amyloidogenic proteins were taken at various time points during fibril formation and frozen at - 20 °C until required. Samples (1 µg) were spotted on to nitrocellulose membranes (Pall, FL, USA) and allowed to dry, the membranes were then blocked with HDC. The membranes were incubated for 2 h at 37 °C in PBS containing 10 µg/ml Hp or  $\alpha_2$ M, or control protein GST, before being washed with PBS. Bound Hp and  $\alpha_2$ M were detected using specific rabbit polyclonal antibodies (Sigma and DAKO respectively). Rabbit anti-GST (Silenus, Melbourne, Australia) was used to detect any bound control protein. Bound primary antibodies were detected with sheep anti-mouse Ig-HRP or sheep anti-rabbit Ig-HRP (Silenus, Melbourne, Australia) using ECL as described above.

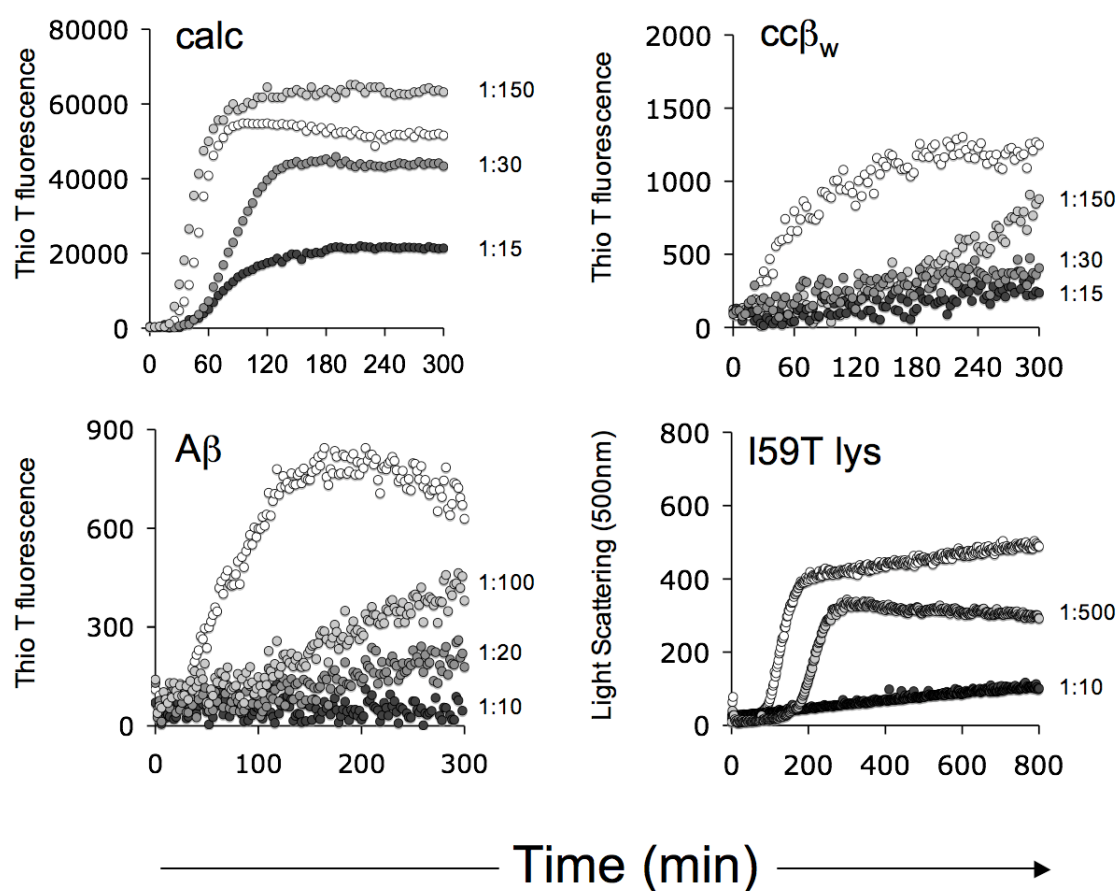
## 5.3 Results

### 5.3.1 The extracellular chaperones $\alpha_2$ M and Hp suppress fibril formation in vitro

The amyloid forming substrates A $\beta$ , cc $\beta_w$ , calcitonin and lysozyme were chosen to reflect a cross section of amyloid forming conditions and cover variables such as whether the substrate is a peptide (A $\beta$ , cc $\beta_w$ , calcitonin) or globular protein (lysozyme), or whether it is disease associated (A $\beta$ , calcitonin, lysozyme) or not (cc $\beta_w$ ). In addition, physiologically-relevant conditions of pH (Ab, cc $\beta_w$ , calcitonin), temperature (A $\beta$ , cc $\beta_w$ , calcitonin) and oxidative stress (A $\beta$ ) were used. In the absence of the extracellular chaperones  $\alpha_2$ M and Hp, aggregation of all substrates tested followed a typical amyloid formation timecourse with a lag phase followed by a rapid growth phase, which eventually approached a plateau. The lag phases varied for each specific substrate being 30, 30, 50 and 150 min for calcitonin, cc $\beta_w$ , A $\beta$  and I59T lys, respectively. Hp dose-dependently inhibited the aggregation of all substrates tested (Figure 5.1). In some cases (calcitonin and A $\beta$ ), although the addition of Hp did not alter the amount of aggregation (Hp:calc ratio of 1:150 and 1:30; Hp:A $\beta$  ratio of 1:100), the lag phase was greatly extended suggesting that Hp affected the nucleation step (Figure 5.1; calc and A $\beta$ ). In each case a substoichiometric ratio of Hp:substrate was enough to inhibit almost all the aggregation (calc and cc $\beta_w$ ; 1:15 and A $\beta$  and I59T lys; 1:10). Similarly the addition of  $\alpha_2$ M to all amyloid forming reactions decreased the amount of measured aggregation in each case (Figure 5.2). As for Hp, the lag phase was extended in all reactions even though the final amount of aggregation was not always greatly reduced (Figure 5.2; cc $\beta_w$ , A $\beta$ , I59T lys). In addition, a substoichiometric ratio of  $\alpha_2$ M:substrate was enough to inhibit almost all aggregation in each case (calc and cc $\beta_w$ ; 1:15 and A $\beta$  and I59T lys; 1:10). The presence of control protein BSA in the amyloid forming reactions had no effect on aggregation for any of the substrates tested (Figure 5.3). In addition, when incubated alone, neither Hp nor  $\alpha_2$ M aggregated under any of the conditions tested (data not shown).



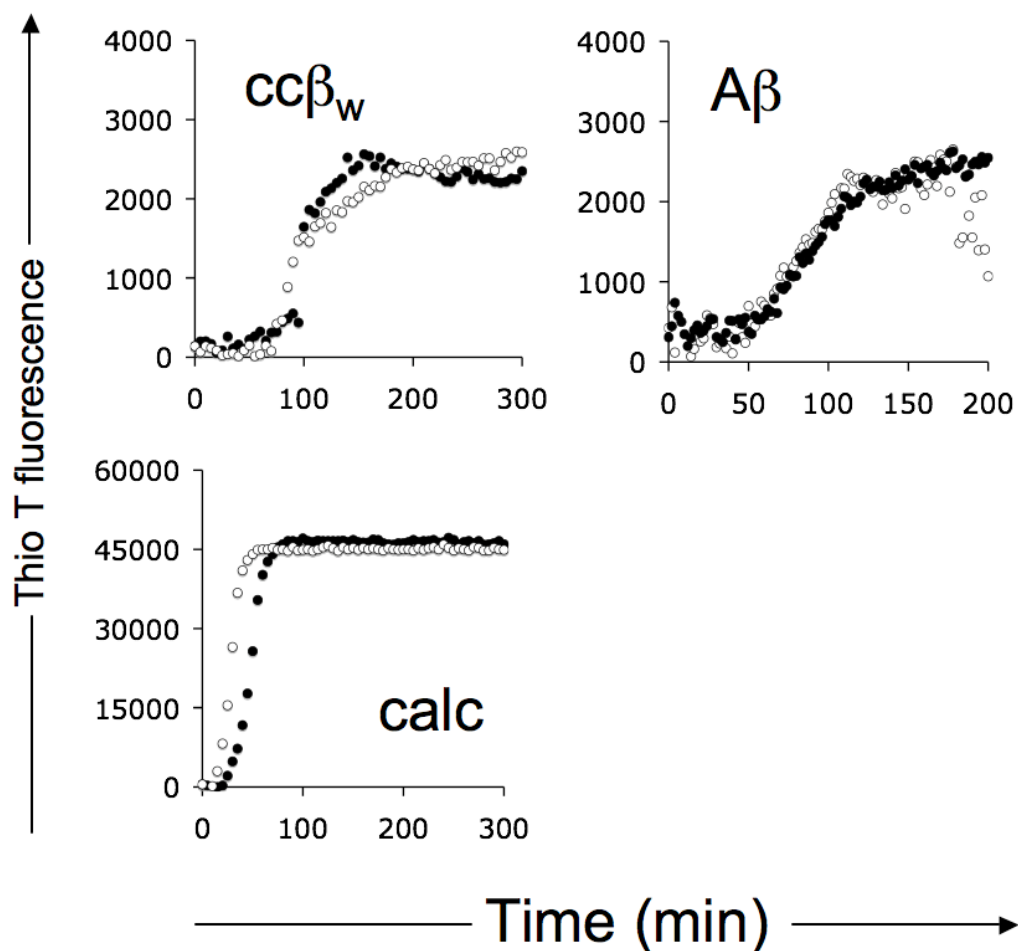
**Figure 5.1 Haptoglobin suppresses the aggregation of amyloid forming proteins.** Calcitonin (calc, white circles; 50  $\mu$ M) and cc $\beta_w$  (white circles; 60  $\mu$ M) were incubated at 37  $^{\circ}$ C, A $\beta$  (white circles; 10  $\mu$ M) was incubated in oxidative buffer at 37  $^{\circ}$ C, and I59T lysozyme (I59T lys, white circles; 6.8  $\mu$ M) was incubated at 48  $^{\circ}$ C, in the presence of various molar ratios of Hp:amyloid substrate (indicated in the individual panels). Aggregation was measured as an increase in thioflavin T fluorescence for calcitonin, cc $\beta_w$  and A $\beta$  while the aggregation of I59T lysozyme was measured as an increase in light scattering ( $A^{500}$ ). Data points shown are individual measurements and are representative of at least three independent experiments.



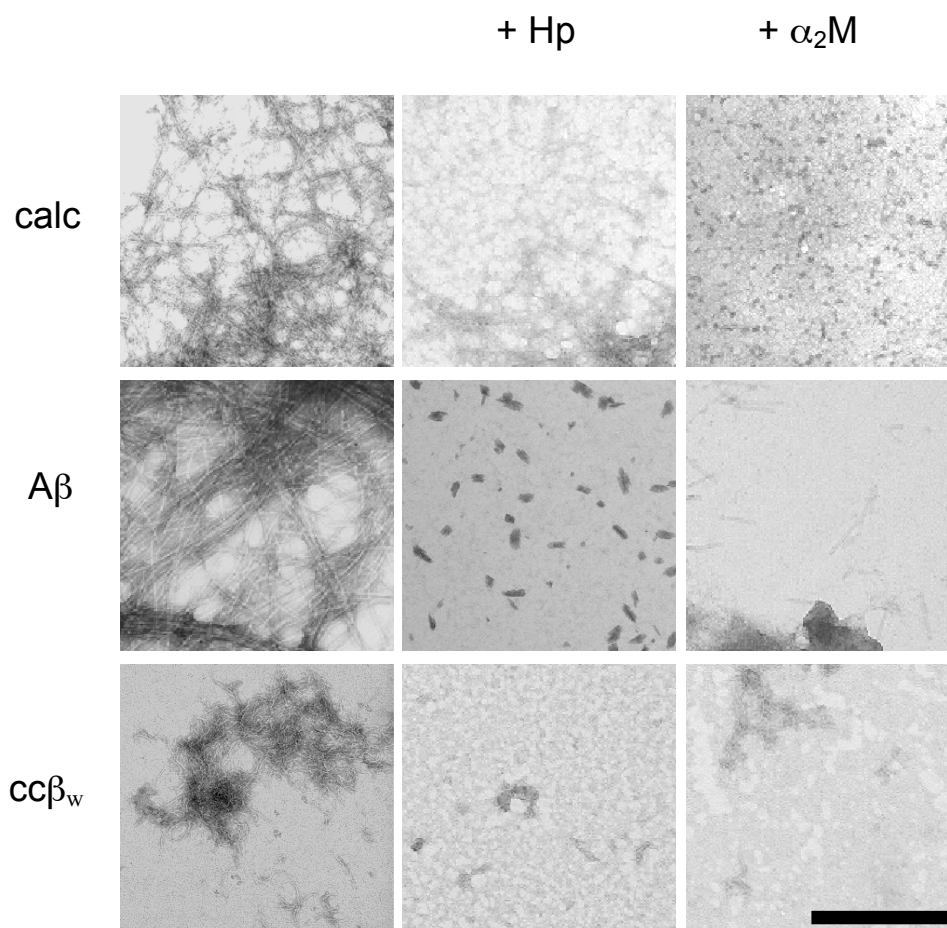
**Figure 5.2**  $\alpha_2$ M suppresses the aggregation of amyloid forming proteins. Calcitonin (calc, white circles; 50  $\mu$ M) and cc $\beta_w$  (white circles; 60  $\mu$ M) were incubated at 37 °C, while A $\beta$  (white circles; 10  $\mu$ M) was incubated in oxidative buffer at 37 °C, and I59T lysozyme (I59T lys, white circles; 6.8  $\mu$ M) was incubated at 48 °C, in the presence of various molar ratios of  $\alpha_2$ M:amyloid substrate (indicated in the individual panels). Aggregation was measured as an increase in thioflavin T fluorescence for calcitonin, cc $\beta_w$  and A $\beta$  while the aggregation of I59T lysozyme was measured as an increase in light scattering ( $A^{500}$ ). Data points shown are individual measurements and are representative of at least three independent experiments.

TEM was used to examine the morphology of proteins at the conclusion of amyloid forming reactions. In the absence of either Hp or  $\alpha_2$ M, samples from all substrate proteins tested contained fibrillar aggregates whose structural appearance was as expected for amyloid fibrils in these well-characterised systems (Figure 5.4; I59T lysozyme data not shown). The presence of Hp in Ab (1:10 Hp:Ab) and cc $\beta_w$  (1:15 Hp:cc $\beta_w$ ) aggregation reactions fully suppressed the formation of fibrils in the same samples small amounts of amorphous aggregates were detected (Figure 5.4). However, although the thioflavin T fluorescence was almost totally suppressed, calcitonin fibrils

could be found in reactions containing Hp ( at Hp:ccb<sub>w</sub> = 1:15). Addition of  $\alpha_2$ M into calcitonin aggregation (at  $\alpha_2$ M:calc = 1:15) reactions inhibited the formation of fibrillar structures, whereas small fibril-like structures were observed in Ab ( $\alpha_2$ M:Ab = 1:10) and ccb<sub>w</sub> ( $\alpha_2$ M:ccb<sub>w</sub> = 1:15) samples containing  $\alpha_2$ M (Figure 5.4).



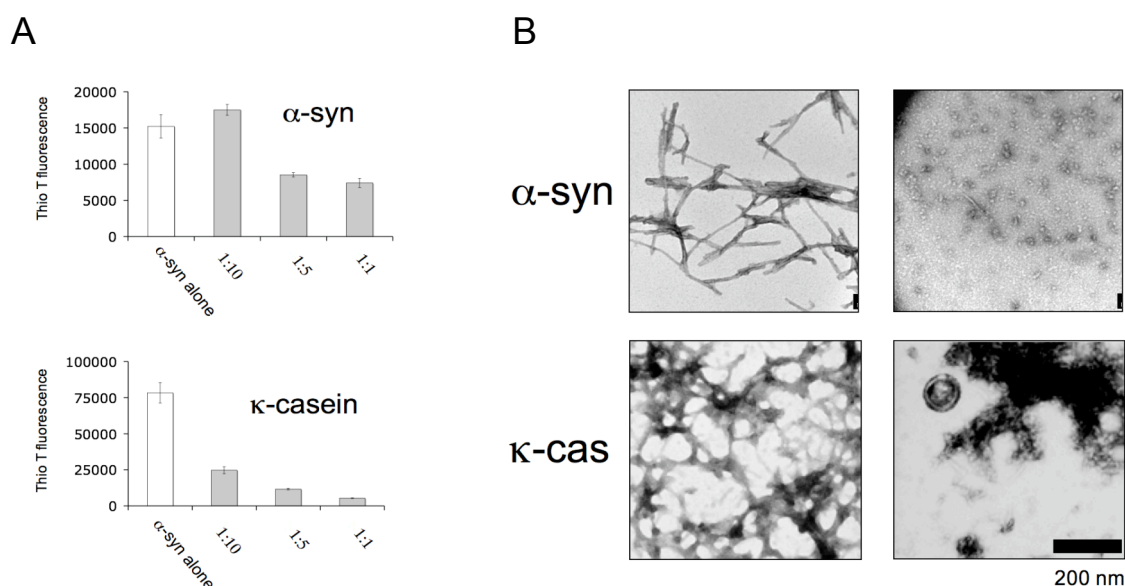
**Figure 5.3** *Control protein BSA does not influence aggregation of amyloid forming proteins.* Calcitonin (calc; 50  $\mu$ M) and ccb<sub>w</sub> (60  $\mu$ M) were incubated at 37 °C, A $\beta$  (10  $\mu$ M) was incubated in oxidative buffer at 37 °C, each in the presence (black circles) or absence (white circles) of a 1:10 ratio of BSA:amyloid substrate. Aggregation was measured as an increase in thioflavin T fluorescence. Data points shown are individual measurements and are representative of at least three 3 independent experiments.



**Figure 5.4** *Hp and  $\alpha_2$ M suppress the formation of fibrillar structures.* TEM images of samples taken from the conclusion of aggregation reactions containing either amyloid forming protein alone or amyloid forming protein in the presence of either Hp or  $\alpha_2$ M. The molar ratio of extracellular chaperone:substrate used was 1:10 for Ab and 1:15 for both calcitonin (calc) and ccb<sub>w</sub>. 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 and represents 500 nm.

Additional experiments were conducted to examine the effects of Hp on amyloid formation by two additional substrates,  $\alpha$ -synuclein and  $\kappa$ -casein. In both cases, Hp reduced the endpoint thioflavin T fluorescence in a dose dependant fashion. Hp was less efficient at reducing the thioflavin T associated with  $\alpha$ -synuclein fibril formation. Even when added at a molar ratio of Hp: $\alpha$ -synuclein = 1:1, Hp only reduced the levels of thio

T fluorescence to just over a third of that measured in the absence of Hp (Figure 5.5A). Although thioflavin T fluorescence was incompletely suppressed, the formation of mature fibrils was completely inhibited. An examination by TEM of species present at the end point of the reaction showed that no mature fibrils were present, however smaller aggregates incorporating fibril-like structures were visible that probably represent protofilaments. Similarly, when incubated alone,  $\kappa$ -casein formed fibrils (Figure 5.5B,  $\kappa$ -cas, -Hp). When incubated in the presence of Hp,  $\kappa$ -casein formed substantially less fibrils but formed some large amorphous aggregates (Figure 5.5B,  $\kappa$ -cas, +Hp).

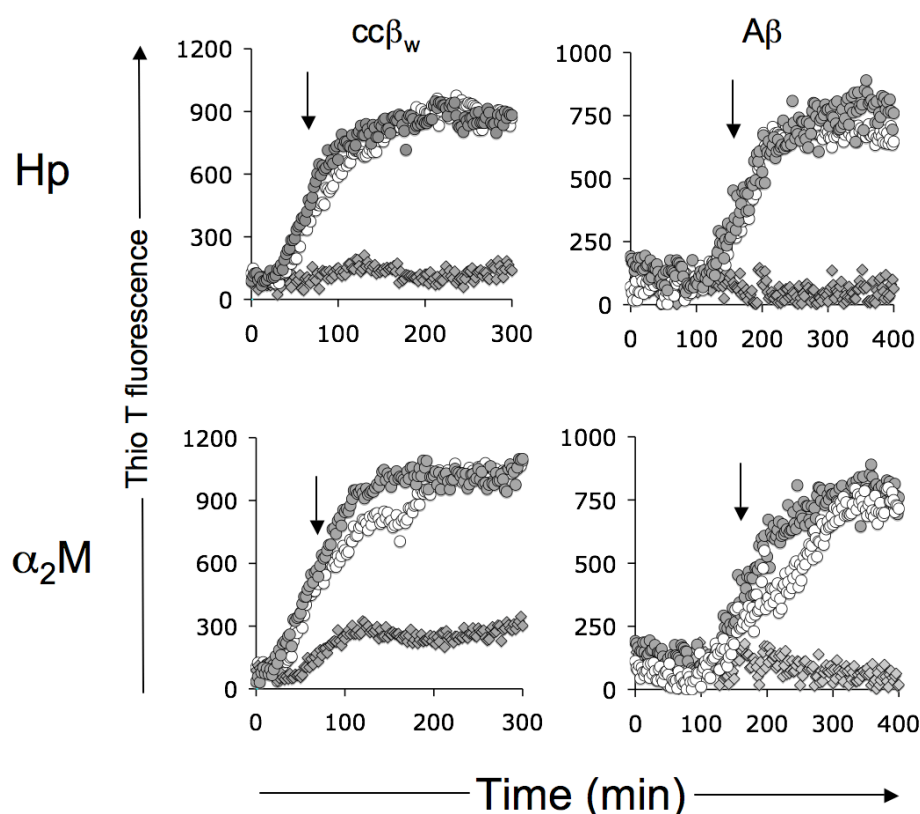


**Figure 5.5 Effect of Hp on  $\alpha$ -synuclein and  $\kappa$ -casein fibril formation.** A) Thioflavin T fluorescence (in arbitrary units) of protein species at the end of aggregation reactions in the presence (grey bars) and absence (white bars) of various molar ratios of Hp:amyloid substrate (indicated below the bars). In each case the data shown are means of triplicates and the error bars are standard errors of the mean. B) TEM images of samples taken from the end of the aggregation reactions containing either no Hp (-Hp) or a 1:1 molar ratio of Hp:amyloid substrate. The scale bar shown in the bottom right of the panel applies to all TEM images shown. In each case the results are representative of two or more individual experiments.



### 5.3.2 $\alpha_2$ M and Hp do not influence fibril elongation

Two main processes occur during amyloid formation, nucleation and fibril elongation. To determine which of these processes Hp and  $\alpha_2$ M acted upon to suppress fibril formation, Hp or  $\alpha_2$ M were added either at the beginning of the reaction (0 min) or mid-way through the elongation phase of  $cc\beta_w$  and A $\beta$  fibril formation (75 min and 150 min, respectively). Addition of either Hp or  $\alpha_2$ M (both at a 1:10 molar ratio of chaperone:substrate) at the beginning of the experiment resulted in almost total inhibition of aggregation over the entire reaction (Figure 5.6). In contrast, when the same ratios of Hp or  $\alpha_2$ M were added to the reaction mid-elongation phase there was no measurable effects on aggregation (Figure 5.6).



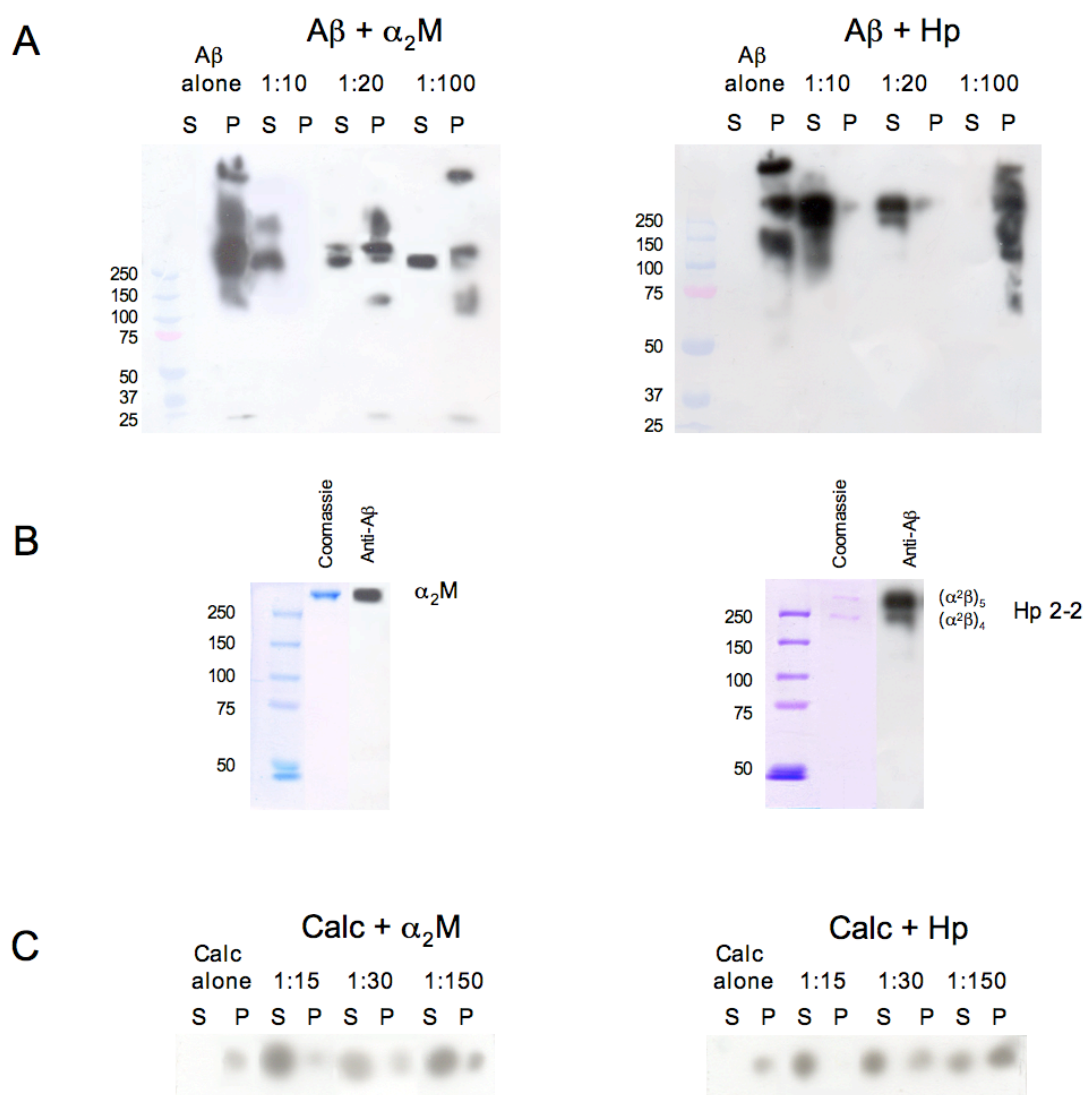
**Figure 5.6 Hp and  $\alpha_2$ M are most effective when added at the start of the aggregation reaction.**

Thioflavin T measurements of A $\beta$  and  $cc\beta_w$  aggregation in the absence of Hp and  $\alpha_2$ M (white circles), and following addition of Hp or  $\alpha_2$ M to the reaction in sub-stoichiometric quantities (molar ratio of Hp or  $\alpha_2$ M to substrate of 1:10) at time zero (grey diamonds) or at mid growth phase (grey circles) (addition of Hp and  $\alpha_2$ M indicated by arrows). Each symbol is an individual measurement and is representative of at least 2 independent experiments.



### 5.3.3 Both $\alpha_2$ M and Hp affect the sedimentation properties of amyloid forming proteins

Hp and  $\alpha_2$ M were added at various concentrations to A $\beta$  and calcitonin fibril formation reactions and at the end of the time-course the samples were centrifuged to obtain supernatant and pellet fractions. The Ab samples were then examined by western blot to determine the fraction containing A $\beta$ , while calcitonin samples were examined by immunoblotting techniques due to difficulties with the peptide transferring onto the membrane. In the absence of either extracellular chaperone, A $\beta$  was found exclusively in the pellet fraction and consisted of high molecular mass aggregates (Figure 5.7A; A $\beta$  alone). When  $\alpha_2$ M was added to the reaction to give a molar ratio of  $\alpha_2$ M:A $\beta$  = 1:10 all of the A $\beta$  was found in the supernatant fraction, and subsequent decreases in the concentration of  $\alpha_2$ M added to the reaction resulted in a gradual shift of A $\beta$  from supernatant fraction to pellet fraction (Figure 5.7A). When  $\alpha_2$ M was present in the reaction, any A $\beta$  that was found in the soluble fraction co-migrated with  $\alpha_2$ M (Figure 5.7A and B). This suggests that A $\beta$  had formed an SDS-resistant complex with  $\alpha_2$ M. Similarly, the addition of Hp (at a molar ratio of Hp:A $\beta$  = 1:10) to an A $\beta$  amyloid formation reaction resulted in A $\beta$  being found in the supernatant fraction; as the concentration of Hp added was decreased, more A $\beta$  was found in the pellet fraction (Figure 5.7A). In addition, A $\beta$  co-migrated with the most common polymers of Hp (2-2), suggesting that there was an SDS-resistant complex formed between Hp and A $\beta$  (Figure 5.7B). In the absence of either extracellular chaperone, calcitonin was detected only in the pellet fraction (Figure 5.7C). In contrast, when  $\alpha_2$ M was present in the reaction (at calc: $\alpha_2$ M = 1:15) most of the calcitonin was detected in the supernatant fraction. The proportion of calcitonin in the pellet fraction increased as the amount of  $\alpha_2$ M in the reaction decreased (Figure 5.7C). Similarly, in the presence of Hp (calc:Hp = 1:15) all of the calcitonin was detected in the supernatant fraction and subsequent decreases in Hp concentration resulted in increased calcitonin detected in the pellet fraction (Figure 5.7C).

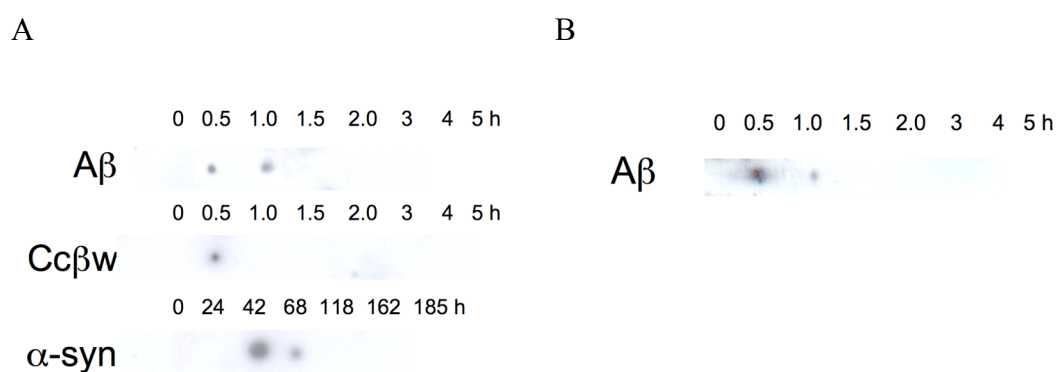


**Figure 5.7  $\alpha_2M$  and Hp form stable complexes with  $A\beta$  and maintain its solubility.** (A) Images of  $A\beta$  immunoblot showing supernatant (S) and pellet (P) fractions prepared by centrifugation of final samples containing various ratios of Hp and  $\alpha_2M$  to substrate (indicated above the corresponding lanes). (B) Composite image of both Coomassie blue stained gel and western blot probed with anti- $A\beta$  of an  $A\beta$  supernatant fraction containing extracellular chaperones taken from fibril formation reactions. Values for molecular weight markers are shown on the left of each panel. (C) Images of immuno dot blots showing supernatant (S) and pellet (P) fractions separated by centrifugation of final samples containing calcitonin with various molar ratios of both haptoglobin and  $\alpha_2M$  (indicated above corresponding spots).

#### 5.3.4 $\alpha_2M$ and Hp bind transient amyloid pathway species

Samples taken at various points in time from fibril formation reactions (performed in the absence of Hp and  $\alpha_2M$ ) for the same panel of substrates previously examined were spotted on to nitrocellulose membranes. When assayed by immuno dot blot there was

no binding of Hp detected to species present at any time in calcitonin and k-casein fibril formation reactions (data not shown). In addition, there was no detectable binding of  $a_2M$  to species present in calcitonin or  $ccb_w$  reactions (data not shown). However, binding of Hp was detected to species present in Ab,  $\alpha$ -synuclein and  $ccb_w$  timecourses, while  $a_2M$  was detected binding to samples in the Ab timecourse. In the case of  $A\beta$ , maximum binding was detected to species present in samples taken at 0.5 and 1 hour (Figure 5.8). This corresponds to the transition between the lag and growth phases (see Figure 5.3). For  $\alpha$ -synuclein and  $ccb_w$ , Hp bound to species present before the elongation phase ( $ccb_w$  see Figure 5.3;  $\alpha$ -syn data not shown). Haptoglobin and  $a_2M$  did not bind to the monomeric substrates present in samples prior to incubation, nor did they bind to final samples (Figure 5.8). There was no detectable binding of the control protein GST to any of the substrates tested (data not shown).



**Figure 5.8 Hp and  $a_2M$  bind transient early species during amyloid formation.** Results of immuno dot blot assays measuring the binding of Hp (A) and  $a_2M$  (B) to protein species present at different times during fibril formation. The times at which individual samples were taken are indicated above the images. The results shown are representative of two or more individual experiments.

## 5.4 Discussion

Previous work has indicated that  $\alpha_2$ M can inhibit the formation of fibrils by A $\beta$  (Hughes *et al.*, 1998). In addition, both  $\alpha_2$ M and Hp have been found associated with A $\beta$  plaques in Alzheimer's patients (Powers *et al.*, 1981; Fabrizi *et al.*, 2001). Results shown here demonstrate that both  $\alpha_2$ M and Hp can potently suppress the *in vitro* formation of amyloid by a broad panel of substrates. Both Hp and  $\alpha_2$ M, at substoichiometric levels, inhibited the aggregation of peptides and a globular protein, and were potently anti-amyloidogenic under both oxidative stress and non-oxidative physiological conditions. This activity was seen even at the elevated, non-physiological, temperature of 48 °C. These data suggest that the anti-amyloidogenic activity of  $\alpha_2$ M and Hp is not dependant on the identity of the polypeptide substrate or the conditions under which it is incubated. In all cases tested, significantly substoichiometric molar ratios of  $\alpha_2$ M or Hp to substrate (from 1:10 to 1:15) were enough to almost totally inhibit the formation of amyloid fibrils as judged by thioflavin T fluorescence, light scattering and TEM. This activity was specific since similar levels of control protein BSA (a molar ratio of BSA:substrate = 1:10) had no effect on aggregation of any of the substrates tested. In some experiments, lower levels of Hp and  $\alpha_2$ M (1:500 to 1:30) failed to reduce the final level of protein aggregation. However, in these cases the lag phase of the reaction was slowed. This suggests that when present at low levels, the effects of the ECs are eventually overwhelmed, but that they delay the establishment of a nucleation event and subsequent rapid elongation phase.

Both  $\alpha_2$ M and Hp were more effective at suppressing aggregation of amyloid forming substrates when added at the initiation of the reaction rather than when added during the elongation phase. This suggests that Hp and  $\alpha_2$ M both 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  $\alpha_2$ M and Hp effectively extended lag phase, these data suggest that the ECs interact with protein species that are either precursors to nuclei or that are functional nuclei themselves. Immuno-dot blot analysis showed that for substrates where binding could

be detected, Hp and  $\alpha_2$ M bound to transient species present early in the timecourse. The ECs Hp and  $\alpha_2$ M appear not to bind to samples taken before the start of the reaction (Time 0) or those taken at the end of the reaction (Figure 5.8). This demonstrates that Hp and  $\alpha_2$ M do not detectably bind to the native monomer or to mature fibrils formed from them. This is consistent with the idea that both Hp and  $\alpha_2$ M are binding to species that are more abundant during fibril nucleation. The fact that there was no detectable binding to any species in the calcitonin timecourse suggests that the interacting species are at very low levels.

When increasing amounts of Hp and  $\alpha_2$ M were added to amyloid formation reactions containing either A $\beta$  or calcitonin a progressive increase in the proportion of substrate was found in the supernatant fraction. In addition, in the case of A $\beta$ , western blot analysis detected soluble A $\beta$  in positions that corresponding to both  $\alpha_2$ M and Hp, suggesting that there was an SDS-stable interaction between A $\beta$  and both Hp and  $\alpha_2$ M. This is consistent with Hp and  $\alpha_2$ M forming very stable complexes with A $\beta$  to maintain aggregation-prone conformations of the peptide in solution.

Collectively, the results indicate that substoichiometric levels of Hp and  $\alpha_2$ M effectively suppress amyloid fibril formation by a range of substrates under varying conditions. The data suggests that both Hp and  $\alpha_2$ M suppress fibril formation by interacting with one or more transient species on the amyloid forming pathway which are populated most heavily before fibril elongation begins. The interaction between EC and substrate appears to be stable and maintains the solubility of the substrate. This is consistent with the hypothesis that like clusterin,  $\alpha_2$ M and Hp bind to species sharing common structural features present during amyloid formation of a range of substrates (Yerbury *et al.*, 2007). These findings suggest that Hp and  $\alpha_2$ M along with clusterin make up a small family of ECs that may be an important part of an extracellular protein folding quality control system *in vivo*. It has been proposed that protein aggregation *in vivo* occurs when the protein quality control machinery is overwhelmed (Muchowski and Wacker 2005). In the extracellular space, these ECs are the only currently known components of such a system with the potential to be overwhelmed. It has been proposed that ECs respond to misfolded or aggregating proteins in the extracellular

space by stable binding to exposed hydrophobic regions, maintaining the solubility of the substrate and promoting its removal from the extracellular space *via* receptor mediated endocytosis. This is consistent with data presented here, and with data that shows that the removal of radiolabelled A $\beta$  from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against the  $\alpha_2$ M receptor (LRP-1) and  $\alpha_2$ M (Shibata *et al.*, 2000). This hypothesis is also supported by data showing that, when complexed with clusterin, the rate of clearance of A $\beta_{1-42}$  from the mouse brain across the BBB into plasma is increased by more than 80% and that this transport is significantly inhibited by anti-megalin antibodies (Bell *et al.*, 2007).

In conclusion, the small family of known ECs (Hp,  $\alpha_2$ M and clusterin) are likely to be an important part of a system defending the human body against inappropriate aggregation of extracellular proteins. Greater understanding of this system is likely to lead to the development of new therapeutic weapons against extracellular protein deposition disorders.

Chapter 6

## Conclusions

### Preface

As a consequence of this thesis having been prepared as a series of manuscripts written for publication, each chapter stands alone and their individual discussions and conclusions are detailed, citing relevant literature and addressing the issues raised for each chapter. To avoid overlap of material between the Discussion sections of each individual chapter and that contained here, the present Chapter will focus on what the findings of this thesis mean as whole, and possible future directions for the field.

As the population ages, Alzheimer's disease threatens to become one of the world's most prevalent and costly diseases (Dobson 2002). However, Alzheimer's disease is only one of a group of many *protein deposition disorders*, closely related to each other because they involve the inappropriate deposition of proteins in the brain, or other organs depending on the specific disease. There are over 40 diseases in this group including some that are sporadic and familial (e.g. Alzheimer's, Parkinson's, ALS) and some that are transmissible (Creutzfeldt-Jakob disease). The protein deposits can range from microscopic to some that are large enough to disrupt organs. The deposits are composed mainly of a single predominant protein, specific for each disease, which has generally formed rope-like fibrils. Under normal conditions proteins are generally soluble and have a unique 3D conformation that is specific for their function. However, in protein deposition disorders the specific protein involved must somehow unfold from its native conformation and be refolded in to a conformation high in b-sheet content that has the propensity to form fibrils (Dobson 1999).

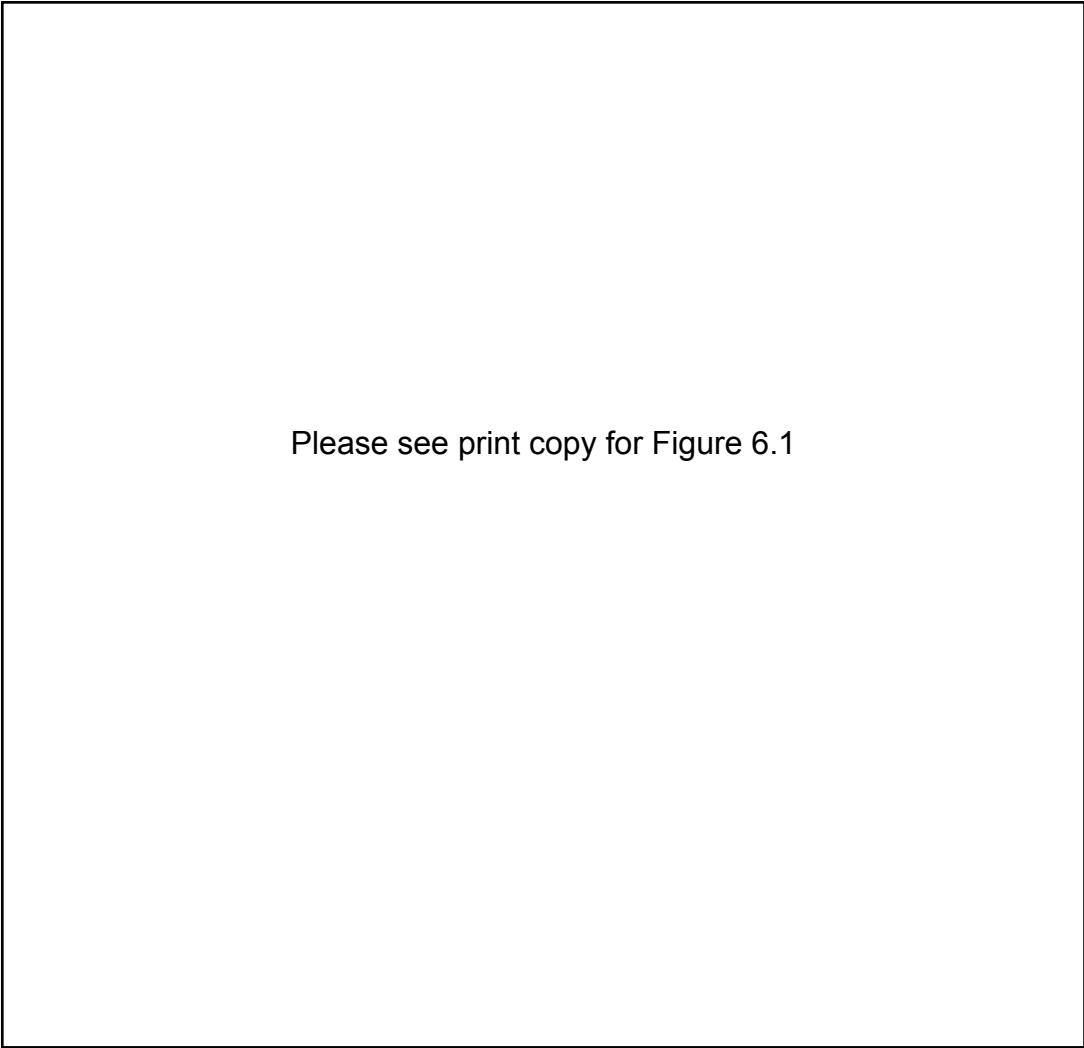
When it is considered that cells and their surrounds (extracellular spaces) are densely packed with thousands of different proteins, and are regularly exposed to various stresses capable of unfolding proteins, it seems miraculous that there are not more protein deposition disorders. Inside cells there is a large amount of energy invested into making sure that proteins reach and maintain their native shape. This quality control machinery includes molecular chaperones, which bind to hydrophobic regions normally buried inside the normal three-dimensional structure of a protein, and sophisticated degradation machinery such as the proteasome (Wickner *et al.*, 1999). The ability of this system to efficiently deal with non-native proteins may decrease with age, and thus explain the positive correlation between protein deposition and ageing (Dobson 2002). But although there are many diseases, including Alzheimer's disease, that are associated with extracellular protein deposits, until recently there has been almost no work focussed on mechanisms that prevent protein aggregation and deposition in the extracellular space. This thesis characterises a small group of proteins that have functional similarities. They have in common an ability to stably bind misfolded proteins and thus inhibit inappropriate protein-protein interactions, preventing



aggregation and maintaining proteins in solution. Each of these “extracellular chaperones” (clusterin, haptoglobin and  $\alpha_2$ -macroglobulin) has specific cell surface receptors that recognise and internalise them in complex with ligands for subsequent degradation in lysosomes (Hammad *et al.*, 1997; Shibata *et al.*, 2000; Graversen *et al.*, 2002). The data presented in this thesis suggests that these proteins and their receptors may be the foundation of an extracellular system for protein quality control.

Moreover, the data is consistent with the model proposed by Margineanu and Ghetie (see section 1.7 An ‘ageing’ model) that proposed that clearance of proteins from the extracellular space was a simple two-step process whereby a protein would undergo molecular modification leading to a form recognisable by catabolic cells, and then this latter form would be internalised and degraded (Margineanu and Ghetie 1983). The data presented here suggests that the exposed hydrophobicity on modified or non-native conformations of extracellular proteins is recognised by ECs which stably bind preventing aggregation. In this scenario, the complex between EC and substrate would be recognised by catabolizing cells via specific cell surface receptors which would internalise the complex for subsequent degradation (Figure 6.1).

Since it is thought that pathological aggregation occurs due to an overwhelming of quality control systems (Muchowski and Wacker 2005) it may be that in diseases associated with extracellular protein deposits this proposed quality control system is unable to cope with the imposed pathological load. If this was the case then it would be logical that inhibiting these pathways may lead to conditions more likely to induce extracellular protein deposition disorders such as Alzheimer’s disease. Evidence in mice suggests that this could be the case; the normally rapid removal of radiolabelled A $\beta$  from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against LRP-1 and  $\alpha_2$ M (Shibata *et al.*, 2000). Furthermore, when complexed with clusterin, the rate of clearance of A $\beta_{1-42}$  from the mouse brain across the BBB into plasma is increased by more than 80% and this transport is significantly inhibited by anti-megalin antibodies (Bell *et al.*, 2007).



Please see print copy for Figure 6.1

**Figure 6.1 Possible extracellular quality control mechanisms and the consequences of its failure.** An extracellular quality control process has recently been proposed whereby extracellular chaperones (EC) facilitate the clearance of non-native protein structures via receptor-mediated endocytosis and intracellular, lysosomal degradation. The existence of such a quality control mechanism ensures that proteins do not persist beyond their intended lifespan in the extracellular space. Failure of this system, at any of its stages, may potentially result in the onset of disease caused by the accumulation and/or deposition of i) cytotoxic species generated through the amyloid assembly pathway, ii) aberrantly folded structures and aggregates such as insoluble complexes of ECs and fibrillar material, or iii) degradation-resistant complexes (as exemplified by A $\beta$ <sub>1-42</sub> amyloid fibrils). Reproduced from (Wilson *et al.*, 2008).

The extracellular protein quality control field is in its infancy, but in time may provide important insights into the mechanisms underlying extracellular deposition disorders. However, much work is required in this burgeoning field before that point is reached. In particular, research demonstrating that the processes described here occur *in vivo* are necessary. In addition, further research into other possible components of the quality control system is required. This would entail further work focussing on identifying receptors that recognise extracellular chaperone-substrate complexes, and following these complexes inside cells. It would also be advantageous to identify which cells in the body are responsible for EC mediated uptake of non-native protein. This will need to be followed by important work determining the role of these ECs in disease. Information gained from these future studies is likely to identify new therapeutic targets for a range of diseases that will have an increasing impact on an ageing world population.

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## Appendix 1

### Review Publications

#### **Preface**

Since the present thesis is composed of experimental chapters that were written for publication, to ensure a logical flow of ideas and experimental concepts the manuscripts in this appendix were left out of the main body of work. However, it is important that they are included since they reflect a conceptual and theoretical contribution to the field. This appendix is comprised of two review articles, one published at the beginning of the project and one published at completion.

#### Publications:

**Yerbury Justin J.**, Stewart Elise M., Wyatt Amy R., Wilson Mark R. (2005) Quality control of protein folding in extracellular space. *EMBO Reports* 6: 1131-1136

Wilson Mark R., **Yerbury Justin J.**, Poon Stephen. (2008) The role of extracellular chaperones in amyloid formation. *Molecular BioSystems* 4: 42–52

Please see print copy for Review Publications