The role of clusterin (CLU) in extracellular protein folding quality control

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The Role of Clusterin (CLU) in Extracellular Protein Folding Quality Control

By

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Bachelor of Biotechnology (Advanced) Honours 1

This thesis is presented as part of the requirements for the degree of

Doctor of Philosophy

School of Biological Sciences
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September 2009
DECLARATION OF AUTHENTICITY

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the Degree of Doctor of Philosophy. It does not include any material published by another person except where due reference is made in the text. The experimental work described in this thesis is original and has not been submitted for a degree to any other university.

Amy Ruth Wyatt
ACKNOWLEDGEMENTS

Foremost thanks must go to my supervisor Mark Wilson for putting up with my many idiosyncrasies and guiding me safely towards the completion of my PhD. I am very appreciative of the many opportunities you have given me over the years. Also, I am extremely grateful for the help of Justin Yerbury, Elise Stewart, Danielle Murphy-Durland, Stephen Poon, Kara Perrow, Christine Gillen, Rebecca Dabbs and Natalie Farrawell - who were not only fellow lab members (past, present and honorary) but remain tremendous friends.

For the work carried out at ANSTO, sincere thanks to Andrew Katsifis, Ivan Greguric, Paula Berghofer and the many other individuals who assisted me from time to time.

Special thanks to my ‘theatre family’, who are too numerous to mention in full here. Of them, I am especially thankful to my dear friend, business partner, confidant, counsellor, accompanist and IT support person, Adam Vujic. The numerous productions I have been involved in over the years have provided me with the perfect distraction from my day job.

Lastly, I thank my parents for nurturing my unending curiosity as a child and for providing me with limitless support throughout my PhD candidature.
ABSTRACT

Processes to attain and maintain the correct three-dimensional shape, known as the native conformation, of proteins are vital. However, certain conditions including thermal and oxidative stress may cause proteins to partially unfold and aggregate. Intracellular and/or extracellular protein aggregates have been identified in a large number of diseases, including Alzheimer’s disease, arthritis and type II diabetes. While intracellular quality control for the folding state of proteins is well characterized, corresponding mechanisms for extracellular protein folding quality control have yet to be described.

Clusterin (CLU) is an abundant extracellular chaperone that can stabilize proteins and prevent their precipitation during exposure to elevated temperatures or oxidative stress in vitro. The work described here demonstrates that CLU stabilizes stressed client proteins by forming soluble, high molecular weight (HMW) complexes with them. The maximum loading of CLU appears to be at a mass ratio of CLU:stressed client protein of approximately 1:2 (irrespective of the identity of the client protein or the temperature used to induce heat stress). It was demonstrated that various human plasma proteins show increased association with CLU after plasma is subjected to mild shear stress or oxidative stress at 37°C - the most abundant of these was fibrinogen (FGN) which co-purified with CLU from stressed plasma. In vitro, using purified proteins, heat stress of 45°C for 12 h was required to induce FGN precipitation and the formation of HMW CLU-FGN complexes. Size exclusion chromatography (SEC) of the stressed plasma suggested that a portion of the complexes formed in plasma between CLU and FGN may be similar in mass to those formed in vitro.

Using surface plasmon resonance, although CLU was found to bind to megalin, only minimal (or no) binding of HMW complexes formed between CLU and glutathione-S-transferase, citrate synthase or lysozyme was detected. Similarly, negligible binding of these complexes to low density lipoprotein receptor superfamily members expressed on the surface of the rat yolk sac cell line BN was detected. However, the complexes were shown to preferentially bind to the surface of BN cells, peripheral human monocytes and rat hepatocytes (more so than uncomplexed CLU or client proteins). In all cases, this binding was inhibited by fucoidin (a scavenger receptor inhibitor). Confocal microscopy suggested that binding of HMW CLU-stressed protein complexes to the
surface of BN cells or rat hepatocytes was followed by their internalization into lysosomes. Furthermore, Western blotting showed that hepatocytes were able to degrade the HMW CLU-stressed protein complexes and that the degradation was almost completely abolished by inhibiting lysosomal proteases with chloroquine. The results of in vivo biodistribution studies in Sprague Dawley rats were highly consistent for several different HMW CLU-stressed protein complexes. Intravenous $^{123}$I-labelled HMW CLU-stressed protein complexes were cleared more efficiently from circulation compared to free CLU and the uncomplexed client proteins. The liver and to a lesser degree the spleen appeared to be the key organs responsible for the uptake of complexes and this uptake was inhibited by pre-injection of the animals with fucoidin.

The findings of this study suggest an important role for CLU in global quality control of extracellular protein folding. It appears likely that stressed (partially unfolded) extracellular proteins are stabilized and held in solution by CLU and that CLU-stressed protein complexes are subsequently taken up by fucoidin-inhibitable cell surface receptors for subsequent degradation within lysosomes. The precise physical characteristic or binding site that targets CLU-stressed protein complexes for receptor-mediated uptake remains to be identified and further work is needed to determine the particular receptor(s) involved. The findings of this study support a model in which complexation with CLU is an important first step in the targeted disposal of stressed proteins via scavenger-like endocytic receptors.
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<tr>
<td>ºC</td>
<td>degrees celsius</td>
</tr>
<tr>
<td>41D</td>
<td>monoclonal anti-clusterin antibody</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>α₂M</td>
<td>α₂-macroglobulin</td>
</tr>
<tr>
<td>A280</td>
<td>absorbance at 280 nm</td>
</tr>
<tr>
<td>A360</td>
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</tr>
<tr>
<td>A490</td>
<td>absorbance at 490 nm</td>
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<td>ACID GLY</td>
<td>α₁ acid glycoprotein</td>
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<tr>
<td>AcLDL</td>
<td>acetylated low density lipoprotein</td>
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<td>arbitrary fluorescence units</td>
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<td>advanced glycation end product</td>
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</tr>
<tr>
<td>ASF</td>
<td>asialofetuin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Az</td>
<td>sodium azide</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid-beta</td>
</tr>
<tr>
<td>BD</td>
<td>Becton, Dickinson and Company</td>
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<tr>
<td>bisANS</td>
<td>4,4'-Bis(1-anilino-8-naphthalene sulfonate)</td>
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<td>bovine serum albumin</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
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<td>CS</td>
<td>citrate synthase</td>
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<td>cerebral spinal fluid</td>
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<td>Da</td>
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</tr>
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<td>DMEM:F-12</td>
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<tr>
<td>dSR-CI</td>
<td>drosophila scavenger receptor class C type I</td>
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<tr>
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<tr>
<td>Term</td>
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<td>E. coli</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>ethylene diamine tetraacetic acid</td>
</tr>
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<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
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<td>ethylene glycol tetraacetic acid</td>
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<td>enzyme linked immunosorbent assay</td>
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<td>emission</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>endoplasmic reticulum-associated protein degradation</td>
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<td>Ex</td>
<td>excitation</td>
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<td>F</td>
<td>F statistic</td>
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<td>FPLC</td>
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<td>g</td>
<td>relative centrifugal force (9.8 m.s⁻²)</td>
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<td>monoclonal anti-CLU antibody</td>
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<td>glutathione-S-transferase-receptor-associated protein</td>
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<td>(fusion protein)</td>
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<td>hour(s)</td>
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<td>Hank’s binding buffer</td>
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<td>heat-shock cognate protein</td>
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<td>HSD</td>
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*Abbreviations and acronyms are provided for clarity and ease of reading.*
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<td>lysosome-associated membrane protein type 2a</td>
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<td>lactate dehydrogenase</td>
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<td>low density lipoprotein</td>
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<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
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<td>LOX-1</td>
<td>lectin-like oxidized low density lipoprotein receptor</td>
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<td>LRP</td>
<td>low density lipoprotein receptor-related protein</td>
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<tr>
<td>ly-hsc73</td>
<td>lysosomal hsc73</td>
</tr>
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<td>LYS</td>
<td>lysozyme (hen egg)</td>
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<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MARCO</td>
<td>macrophage receptor with collagenous structure</td>
</tr>
<tr>
<td>mBSA</td>
<td>maleylated bovine serum albumin</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
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<td>min</td>
<td>minutes</td>
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<td>oxLDL</td>
<td>oxidized low density lipoprotein</td>
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<tr>
<td>PARP</td>
<td>poly ADP ribose polymerase</td>
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<tr>
<td>p</td>
<td>statistical significance</td>
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<tr>
<td>p.i.</td>
<td>post-injection</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDDs</td>
<td>protein deposition diseases</td>
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xxiii
PEG  polyethylene glycol
pH  power of hydrogen
PI  propidium iodide
RAGE  receptor for advanced glycation end products
RAP  receptor-associated protein
ROS  reactive oxygen species
rpm  revolutions per min
s  seconds
SA  streptavidin
SAP  serum amyloid P component
SDS  sodium dodecyl sulphate
SDS-PAGE  sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC  size exclusion chromatography
sHsps  small heat-shock proteins
SOD  superoxide dismutase
SPECT  single photon emission computed tomography
SR-AI  scavenger receptor class A type I
SR-AII  scavenger receptor class A type II
SR-AIII  scavenger receptor class A type III
SR-BI  scavenger receptor class B type I
SR-CR  scavenger receptor cysteine-rich domain
SREC-1  scavenger receptor expressed by endothelial cells
SR-PSSX  scavenger receptor for phosphatidylserine and oxidized low density lipoprotein
TAE  TRIS acetate EDTA
TEM  transmission electron microscopy
TMED  N,N,N',N'-tetramethylethylenediamine
TRANS  transferrin
TRIS  2-amino-2-hydroxymethyl-propane-1,3-diol
tRNA  transfer ribonucleic acid (not defined in text)
TRYP  α1 antitrypsin
UPR  unfolded protein response
UV  ultraviolet
V  volts
v/v  volume per volume
VLDL  very low density lipoprotein
VLDLR  low density lipoprotein receptor
V_o  void volume, exclusion limit
w/v  weight per volume
Z  Z score
PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications


Conference Presentations

2006 International Biotechnology and Medical Science Student Forum, Beijing, China.
Oral presentation titled: The role of clusterin in extracellular protein quality control.

2007 World Conference of Stress, Budapest, Hungary.
Poster presentation titled: Its time to take out the trash! – Chaperone-dependent disposal of unfolded proteins via LDL superfamily receptors.

2007 International Society of Neurochemistry, Protein Misfolding and Neurodegenerative Disease Meeting, Dunk Island, Australia.
Oral presentation titled: The role of clusterin in extracellular protein quality control.
2008  5th Clusterin/ApoJ Workshop, Spetes, Greece
Oral presentation titled: The role of clusterin in extracellular protein quality control.

2008  AINSE 50th Anniversary Seminar Series, Lucas Heights, Australia
Oral presentation titled: The role of clusterin in extracellular protein quality control.
1 INTRODUCTION

Correct protein folding and the regulation of protein unfolding are fundamental biological processes that are carried out by elaborate cellular machinery. Much is known regarding the sophisticated intracellular systems for protein folding quality control, however, to date, little is known about corresponding systems for the extracellular environment. This review summarizes our current understanding of intracellular protein folding quality control and considers the likely importance of normally secreted extracellular chaperones including clusterin (CLU), in stabilizing extracellular proteins during stress-induced unfolding and preventing potentially pathological protein deposition.

1.1 Protein folding and unfolding

1.1.1 Protein folding

Proteins form the molecular machinery that controls almost every biological process and as such, their proper functionality is vital. For proteins, structure and function are inherently linked and this is governed by the unique sequence of amino acids that form the polypeptide backbone. This was demonstrated in the early 1970s using the model protein ribonuclease (Anfinsen, 1973). Anfinsen (1973) showed that complete reduction and reoxidation of ribonuclease in the presence of a strong denaturant results in the formation of numerous inactive disulfide-bonded structures. The restoration of thiol linkages and the removal of the denaturant returned the enzyme to its native conformation. From this, it was suggested that refolding is thermodynamically driven by interactions between amino acids resulting in the lowest free energy state and that the native conformation of a protein corresponds to the lowest free energy state under normal physiological conditions.

Interactions contributing to the stability of the native conformation include hydrogen bonding, ionic interaction, disulfide bonding and the packaging together of hydrophobic side-chains. Interactions between locally adjacent amino acids determine secondary structural elements including $\alpha$-helices and $\beta$-sheets while interactions between distant amino acids determine the three-dimensional or tertiary structure. An additional level of
Introduction

Structural complexity (quaternary structure) includes the assemblage of multiple polypeptide chains to form subunits of a larger entity, but is not seen in all proteins. Exactly how a protein reaches its native conformation is not completely understood. Small polypeptides can reach their native conformation in less than 50 μs (Mayor et al., 2003; Yang and Gruebele, 2003). Although it is generally accepted that proteins follow a folding pathway that leads to the most thermodynamically stable state, it is not known what processes enable a polypeptide to find this conformation rapidly when theoretically there are an enormous number of different non-native states that a polypeptide can adopt. It has been proposed that sampling of the conformational space becomes increasingly refined with each fluctuation in shape since native-like interactions are relatively more stable than non-native interactions and this ‘funnels’ the protein towards the most favourable form (Sali et al., 1994; Honig, 1999; Dinner et al., 2000; Dobson, 2003). While conditions to promote spontaneous protein folding may be generated in vitro, in reality, in vivo the crowded macromolecular environment does not favor successful spontaneous protein folding (Zimmerman and Minton, 1993). Consequently many proteins require help from molecular chaperones to reach their native conformation (Hartl, 1996). Molecular chaperones function by interacting with hydrophobic regions of the various partially unfolded intermediates, known as molten globule states, that a protein may form on its way towards the native conformation. Cycles of controlled binding and release prevent the aggregation of partially unfolded intermediates but do not contribute information to guide the folding process.

1.1.2 Protein unfolding

Considering the importance of adopting the native conformation, cells have extensive quality control mechanisms to monitor the progression of nascent polypeptides to mature proteins. However, nascent polypeptides may irreparably misfold if ribonucleic acid (RNA) modification, translational amino acid misincorporation or genetic mutations alter the primary sequence of the polypeptide. Additionally, native proteins are relatively unstable and may partially unfold when exposed to physiologically relevant stresses. Loss of conformational entropy is the major force opposing adoption of the native conformation. Given that protein stability is collectively determined by all of the interactions contributing to the native conformation, the stability of different proteins is highly variable and may change over the lifetime of a protein if it becomes modified.
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For example, damage of amino acids by reactive oxygen species (ROS) causes modifications including the formation of carbonyl derivatives which distort secondary and tertiary protein structure and result in unfolding, increased exposed hydrophobicity, aggregation and susceptibility to proteolysis (Davies and Delsignore, 1987). Cysteine, histidine, lysine and arginine residues are particularly susceptible to oxidation (Levine et al., 1994; Stadtman and Levine, 2003). There are numerous sources of ROS including mitochondria, peroxisomes, lipoxygenases and various other redox enzymes, inflammatory cytokines, exposure to ultraviolet light, ionizing radiation, chemotherapeutics and toxins (Finkel and Holbrook, 2000). While many antioxidant defenses exist within cells, aging and diseases such as atherosclerosis, arthritis, muscular dystrophy, cataract, pulmonary dysfunction, certain neurological disorders and some cancers are believed to be the result of free radical damage impairing cellular functions (Stadtman and Oliver, 1991). Additionally, heat stress (such as that observed in muscles during exercise) markedly increases the production of intracellular and extracellular ROS (Zuo et al., 2000). Aside from participating in reactions that produce ROS, heavy metals may disrupt salt bridges and disulfide bonds by ionic interactions. Salt bridges are susceptible to interference by many compounds including acids and bases due to their ability to exchange ionic partners. Mechanical stress may also disrupt amino acid interactions sufficiently enough to induce protein unfolding (Ker and Chen, 1998).

Protein thermostability is only partly understood despite its obvious importance to all living organisms. Efforts to increase our understanding have included sequence analysis and investigations of the structures of proteins from extreme thermophiles (Argos et al., 1979) and site-directed mutagenesis (Matthews et al., 1987). Using these strategies a number of physiochemical factors have been implicated in increased thermostability (Querol et al., 1996; Vogt et al., 1997). Regardless of their relative stability all proteins may be induced to unfold providing that the kinetic energy (e.g. heat) supplied to the system is sufficient. The events of protein unfolding are consistent regardless of temperature however, the rate at which unfolding can occur is highly dependent on the amount of heat supplied (Day et al., 2002). There are many reasons why proteins may experience elevated temperatures the human body, several examples are provided in Table 1.1.
Introduction

Table 1.1 Examples of conditions causing elevated temperatures in humans.
A number of conditions may cause heat stress in vivo. This table provides several examples of elevated body temperature in human patients.

<table>
<thead>
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<th>Condition</th>
<th>Example</th>
<th>Reference</th>
</tr>
</thead>
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<td>Infection</td>
<td>Persistent 38.5°C fever after Coxiella burnetii infection</td>
<td>(Pape et al., 2007)</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>Spiking fever in rheumatoid arthritis</td>
<td>(Gadoth and Hershkovitch, 1979)</td>
</tr>
<tr>
<td>Hormonal dysfunction</td>
<td>Fever of 39.4°C during thyroiditis</td>
<td>(Porsche and Brenner, 2006)</td>
</tr>
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<td>Environmental heat exposure</td>
<td>Tympanic and rectal temperatures of 40.3°C after a 3 day heat wave</td>
<td>(McGugan, 2001)</td>
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<tr>
<td>Exercise</td>
<td>Muscle temperatures as high as 40°C after 60 min of cycling</td>
<td>(Saltin and Hermansen, 1966)</td>
</tr>
<tr>
<td>Some medicines</td>
<td>Fevers between 40-42°C after administration of bleomycin</td>
<td>(Kumar and Reuler, 1986)</td>
</tr>
<tr>
<td>Severe trauma</td>
<td>Fever as high as 38.3°C after acute stroke</td>
<td>(Azzimondi et al., 1995)</td>
</tr>
<tr>
<td>Certain cancers</td>
<td>Temperatures up to 40°C in granulocytopenic cancer patients</td>
<td>(Viscoli et al., 1996)</td>
</tr>
</tbody>
</table>

The results of in vitro refolding studies have identified that, after the native conformation is lost, a protein may transition between many possible partially unfolded intermediate states. However, energy constraints favor the formation of certain non-native intermediates (Radford, 2000). These intermediate states are often partially folded, but have regions of unstructured polypeptide backbone and exposed hydrophobicity (Kopito, 2000). The coalescing together of exposed hydrophobic regions on partially unfolded proteins and the consequent formation of oligomeric aggregates is thermodynamically favorable. Protein aggregation is also promoted by the crowded nature of the intracellular environment, which influences the kinetics and equilibria of elementary isomerization and protein association reactions (Minton, 2000a). The aggregates formed may be amorphous or have an ordered arrangement, such as in amyloid fibrils (Sipe, 1994). The ‘on pathway’ and the ‘off pathway’ describe the possible
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fates of unfolding proteins (Kopito, 2000) (Figure 1.1). Under certain conditions, unfolding proteins may follow the ‘on pathway’ and be rescued and refolded back into their native conformation by molecular chaperones. Alternatively, unfolding proteins may follow the ‘off pathway’, which ends in the formation of ordered or amorphous protein aggregates that eventually precipitate when their solubility limit is reached.

Figure 1.1 Potential fates of unfolding proteins.
Unfolding proteins may under certain conditions: (i) follow the ‘on pathway’ (blue arrows) and be rescued and refolded back into their native conformation by molecular chaperones, or (ii) follow the ‘off pathway’ (red arrows) ending in the formation and precipitation of ordered or amorphous aggregates. Figure adapted from Kopito (2000).

1.2 Protein aggregation and disease

Controlled unfolding is important in many biological processes including protein translocation, degradation by proteases and regulation of enzyme activity. Additionally, it has been proposed that some proteins are dynamic and may constantly transition between less ordered forms and the native conformation (Dunker et al., 2001). However, uncontrolled unfolding and the consequent accumulation of protein aggregates are implicated in the pathology of many diseases collectively known as
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Protein Deposition Diseases (PDDs) (Table 1.2). PDDs include diseases arising from genetic errors (where proteins never attain their native conformation) and diseases arising from the accumulation of damaged proteins (where native proteins unfold). Many PDDs are late-onset (Carrell, 2005), suggesting that the underlying cause of the disease may be disruption or overwhelming of protein folding quality control mechanisms. Although the reasons for progressive impairment of fundamental physiological processes in aging is not fully understood, it is likely that the combination of declining protein folding quality control and the cumulative effects of exposure to thermal, ionic, heavy metal, shear and oxidative stress may be responsible for late-onset PDDs.

Table 1.2 Examples of PDDs and the proteins implicated in their pathology.

Protein unfolding and the deposition of protein aggregates are implicated in the pathology of many serious human diseases (Kelly, 1996; Carrell and Lomas, 1997; Hamidi et al., 1997; Carrell and Gooptu, 1999; Thomas et al., 1999; Soto, 2001). This table list some but not all known PDDs. * Denotes extracellular location.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>Amyloid-β *</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>Human islet amyloid polypeptide *</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>Haemodialysis-related amyloidosis</td>
<td>β2-Microglobulin *</td>
</tr>
<tr>
<td>Reactive amyloidosis</td>
<td>Amyloid-α *</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin</td>
</tr>
<tr>
<td>Creutzfeldt-Jakob disease</td>
<td>Prion protein *</td>
</tr>
<tr>
<td>Primary systemic amyloidosis</td>
<td>Immunoglobulin light chain *</td>
</tr>
<tr>
<td>Secondary systemic amyloidosis</td>
<td>Serum amyloid-β *</td>
</tr>
<tr>
<td>Hereditary non-neuropathic systemic amyloidosis</td>
<td>Lysozyme *</td>
</tr>
<tr>
<td>Corneal dystrophy</td>
<td>Kerato-epithelin *</td>
</tr>
<tr>
<td>Non-amylloidotic monoclonal IgG deposition disease</td>
<td>Immunoglobulin G *</td>
</tr>
<tr>
<td>Age-related macular degeneration (Drusen)</td>
<td>62 different proteins *</td>
</tr>
<tr>
<td>Renal disease</td>
<td>Tamm-Horsfall protein *</td>
</tr>
<tr>
<td>Hereditary renal amyloidosis</td>
<td>Fibrinogen *</td>
</tr>
</tbody>
</table>
1.2.1 Types of protein deposits

In many PDDs including Alzheimer’s disease, type II diabetes, systemic amyloidosis and transmissible spongiform encephalopathy, the deposition of insoluble aggregates occurs as fibrillar structures called amyloid. Amyloid fibrils consist of a helical arrangement of β-sheets parallel to the long axis of the fibril (diameter 70-120 Å) with their component β-strands perpendicular to the long axis (Sunde et al., 1997). Many structurally diverse proteins are known to form amyloid suggesting that this type of fibril may be formed by interactions between the polypeptide backbone of all proteins (Dobson, 2001). In other PDDs the nature of the protein deposits is fibrillar, but not amyloid. Non-amyloid fibrillar structures include Lewy bodies, which are found in Parkinson’s disease, Alzheimer’s disease, some other forms of dementia and occasionally Shy-Drager’s syndrome. Lewy bodies generally consist of a dense core of filaments and granular material radially surrounded by additional filamentous structures. However, randomly arranged Lewy body filaments have been identified in the cerebral cortex of dementia patients (Kosaka, 1978). In Pick’s disease, non-amyloid fibrillar deposits known as Pick bodies accumulate in specific regions of the central nervous system and contribute towards neuronal damage (Hof et al., 1994). Pick bodies consist of randomly oriented straight filaments and periodical, paired, twisted filaments of abnormal tau protein (Murayama et al., 1990).

Amorphous, non-filamentous extracellular aggregates formed by immunoglobulin G (IgG) light chain and/or IgG heavy chain are characteristic of non-amyloidotic monoclonal IgG deposition disease (NAMIDD) (Buxbaum and Gallo, 1999; Lin et al., 2001). The clinical manifestations of NAMIDD are similar to those of amyloid-forming IgG deposition disease and include glomerulonephritis as result of an organ-compromising accumulation of IgG in the kidneys. Pathologic non-amyloid deposits have also been identified in certain corneal dystrophies. In granular Groenouw type I corneal dystrophy, the progressive accumulation of non-amyloid deposits contributes to corneal opacity and the loss of vision (Korvatska et al., 1999). In Avellino corneal dystrophy both amyloid and non-amyloid material are found co-localized in the cornea (Korvatska et al., 1999). Drusen are amorphous extracellular deposits that accumulate in patients with age-related macular degeneration (AMD). In healthy eyes drusen are not found in the macula, however they may exist in the retinal periphery and their size and
number are considered a risk factor for developing AMD later in life (Lewis et al., 1986). Many different proteins are found in drusen with crystallins, lactoglobulin, CLU, complement component 9, human serum albumin (HSA), haemoglobin and vitronectin being some of the most abundant and common (Crabb et al., 2002).

Oxidized IgG aggregates are found in many acute and chronic inflammatory states including rheumatoid arthritis (Jasin, 1983). These diseases may be considered a special class of PDDs since oxidized IgG may persist in extracellular fluids as soluble aggregates. IgG aggregates have immune complex-like properties and stimulate neutrophils to release ROS thereby inducing the aggregation of previously unaggregated IgG (Lunec et al., 1985). The self-perpetuating cycle of neutrophil activation and oxidized IgG aggregation and the creation of damaging ROS are likely to be very important in the pathology of rheumatoid diseases. Protein deposits are also a hallmark of many cancers. For example the deposition of fibrinogen (FGN) has been reported in breast cancer (Costantini et al., 1991), mesothelioma (Wojtukiewicz et al., 1989a), colon cancer (Wojtukiewicz et al., 1989b) and lymphoma (Costantini et al., 1992). Immunoglobulins are also known to form deposits in the nodes of immunoblastic lymphoma patients (Ough et al., 1983). In cancer, extracellular protein deposition forms a scaffold to support the processes of proliferation, migration and angiogenesis that are necessary for tumour growth (Nagy et al., 1988; Rybarczyk and Simpson-Haidaris, 2000).

1.2.2 Cytotoxicity

While the cytotoxicity of protein aggregates is the focus of many studies, it is unknown whether the deposition in tissues of protein aggregates is, in fact, the critical event in pathogenesis. Temporal studies of transgenic animals that express abnormal aggregating human proteins have not been very successful at identifying the precise pathogenic species. In diseases such as non-neurological systemic amyloidoses, it appears likely that the large quantity of protein aggregates deposited in tissues cause the clinical symptoms of the disease. However, at least in the case of amyloid diseases, there is increasing evidence to suggest that the toxic species may occur early in the aggregation prior to the formation of mature fibrils. One study of amyloid formation has reported that amyloid-beta (Aβ) peptide, which is important in Alzheimer’s disease pathogenesis, was cytotoxic once amyloidogenic fibrils were formed, but not while it was present as an
Introduction

early-stage amorphous aggregate (Lorenzo and Yanker, 1994). Conversely, evidence of tissue damage before amyloid fibrils were formed was documented in a study of transgenic mice expressing human islet amyloid polypeptide, which is relevant in type II diabetes (Jason et al., 1996). Similarly, a study of transgenic mice expressing different mutant forms of Aβ precursor protein showed that behavioural and cognitive defects in the mice preceded amyloid plaque formation (Moechars et al., 1999). Another recent report provided evidence that misfolded proteins are inherently cytotoxic during the early stages of amyloid formation, but that the mature aggregates themselves are not cytotoxic (Bucciantini et al., 2002). If mature aggregates are not cytotoxic then it is possible that the formation of mature aggregates may indeed be cytoprotective. This could be particularly significant in the case of intracellular aggregates, since the localization of unfolding protein species may increase the efficiency of their autophagic capture and subsequent degradation.

Perhaps the most popular theory regarding the cytotoxicity of protein aggregates involves the production of ROS. It has been suggested that cumulative oxidative damage may be responsible for many PDDs including Alzheimer’s disease (Practico, 2002) and AMD (Imamura et al., 2006). While oxidative stress is a known factor contributing to the unfolding and aggregation of proteins, there is increasing evidence that the accumulation of protein aggregates causes elevated ROS (Davis, 1996; Matsuoka et al., 2001; Bucciantini et al., 2004). The mechanism by which this occurs remains unclear, however, it may involve the disruption of ion gradients across cell membranes. For example, pre-fibrillar amyloid aggregates share certain structural similarities with other membrane pore-forming proteins and consequently the formation of unregulated ion channels has been proposed as a possible mechanism for their cytotoxicity (Kourie and Shorthouse, 2000; Kourie and Henry, 2002; Stefani and Dobson, 2003). One suggestion is that ROS are elevated as a result of increased oxidative metabolism to produce adenosine triphosphate (ATP) needed for pumping excess calcium out of the cells (Squier, 2001). Alternatively, it has been proposed that Aβ peptide interacts directly with metal ions such as Cu^{2+} and Fe^{3+} (which are found at particularly high levels in amyloid plaques (Lovell et al., 1998)) to produce damaging H_2O_2 (Huang et al., 1999). As previously mentioned, a likely possibility for the generation of extracellular ROS in rheumatoid arthritis is stimulation of neutrophils by aggregated IgG (Lunec et al., 1985). Similarly, the activation of microglia by amyloid-forming Aβ
Introduction

peptide or prion peptides and the subsequent generation of ROS have been implicated in neurodegenerative diseases (Marella and Chabry, 2004; Veerhuis et al., 2005; Garcao et al., 2006). The release of cytokines, such as interleukin (IL)-6 and IL-1β, have also been linked to neuronal damage (Marella and Chabry, 2004). Moreover, minocycline (an inhibitor of microglia activation) was found to be neuroprotective in a murine model of Parkinson’s disease (Wu et al., 2002). While autoantibodies against amyloid forming structures have been found in Alzheimer’s patients (Gruden et al., 2004), it is unclear whether classical autoimmunity contributes to the pathology of this disease although this has been suggested for other PDDs including AMD (Umeda et al., 2005) and renal disease (Fasth et al., 1981).

Whether the causes of protein aggregate toxicity in individual diseases relate to physical organ/tissue disruption resulting from the deposition of large insoluble aggregates, or to cytotoxic effects of smaller soluble oligomeric aggregates, to develop new therapies, strategies will need to target the relevant underlying molecular mechanism(s). Regardless of the mode of toxicity, it appears likely that PDDs arise when normally efficient protein folding quality control mechanisms are overwhelmed. Therefore, increasing our understanding of protein folding quality control mechanisms will be critical in efforts to combat PDDs.

1.3 Protein folding quality control

A great deal of cellular energy is invested in processes that establish and maintain a protein’s native conformation, or degrade damaged or aged proteins. This includes a post-translational control system to deal with mistakes in the transfer of genetic information from genes to functional proteins, and mechanisms to repair or degrade proteins that have lost their native conformation. Since the native conformation of a protein is determined by its amino acid sequence, quality control of the correct transcription of RNA and translation of the polypeptide sequence is extremely important. The exonuclease ability of deoxyribonucleic acid (DNA) polymerase and the proof-reading activity of transfer ribonucleic acid (tRNA) are the first line of defence in correct protein folding. However, mistakes in transcription or translation are not always rectified and environmental conditions may promote the unfolding of correctly folded
proteins. Under these circumstances, the cells post-translational protein folding quality control system may rescue abnormal proteins or target them for destruction.

1.3.1 Intracellular protein folding quality control

In a healthy cell the major fates of stressed proteins are degradation or rescue by refolding. Molecular chaperones are central to refolding pathways but also play an important role in the targeting of stressed proteins to the proteasome or lysosomes for degradation. Together these processes compensate for the relative instability of proteins ensuring that the current pool of proteins remain in their native conformation and preventing their potentially pathological accumulation (Figure 1.2). Extracellular proteins exit the cell after folding, maturation and modification in the endoplasmic reticulum (ER). As it is an important site of protein synthesis and processing, the ER possesses extensive protein folding quality control. Once polypeptides reach the ER, mistakes in folding or processing are recognized by an intricate system of chaperones and resident proteases. If non-native proteins accumulate, an unfolded protein response (UPR) may be elicited via Ire1p (a transmembrane ER protein), which ultimately results in activation of Hac1p (a transcription factor) and the upregulation of molecular chaperones and translocation proteins (Brodsky and Mc Cracken, 1999). Alternatively, non-native proteins may be targeted for immediate degradation and trafficked to lysosomes or the proteasome. Non-native proteins destined for secretion that evade the ER protein folding quality control may be recognized as abnormal after reaching the Golgi apparatus. This results in retrograde transport of the protein back to the ER or re-targeting of the protein to a lysosome for degradation (Arvan et al., 2002). Together these actions ensure that only correctly folded proteins are secreted from normal healthy cells. However, little is known regarding corresponding mechanisms that may prevent these native exported proteins from accumulating in extracellular spaces after stress-induced unfolding.
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1.3.1.1 The ubiquitin-proteasome system

The degradation of non-native proteins via the ubiquitin-proteasome system is one mechanism that prevents the intracellular accumulation of protein aggregates. In this system, abnormal or aged proteins are tagged with four or more molecules of ubiquitin by the ubiquitin activating enzyme (E1), a ubiquitin carrier protein (E2) and the ubiquitin-protein ligase (E3) (Hershko and Ciechanover, 1989). This ubiquitin tail interacts with a regulatory subunit of the 26S proteasome leading to translocation of the protein into the central proteasomal chamber, which contains six proteolytic sites (Hershko and Ciechanover, 1989; Sherman and Goldberg, 2001). Of the six proteolytic sites, two are trypsin-like, two are chymotrypsin-like and two cleave after acidic amino acid residues; their ATP-dependent activity cleaves the protein into peptides that are small enough to diffuse out of the proteasome (Kisselev et al., 1999). These peptides are
Introduction

Further hydrolyzed by peptidases as they enter the cytosol and the free amino acids are recycled into new proteins (Hershko and Ciechanover, 1989; Kisselev et al., 1999; Sherman and Goldberg, 2001).

Proteins in the ER are subjected to extensive quality control during their maturation. Non-native proteins are subjected to ER-associated protein degradation (ERAD) which culminates in retro-translocation of the non-native structures to the proteasome after ubiquitination. The efficiency and selectivity of the ubiquitin-proteasome system is attributed to the properties of ubiquitin-protein ligases including CHIP, a ubiquitin-protein ligase that specifically targets non-native proteins for ubiquitination with the assistance of the molecular chaperones, heat-shock protein (Hsp)90 and heat-shock cognate protein (Hsc)70 (Murata et al., 2001). BAG-1, another co-factor of Hsc70, is responsible for recruiting the chaperone-ubiquitinated protein complex to the proteasome and directing the release of the chaperone substrates (Luders et al., 2000). Together CHIP, BAG-1 and molecular chaperones play an important role in the selective proteolysis of non-native proteins via the ubiquitin-proteasome system (Figure 1.3). Defects in the 26S proteasome have been identified in the brains of sufferers of Alzheimer’s disease (Keller et al., 2000), Parkinson’s disease (McNaught et al., 2001) and amyotrophic lateral sclerosis (Johnston et al., 2000). In these same diseases, ubiquitinated proteins have been recognized as participating in the formation of insoluble aggregates (Johnson, 2000). In an in vitro system, protein aggregation was observed to significantly disrupt the normal function of the ubiquitin-proteasome system (Bence et al., 2001). The investigators suggested that inhibition of the ubiquitin-proteasome system by aggregated proteins may promote the accumulation of further aggregates by positive-feedback and explain the progression of some PDDs.
Introduction

Figure 1.3 Targeted degradation of non-native proteins via the ubiquitin-proteasome pathway.
(1) During conditions of cellular stress native proteins become partially unfolded. (2) Non-native proteins are rescued by (3) the intracellular chaperones Hsp90 or Hsc70. (4) The cofactor chaperone known as CHIP and E2 ubiquitin conjugating enzyme are recruited to the complex. (5) CHIP and E2 facilitate the ubiquitination of the stressed client protein. (6) Association of BAG-1, a ubiquitin domain protein, to the chaperone targets the complex to the proteasome for degradation. Figure adapted from Hohfeld et al. (2001).

1.3.1.2 Lysosomal degradation

There are two major intracellular routes for the trafficking of non-native proteins to lysosomes: chaperone-mediated autophagy and the autophagic capture of proteins sequestered in aggresomes (cytoplasmic microtubule-dependent inclusion bodies) (Figure 1.4). In chaperone-mediated autophagy, proteins containing in their sequence a lysine-phenylalanine-glutamine-arginine-glutamic acid motif are targeted to lysosomes for degradation by Hsc73 (Dice, 1990). The Hsc73-protein complex binds to lysosome-associated membrane protein type 2a (Lamp 2a) and interacts with a second molecular chaperone called lysosomal Hsc73 (ly-Hsc73), which facilitates the transport of the protein into the lysosome where it is enzymatically degraded (Curevo et al., 1997).
Cytoplasmic components including aggresomes may be captured in autophagosomes, which are membranous structures formed by ER cisternae, and degraded after maturation of the autophagosomes into lysosomes (Kopito, 2000). Aggresomes form around microtubule organizing centres by directed transport of misfolded proteins along microtubules (Kopito and Sitia, 2000). They are characterized by the presence of proteinaceous deposits that contain ubiquitin, proteasome subunits and molecular chaperones (Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999). Aggresome formation may be induced by disruption of the proteasome (Wojcik et al., 1996) and is prevented by over-expression of the chaperone Hsp70 (Dul et al., 2001). This indicates that overwhelming or impaired performance of protein folding quality control mechanisms may be the trigger for aggresome formation. Moreover, it has been suggested that the formation of aggresomes increases the efficiency of autophagic capture and the subsequent degradation of protein aggregates in lysosomes (Johnston et al., 1998; Kopito, 2000). The association of chaperones with aggresomes is likely to be due to failed attempts to rescue proteins on the unfolding pathway, however there is also evidence that some chaperones may be targeted to aggresomes via interactions with the motor protein known as dynein (Harrell et al., 2002; Harrell et al., 2004).

*Introduction*
Figure 1.4 Lysosomal degradation of non-native proteins.
Non-native proteins may be directed to form aggresomes via transport along microtubules and captured in membranous structures formed by the ER during autophagy. After pinching off from the ER the vesicle containing the aggresome fuses with a lysosome where the protein aggregates are degraded. Alternatively, stressed proteins may be targeted to the lysosome by the chaperone Hsc73. Hsc73 binds to Lamp 2a embedded in the lysosomal membrane and transport of the non-native protein into the lysosome is facilitated by another chaperone ly-Hsc73.

1.3.1.3 Molecular chaperones

Although all the information required for a nascent polypeptide to adopt its native conformation is encoded in the amino acid sequence, protein folding is not necessarily a spontaneous process. Cellular machinery and the input of metabolic energy are required to facilitate the folding of many proteins. Molecular chaperones are a diverse group of proteins that may assist the folding of nascent polypeptides, help translocate proteins across cellular membranes and mediate protein trafficking, participate in signal transduction cascades, act to prevent protein aggregation during conditions of cellular stress or facilitate the refolding of stressed (partially unfolded) proteins (Hartl and Hayer-Hartl, 2002; Muchowski, 2002). Molecular chaperones that directly assist in protein folding, such as Hsp70 and chaperonins, function by transiently binding to exposed hydrophobic regions on partially unfolded proteins, which are usually buried within the core of a protein in its native conformation (Minton, 2000a). The binding and
Introduction

The Hsp family include several molecular chaperones, antioxidant enzymes and components of the ubiquitin-proteasome pathway that function together to defend the cell against the accumulation of non-native proteins and help restore cellular homeostasis after conditions of stress. Large numbers of Hsps have been identified in protein aggregates and aggresomes, which reflects their affinity for partially unfolded hydrophobic polypeptide domains (Kopito, 2000; Sherman and Goldberg, 2001). Small heat-shock proteins (sHsps) are a subclass of the Hsp family and consist of an array of 15-30 kDa proteins that all have a tendency to aggregate (de Jong et al., 1993). This family of proteins is structurally diverse and monomeric units of sHsps are usually found assembled into large oligomers, depending on the specific protein. These proteins are molecular chaperones, although they do not directly participate in the folding of partially unfolded polypeptides. It appears that sHsps prevent protein precipitation by forming soluble high molecular weight (HMW) complexes with stressed (partially unfolded) proteins; however, this action does not protect the function of denatured enzymes or restore their native conformation (Horwitz, 1992; Carver et al., 1994). Stabilization of partially unfolded proteins (in a soluble state) by sHsps may allow other molecular chaperones, such as Hsp70, to assist in their refolding once cellular conditions have stabilized (Lee et al., 1997). Overexpression of sHsps is cytoprotective under conditions of thermal, ionic or oxidative stress (Arrigo and Landry, 1994; Arrigo, 1998).
1.3.2 Extracellular protein protein folding quality control

A 70 kg human contains around 5 L of blood and 10 L of other extracellular fluids including interstitial fluid, cerebrospinal fluid and intraocular fluid. Proteins are a significant component of extracellular fluids, for example whole plasma contains around 7.5% protein by mass (Georgiou et al., 2001). This includes secreted proteins and proteins that may have been shed from the cell surface or lost from damaged tissues. It has been suggested that over 10,000 different proteins may be normally present in plasma at low levels (Wrotnowski, 1998). In the extracellular environment proteins are exposed to various stresses that may contribute to extracellular protein unfolding, which do not affect their intracellular counterparts. This includes the hydraulic force of plasma being pumped around the body, since hydrodynamic shear stress is known to contribute to protein unfolding (Ker and Chen, 1998; Schneider et al., 2007). Additionally, the extracellular environment is relatively more oxidising compared to the cytosol (Sitia and Braakman, 2003). Increased plasma protein oxidation (as determined by measuring protein carbonyl formation) is a characteristic of many disease states including Alzheimer’s disease (Conrad et al., 2000), coronary artery disease (Kaneda et al., 2002) and uremia (Himmelfarb and McMonagle, 2001). The most abundant blood protein, HSA is known to be vulnerable to damage by ROS (Davies and Delsignore, 1987; Himmelfarb and McMonagle, 2001).

To date, little is known about what (if any) specific mechanisms for protein folding quality control operate in extracellular space. However, evidence has been gathered suggesting that they are likely to exist. This includes the observation that unfolded proteins are degraded more rapidly than native proteins in vivo (Margineanu and Ghetie, 1981). Additionally, polymorphonuclear leukocytes preferentially catabolize denatured proteins compared to native proteins (Bocci et al., 1968) and lysosomal enzymes are implicated in this process (Coffey and de Duve, 1968). Furthermore, liposomes with exposed surface hydrophobicity are cleared from circulation more rapidly than those with a hydrophilic outer layer (Senior et al., 1991) and certain modifications of HSA including oxidation and mutations that increase surface hydrophobicity and negative charge enhance its clearance from circulating blood (Iwao et al., 2006). These findings suggest that shared characteristics of non-native proteins may target them for clearance from extracellular spaces and/or degradation.
1.3.2.1 Extracellular proteolytic systems

There are several classes of extracellular proteases with diverse biological functions including matrix degradation, angiogenesis, growth factor bioavailability, cytokine modulation, receptor shedding, cell migration, proliferation and apoptosis. Despite this, extracellular mechanisms for the targeted degradation of structurally abnormal proteins have not yet been identified. Some evidence for the existence of extracellular ubiquitin-proteasome-like elements has been reported. Extracellular ubiquitination and proteasome-mediated degradation of proteins has been reported for sperm in the seawater surrounding an ascidian (Urochordata) during fertilisation (Sawada et al., 2002). However, the relevance of this in the context of mammalian extracellular protein folding quality control is probably negligible. Interestingly, an *in vitro* study of the Aβ precursor protein has found that extracellular, but not intracellular forms of Aβ precursor protein are targeted to the ubiquitin-proteasome pathway (Gregori et al., 1994). Furthermore, extracellular proteasome-like structures have been isolated from serum-free media conditioned by C6 astrocytoma cells (Vaithilingam et al., 1995) and non-lysosomal proteolytic structures have been measured in normal human plasma and diseased patients at 7.7-200 ng/mL (Akarsu et al., 2001; Savas et al., 2003) and 2.1 – 2.4 μg/mL (Lavabre-Bertrand et al., 2001), respectively. Significantly elevated levels of circulating proteasome have been detected in patients with solid tumours or hemopoietic cancer (Lavabre-Bertrand et al., 2001) autoimmune myositis, systemic lupus erythematosus, primary Sjogren’s syndrome, rheumatoid arthritis, and autoimmune hepatitis (Egerer et al., 2001). Egerer et al. (2002) reported that the circulating proteasome subunits were present in native-associated, native-disassociated or degraded forms and suggested that the level of circulating proteasome may be the result of autoimmune inflammatory processes and/or reflect the magnitude of cellular damage. The reported levels of plasma proteasome are around 300 times less than that observed within cells. Moreover, an extracellular ubiquitin-proteasome-like system would require ATP, which is around 1,000 times less abundant in extracellular fluids compared to the cytosol. Thus, it is unlikely that such a system (if it existed) could efficiently deal with large-scale presentation of stressed extracellular protein that might accompany a pathological event.
1.3.2.2 Normally intracellular chaperones found extracellularly

Until recently, molecular chaperones had only been considered in the intracellular context. Interest in their possible extracellular roles was fuelled by the observation that molecular chaperones that are primarily sequestered in intracellular compartments may be released into extracellular fluids from necrotic cells. Supporting this idea, six cell surface receptors have been identified as binding to predominately intracellular Hsp chaperones (Table 1.3). These receptors are located on antigen presenting cells and their interactions with Hsps have two distinct consequences: (i) re-presentation of Hsp-chaperoned peptides (Suto, 1995) and (ii) the release of pro-inflammatory cytokines (Asea et al., 2000; Kol et al., 2000). While this is likely to have importance in vivo, it is probable that in the instance of mass presentation of partially unfolded protein, such as might be encountered during chronic inflammation, the chaperone capabilities of normally intracellular chaperones (e.g. Hsp70 present in plasma at < 10 ng/mL) would be quickly overwhelmed. Furthermore, intracellular chaperones require ATP to effect protein refolding which as previously mentioned is scarce in extracellular fluids.

Table 1.3 Receptors for normally intracellular chaperones.
A number of intracellular chaperones interact with cell surface receptors. This may have physiological significance since intracellular chaperones may reach extracellular spaces during disease or cell damage. Table adapted from Binder et al. (2004) and Calderwood et al. (2007).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
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</thead>
<tbody>
<tr>
<td>LRP</td>
<td>gp96</td>
</tr>
<tr>
<td></td>
<td>Hsp70</td>
</tr>
<tr>
<td></td>
<td>Calreticulin</td>
</tr>
<tr>
<td></td>
<td>Hsp90</td>
</tr>
<tr>
<td>Lox-1</td>
<td>Hsp70</td>
</tr>
<tr>
<td>SR-A</td>
<td>gp96</td>
</tr>
<tr>
<td>TLR-2 and TLR-4</td>
<td>Hsp70</td>
</tr>
<tr>
<td></td>
<td>gp96</td>
</tr>
<tr>
<td></td>
<td>Hsp60</td>
</tr>
<tr>
<td>CD14</td>
<td>Hsp70</td>
</tr>
<tr>
<td>CD40</td>
<td>Hsp70</td>
</tr>
<tr>
<td>SREC-1</td>
<td>Hsp70</td>
</tr>
<tr>
<td>FEEL-1</td>
<td>Hsp70</td>
</tr>
</tbody>
</table>
1.3.2.3 Extracellular chaperones

The discovery of three normally secreted extracellular proteins with sHsp-like chaperone function, namely CLU, haptoglobin (Hp) and α₂-macroglobulin (α₂M) is an important advance towards understanding extracellular protein folding quality control. While CLU, Hp and α₂M do not appear to possess independent protein refolding activity, their ability to stabilize stressed (partially unfolded) proteins is likely to be very important in preventing potentially pathological protein deposition. Recently, interactions between these extracellular chaperones and receptors that are capable of internalizing and degrading their ligands have been suggested as a possible route for the disposal of stressed extracellular proteins.

1.3.2.3.1 Clusterin (CLU)

The heat-stable protein CLU was named for its propensity to cause cell clustering in vitro (Fritz et al., 1983). CLU is found in human blood plasma at around 35-105 μg/mL (Murphy et al., 1988), in cerebral spinal fluid (CSF) at 1.2-3.6 μg/mL (Choi et al., 1990), and in seminal plasma at around 2-15 mg/mL (Choi et al., 1990). Additionally, CLU is also found in numerous other biological fluids including breast milk, ocular fluid and urine (Arnow et al., 1993). CLU has an extremely broad biological distribution and exhibits high sequence homology (70-80%) across a wide range of mammalian species (Jenne and Tschopp, 1992). This suggests that CLU performs some fundamentally important function in vivo. Many biological functions for CLU have been proposed including roles in lipid transport (de Silva et al., 1990), sperm maturation (Hermo et al., 1994), complement regulation (Jenne and Tschopp, 1992), programmed cell death (Buttyan et al., 1989) and membrane recycling (Jordan-Starck et al., 1992). There have been many reports of CLU’s importance in the process of apoptosis however, perplexingly, depending on the model, CLU appears to be either pro-apoptotic or anti-apoptotic (Trougakos and Gonos, 2002).

Sequence analysis has allowed for the prediction of structural elements in CLU (Figure 1.5). Using helical wheel analysis, three amphipathic α-helices (residues 173–184, 234–250 and 424–441) have been predicted (de Silva et al., 1990). In addition, two predicted coiled-coil helices (residues 40-99 and 318-350) were suggested by COILS
Introduction

algorithms (Lupas, 1991). These five predicted \( \alpha \)-helical regions are thought to be significant in the chaperone activity of CLU. It has been proposed that the \( \alpha \)-helical regions form a molten globule-like binding pocket that is the site of interaction for a variety of ligands (Bailey et al., 2001). After proteolytic removal of a 22 amino acid signal peptide and cleavage between arginine\( _{227} \)-serine\( _{228} \), the \( \alpha \) chain (residues 23-227) and \( \beta \)-chain (residues 228-449) of mature human CLU (50 kDa before glycosylation) are formed. Approximately 17-27\% of the mass of CLU is comprised of branched, sialic acid-rich, N-linked carbohydrates (Kapron et al., 1997). The high carbohydrate content of CLU has impeded structural analysis using x-ray crystallography. Interestingly, deglycosylation of CLU does not affect the overall secondary structure of the molecule as determined by circular dichroism analysis (Stewart et al., 2007). Moreover, the binding affinity for many native ligands is improved and deglycosylation does not hinder the chaperone activity of the molecule (Stewart et al., 2007). A mutant form of CLU, which lacks the six N-linked carbohydrate moieties, may be suitable for future structural analysis (Wilson, pers. commun.). Recently, it has been suggested that CLU may also have important intracellular functions after reports of pronuclear (cytoplasmic) and nuclear CLU, which may be expressed through alternative splicing of the CLU gene resulting in an N-terminally truncated mRNA lacking the leader peptide (Leskov et al., 2003).
Introduction

CLU has been identified as an extracellular chaperone that preferentially binds to stressed (partially unfolded) proteins and prevents their slow aggregation on the ‘off’ folding pathway (Humphreys et al., 1999; Wilson and Easterbrook-Smith, 2000; Poon et al., 2002b). The chaperone activity of CLU is similar to, but more potent than, the activity of intracellular sHsps (Poon et al., 2000; Poon et al., 2002b). A unique mode of activation has been suggested for CLU whereby reduced pH increases the extent to which it exposes hydrophobic regions to solvent, thereby increasing the efficiency of its chaperone action (Poon et al., 2002a). It has been observed that CLU-depleted plasma is highly susceptible to protein aggregation and precipitation in vitro (Poon et al., 2000). Other investigations have identified a variety of stressed and unstressed ligands for CLU, which appear to have discrete binding sites (Lakins et al., 2002). Furthermore, interactions between CLU and certain members of the low density lipoprotein (LDL) receptor superfamily have recently been documented (Kounnas et al., 1995; Zlokovic et al., 1996; Mahon et al., 1999; Calero et al., 2000; Lakins et al., 2002; Bajari et al., 2003). A binding site on CLU for megalin, a member of the LDL receptor superfamily, is known.
Introduction

to exist and is distinct from the binding sites on CLU for other ligands, but is not yet fully characterized (Lakins et al., 2002).

CLU is found associated with extracellular protein deposits in numerous diseases including normal peripheral drusen and drusen in AMD patients (Crabb et al., 2002). CLU is also found associated with membrane attack complex (MAC) in renal immunoglobulin deposits (French et al., 1992), prion deposits in Creutzfeldt-Jakob disease (Sasaki et al., 2002; Freixes et al., 2004), and amyloid plaques and soluble Aβ peptide in Alzheimer’s disease (Ghiso et al., 1993; Calero et al., 2000). The presence of CLU in protein deposits may be indicative of a failure in or the overwhelming of the machinery responsible for quality control of extracellular protein folding. CLU binds to soluble Aβ peptide with high affinity ($K_d \sim 2$ nM) in vitro (Matsubara et al., 1995). Binding of CLU to Aβ peptide prevents further aggregation and may be neuroprotective. However, soluble CLU-associated Aβ peptide has been reported to contribute to oxidative stress (Oda et al., 1995). Recently it has been reported that the molar ratio of CLU to fibril forming protein determines whether CLU promotes or inhibits amyloid formation and related cytotoxicity (Yerbury et al., 2007).

The overexpression of CLU has been reported in a diverse range of diseases (Table 1.4). Additionally, CLU is upregulated in experimental models of pathological stress including oxidative stress (Strocchi et al., 2006), shear stress (Ubrich et al., 2000), proteotoxic stress (generated by inhibition of the proteasome) (Loison et al., 2006), heat stress (Michel et al., 1997), ionizing radiation (Criswell et al., 2005) and exposure to heavy metals (Trougakos et al., 2006). In CLU knock-out mice, damage to testicular cells is increased after heat-shock and the removal of damaged cells is impaired (Bailey et al., 2002). After myosin-induced auto-immune myocarditis, cell damage is also more severe in CLU-deficient mice (McLaughlin et al., 2000) and post-ischemic brain injury is more severe (Wehrli et al., 2001). Together this data suggests that stress-induced increase in CLU expression is a cytoprotective response. In an Alzheimer’s disease model, compared to control mice, CLU knock-out or apoE knock-out mice accumulate less fibrillar Aβ peptide with no difference in total Aβ peptide accumulation (DeMattos et al., 2004). In the same study, double CLU/apolipoprotein E (ApoE) knock-out mice
showed early disease onset and a marked increase in Aβ peptide levels and amyloid formation. The researchers concluded that apoE and CLU work synergistically to inhibit the deposition of fibrillar Aβ. A more recent study has demonstrated that CLU knock-out mice develop progressive glomerulopathy which is characterized by the accumulation of insoluble protein deposits in the kidneys (Rosenberg et al., 2002). This directly implicates CLU in the clearance of potentially pathological aggregating proteins, although the precise mechanism underlying this has yet to be described.

**Table 1.4 Overexpression of CLU in disease.**
The expression of the extracellular chaperone CLU is upregulated in numerous serious diseases. Table adapted from Rosenberg and Silkensen (1995).

<table>
<thead>
<tr>
<th>Category</th>
<th>Disease/Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal Disease</td>
<td>Glomerular Nephritis</td>
</tr>
<tr>
<td></td>
<td>Cystic diseases</td>
</tr>
<tr>
<td></td>
<td>Acute renal tubular injury</td>
</tr>
<tr>
<td></td>
<td>Chronic tubulointersital disease</td>
</tr>
<tr>
<td></td>
<td>Allograft rejection</td>
</tr>
<tr>
<td>Neurodegenerative Disease</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td></td>
<td>Pick’s disease</td>
</tr>
<tr>
<td></td>
<td>Scrapie</td>
</tr>
<tr>
<td></td>
<td>AIDS encephalopathy</td>
</tr>
<tr>
<td></td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td></td>
<td>Epilepsy</td>
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<tr>
<td></td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td></td>
<td>Cerebral infarction</td>
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<tr>
<td>Cancer</td>
<td>Breast</td>
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<td></td>
<td>Uterine</td>
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<tr>
<td></td>
<td>Glioma</td>
</tr>
<tr>
<td></td>
<td>Renal</td>
</tr>
<tr>
<td>Other</td>
<td>Diabetes</td>
</tr>
<tr>
<td></td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td></td>
<td>Myocardial Infarction</td>
</tr>
</tbody>
</table>
1.3.2.3.2 Haptoglobin (Hp)

Hp is a secreted glycoprotein found in most body fluids. Its plasma concentration is between 0.3-2 mg/mL (Bowman and Kurosky, 1982) and it is found in CSF between 0.5-2 μg/mL (Sobek and Adam, 2003). Sequence analysis has identified Hp as a chymotrypsinogen-like serine protease homolog, although it has a distinct biological function (Kurosky et al., 1980). Humans may display one of three different Hp phenotypes (Hp 1-1, Hp 1-2 or Hp 2-2) depending on the presence of two principal alleles (Hp1 and Hp2) coding for the α and β subunits which associate covalently via disulphide linkage. The α¹, α² and β chain peptides are 9.2 kDa, 15.9 kDa and 27.2 kDa, respectively (Kurosky et al., 1980). Similar to CLU, Hp is heavily glycosylated.

Hp has many known biological functions, however, it is best known as a haemoglobin binding protein. The non-covalent interaction between Hp and haemoglobin is particularly strong with a reported $K_d \sim 10^{-15}$ M (Dobryszynka, 1997). This interaction prevents the loss of haemoglobin and iron via glomerular filtration by redirecting the Hp-haemoglobin complex to the liver (Giblett, 1968). The interaction of Hp with haemoglobin also reduces the amount of free haemoglobin and iron available to catalyse oxidation reactions (Gutteridge, 1987), and has an inhibitory effect on nitric oxide (Edwards et al., 1986) and prostaglandin synthesis (Lange, 1992). Hp also has a bacteriostatic effect on organisms unable to obtain heme from the haemoglobin-Hp complex (Barclay, 1985) and appears to play an important role in angiogenesis (Cid et al., 1993). Finally, Hp has been implicated in the regulation of lymphocyte transformation (Baskies et al., 1980).

Recently, Hp has been characterized as a molecular chaperone that is capable of inhibiting the stress-induced precipitation of a range of proteins in vitro (Pavlicek and Ettrich, 1999; Yerbury et al., 2005a). It appears to function similarly to shsp and CLU by forming soluble HMW complexes with proteins on the unfolding pathway. Depletion of Hp from human plasma significantly increased stress-induced precipitation of endogenous plasma proteins (Yerbury et al., 2005a). This acute phase protein is upregulated during a variety of conditions including infection, neoplasia, pregnancy, trauma, acute myocardial infarction and other inflammatory conditions (Langlois and
Introduction

Delanghe, 1996). Hp has been found associated with amyloid (Powers et al., 1981) and drusen (Kliffen et al., 1995). Hp knock-out mice display reduced postnatal viability and greater oxidative damage after induced hemolysis (Lim et al., 1998). Hp is a known ligand of the CD11b/CD18 receptor on natural killer cells (El Ghmati et al., 1996). With much lower affinity, Hp also binds to CD4 and CD8 receptors on T lymphocytes (El Ghmati et al., 1996). Neutrophils and monocytes also possess binding sites for Hp and are responsible for Hp uptake in peripheral blood (Wagner et al., 1996). Additionally, the acute-phase macrophage protein CD163 has been identified as a scavenger receptor for haemoglobin-Hp complexes (Kristiansen et al., 2001). This high affinity receptor ligand interaction is Ca\(^{2+}\)-dependent and mediates endocytosis of the haemoglobin-Hp complex (Kristiansen et al., 2001). It is possible that Hp may facilitate the clearance of unfolded proteins via a similar mechanism to the clearance of haemoglobin-Hp complexes, although this is yet to be investigated.

1.3.2.3.3 \(\alpha_2\)-Macroglobulin (\(\alpha_2\)M)

\(\alpha_2\)M is a highly abundant glycoprotein found in many biological fluids. It is comprised of around 10% carbohydrate by mass conjugated to four 180 kDa subunits to form a 720 kDa disulphide-bonded tetramer (Jensen and Sottrup-Jensen, 1986). The approximate concentration of \(\alpha_2\)M in human plasma is between 1.5-2 mg/mL (Sottrup-Jensen, 1989) and in CSF is between 1-3.6 μg/mL (Biringer et al., 2006). A unique mode of activation has been described for \(\alpha_2\)M by which proteolytic cleavage causes a large conformational change that both captures the protease and exposes a recognition site for the low density lipoprotein receptor-related protein (LRP) (Barrett and Starkey, 1973). Members of the LDL receptor superfamily including LRP are capable of facilitating the internalization of a diverse range of ligands including \(\alpha_2\)M (Dolmer et al., 2000).

Aside from its known interaction with proteases, \(\alpha_2\)M binds to a number of structurally different ligands. Among them are ApoE (Krimbou et al., 1998), \(\beta_2\)-microglobulin (Gouin-Charnet et al., 2000) and A\(\beta\) peptide (Du et al., 1997). Moreover \(\alpha_2\)M prevents A\(\beta\) peptide fibril formation and associated neurotoxicity \textit{in vitro} (Du et al., 1998; Hughes
et al., 1998) and is found associated with amyloid fibrils in vivo (Kondo and Tooyama, 2003). Recent evidence indicates that α₂M is as an important extracellular chaperone. α₂M has been shown to have an in vitro sHsp-like chaperone activity very similar to that of CLU and Hp and depletion of α₂M from human plasma renders endogenous plasma proteins more susceptible to precipitation at physiological temperatures (French et al., 2008). The expression of α₂M is known to be increased by shear stress (Chen et al., 2001), which is consistent with it having a role in the clearance of partially unfolded extracellular proteins. Furthermore, complexes formed between α₂M and peptides elicit an immune response in a manner similar to intracellular Hsps (Strivastava, 2002).

1.3.2.3.4 Extracellular refolding chaperones?

To date there is little evidence for the existence of abundant extracellular refolding chaperones. Serum amyloid P component (SAP) is a normally secreted protein that has been reported to have ATP-independent refolding activity (Coker et al., 2000). However, it should be noted that the physiological relevance of this study is questionable since SAP was required at a 10-fold molar excess to elicit an effect. SAP is a member of the pentraxin family and consists of five identical 25 kDa subunits arranged in a ring (Emsley et al., 1994). It is estimated that over 8% of the mass of the molecule is N-linked oligosaccharide (Pepys et al., 1994). SAP is present in human plasma at around 40 μg/mL (Hutchinson et al., 1994) and in CSF at around 8.5 ng/mL (Hawkins et al., 1994). The CSF concentration of SAP is higher in patients with Alzheimer’s disease (Hawkins et al., 1994). SAP is known to interact with a diverse range of molecules suggesting that many biological functions are possible. In recent times there has been much research into its role in inhibiting the formation of autoantibodies against chromatin released after cell death (Bickerstaff et al., 1999). SAP displays an affinity towards amyloid fibrils and is found associated with amyloid deposits (Pepys et al., 1978; Coria et al., 1988). However, the significance of SAP in PDDs is not yet fully understood.
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1.4 A model for the disposal of stressed proteins

The key events in receptor-mediated endocytosis were identified by investigating the transport of cholesterol-rich lipoproteins via the LDL receptor (LDLR) (Brown and Goldstein, 1979). Later studies recognized that the LDL receptor superfamily bind and mediate the endocytosis of a diverse range of ligands and several members also act as signal transducers in important physiologic and pathologic processes (Strickland et al., 2002). When a ligand binds to LDLR, the clathrin-coated pit (in which it is situated) forms a vesicle containing the receptor-ligand complex. The clathrin protein coat subsequently dissociates from the endocytosed vesicle and is recycled back to the plasma membrane. The uncoated vesicle fuses with an early endosome and the acidic conditions cause the receptor and ligand to dissociate. Recycling of the receptor back to the plasma membrane occurs after the part of the endosome containing the membrane bound receptor is pinched off. Maturation of the early endosome into a lysosome is followed by the enzymatic degradation of the endocytosed ligand.

Interactions between sHsp-like extracellular chaperones and endocytic receptors may provide a unique mechanism for the clearance of stressed extracellular proteins. All three known sHsp-like extracellular chaperones (CLU, Hp and α₂M) have previously been implicated in ligand transport across plasma membranes. Moreover, stressed protein/protease/α₂M complexes have been shown to bind to the endocytic receptor LRP (French et al., 2008). The available evidence supports a model in which CLU (and other extracellular chaperones) binds to regions of exposed hydrophobicity on unfolding extracellular proteins to form soluble complexes which are subsequently internalized via receptor-mediated endocytosis (Yerbury et al., 2005b; Appendix 1.1). Once internalized, the extracellular chaperone-stressed protein complexes are most likely disposed of by proteolytic degradation within lysosomes. This model is schematically represented in Figure 1.6. There are many candidate receptors which have not yet been tested for their possible binding to extracellular chaperone-stressed protein complexes. The following discusses the LDL receptor superfamily and scavenger receptors (SRs), two major classes of endocytic receptors that are possible candidates for involvement in the chaperone-facilitated disposal of stressed extracellular proteins. While it is predicted that all three known extracellular chaperones may participate in
similar systems for the disposal of stressed proteins, the majority of discussion in this thesis will be limited to the role of CLU.

Figure 1.6 A model for the disposal of stressed extracellular proteins. When native proteins are exposed to stress and begin to unfold extracellular chaperones hold them in stable complexes. These chaperone-stressed protein complexes are then targeted to endocytic receptors that transport the complexes to lysosomes where the proteins are degraded. Figure from Yerbury et al. (2005b).

1.4.1 Potential receptors for the disposal of CLU-stressed protein complexes

1.4.1.1 The low density lipoprotein (LDL) receptor superfamily

A number of investigations have focused on identifying possible interactions between members of the LDL receptor superfamily and CLU (Kounnas et al., 1995; Zlokovic et al., 1996; Hammad et al., 1997; Calero et al., 1999; Mahon et al., 1999; Bartl et al., 2001; Lakins et al., 2002; Bajari et al., 2003). Cellular internalization of CLU via the LDL receptor megalin was the first reported CLU-LDL receptor superfamily interaction (Kounnas et al., 1995). Subsequent reports described the internalization of free CLU and CLU-Aβ peptide complexes by the same receptor (Zlokovic et al., 1996; Hammad et al., 1997). Recently, two other human members of the LDL receptor superfamily, ApoE receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR), were
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reported to bind and internalize free CLU and leptin-CLU complexes using transfected cell models (Bajari et al., 2003). Interactions of CLU with chicken oocyte-specific LDL receptors have also been described (Mahon et al., 1999).

A recent study has suggested that megalin and LRP are capable of mediating the CLU-dependent clearance of cellular debris into non-professional phagocytes (Bartl et al., 2001). However, the previous report of Kounnas et al. (1995) indicated that megalin, but not LRP, binds CLU. Additional unidentified mechanisms of CLU-dependent internalization were also suggested by Bartl et al. (2001). The binding affinity of CLU to megalin is increased by the association of CLU with lipids (Calero et al., 1999). It is currently unknown how binding interactions with other molecules, such as stressed proteins, affect the binding affinity of CLU for megalin or other members of the LDL receptor superfamily. The finding that CLU has independent binding sites for megalin, stressed proteins and unstressed ligands is consistent with a model in which unfolded extracellular proteins are cleared via CLU-dependent receptor-mediated endocytosis (Lakins et al., 2002). In this model, CLU may bind to stressed proteins and subsequently bind, via a separate site, to megalin on the plasma membrane of cells. Megalin could then mediate the endocytosis and subsequent degradation of CLU-stressed protein complexes and prevent their accumulation in extracellular spaces.

LDLR (95.4 kDa) is the best described member of the LDL receptor superfamily and is highly expressed on hepatocytes where it plays an important role in the maintenance of plasma cholesterol homeostasis (Goldstein and Brown, 1974). Structural analysis of LDLR has identified several important functional regions that are shared by many of the receptors in this superfamily (Figure 1.7). Conserved regions include a cluster of complement-type cysteine-rich repeats, a YWTD consensus tetrapeptide in the epidermal growth factor (EGF) precursor homology domain, and an NPxY sequence on the cytoplasmic tail of the receptor (Nykjaer and Willnow, 2002). The respective functional roles of the aforementioned regions are ligand binding (Russell et al., 1989), acidic ligand uncoupling in endosomes (Davis, 1996) and localization in protein-coated regions of the plasma membrane called clathrin-coated pits (Strickland et al., 2002). All of these functions are relevant to the role of mediating the endocytosis of ligands and are shared by the different members of this receptor family (Figure 1.7).
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Figure 1.7 The structural organization of mammalian LDL superfamily receptors.

All receptors in the LDL receptor superfamily possess a single membrane anchor, EGF precursor homology domains and complement-type repeats. Several also share the NPxY motif and have an O-linked sugar domain. Figure adapted from Nykjaer and Willnow (2002).

Two of the largest LDL receptor superfamily members known as megalin and LRP (~ 600 kDa) are often termed ‘promiscuous’ due to the broad range of diverse ligands with which they interact. This is probably a consequence of the many complement-type repeats present on these members of the LDL receptor superfamily (Figure 1.7) (Nykjaer and Willnow, 2002). Megalin is found primarily located on kidney proximal tubule cells, parathyroid cells, placental cytotrophoblasts, epididymal epithelial cells (Lundgren *et al.*, 1997), brain capillaries and the choroid plexus (Chun *et al.*, 1999). An important role of megalin located in kidney proximal tubules is the uptake of glomerular proteins including vitamin D–binding proteins, enzymes, lipoproteins, the hormone carrier transthyretin (Christensen and Birn, 2001) and also haemoglobin (Gburek *et al.*, 2002). Studies have suggested that abnormal expression of megalin may be important in the pathophysiology of hemoglobinuric acute renal failure and low molecular weight proteinuria-associated tubulointerstitial nephritis (Leheste *et al.*, 1999). Compared to
LDLR, megalin is much larger but shares the same functional domains responsible for the endocytosis of ligands (Nykjaer and Willnow, 2002).

LRP is a multifunctional member of the LDL receptor superfamily with high expression in the liver. LRP is also expressed on neurons and astrocytes, epithelial cells of the gastrointestinal tract, smooth muscle cells, fibroblasts, Leydig cells of the testis, granulosa cells of the ovary, dendritic interstitial cells of the kidney and some monocyte-derived cells from certain tissues including liver, lung and lymphoid tissue (Moestrup et al., 1992). The role of LRP in the liver is well-described and includes the uptake of circulating chylomicron remnants (Rohlmann et al., 1998), serpin-enzyme complexes (Kounnas et al., 1996), and proteinases of the fibrinolytic pathway (Bu et al., 1992; Kounnas et al., 1993). In contrast, the functions of LRP on fibroblasts, macrophages, smooth muscle cells, neurons, and glial cells (activated, not resting) is unclear, but is likely to involve the uptake of extracellular components produced by these cells or surrounding cells.

1.4.1.2 Scavenger Receptors (SRs)

SRs are a family with an important role in the disposal of cellular debris, modified proteins and other undesirable materials including pathogens. While there are no known reports of interactions between CLU and SRs, the ability of SRs to preferentially recognize damaged or modified ligands suggests that they may act to clear stressed proteins. The first known SR was described in the late 1970s and was reported to bind acetyl-LDL (AcLDL) based on recognition of both conformation and charge (Goldstein et al., 1979). In this study the rate of uptake into macrophages was 20 times greater for AcLDL compared to the native molecule and uptake was followed by degradation in lysosomes. The described phenomenon is now known as receptor-mediated endocytosis, a major function of SRs. Later SRs were also implicated in cell adhesion (Fraser et al., 1993), the phagocytosis of microbes (Peiser et al., 2000) and apoptotic cells (Platt et al., 1996). Since AcLDL is not a physiologically relevant ligand in vivo, oxidized LDL (oxLDL) was suggested as an alternative for studies of SR. With a few exceptions, binding to oxLDL is characteristic of all known SRs (Table 1.5). The separate classes of SRs are structurally different (Figure 1.8).
Table 1.5 Classification and ligands of SRs.
Key: + bound; - not bound; NRD no reported data. Table adapted from Horiuchi et al. (2003).

<table>
<thead>
<tr>
<th>Class</th>
<th>Member</th>
<th>AcLDL</th>
<th>OxLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>AGE</th>
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</thead>
<tbody>
<tr>
<td>SR-A</td>
<td>SR-AI</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NRD</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SR-AII</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NRD</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SR-AIII</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NRD</td>
<td>NRD</td>
</tr>
<tr>
<td></td>
<td>MARCO</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NRD</td>
<td>NRD</td>
</tr>
<tr>
<td>SR-B</td>
<td>CD36</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SR-B1/CLA-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>SR-C</td>
<td>dSR-C1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NRD</td>
<td>NRD</td>
</tr>
<tr>
<td>SR-D</td>
<td>Macrosialin/CD68</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NRD</td>
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<tr>
<td>SR-E</td>
<td>LOX-1</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>SR-F</td>
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<td>-</td>
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</tr>
<tr>
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<td>SR-P Sox</td>
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<td>+</td>
<td>-</td>
<td>NRD</td>
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</tr>
<tr>
<td></td>
<td>FEEL-1</td>
<td>+</td>
<td>NRD</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
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<td>NRD</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>RAGE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
</tbody>
</table>
SRs are a diverse receptor family that can be divided into classes A-F depending on their structure and shared sequence homologies. Many SRs share the ability to bind a broad range of anionic proteins, carbohydrates, lipids and polynucleotides. The precise physical characteristics that determine this binding are not fully understood, however, charge interactions are likely to be important. Fucoidin, a polyanionic homopolymer of sulfated L-fucose, is routinely used as an inhibitor of SRs as it is recognized by class A scavenger receptors (Platt et al., 1996), lectin-like oxidized low density lipoprotein receptor (LOX-1) (Oka et al., 1998) and SR expressed by endothelial cells (SREC-1) (Berwin et al., 2004). An overall increase in negative charge is thought to account for the preferential binding of oxidized or acetylated LDL to SRs (Zhang et al., 1993). Additionally, Iwao et al. (2006) reported that the clearance of blood-borne HSA by the liver is enhanced by oxidation or single residue mutation and that increased negative charge and exposed hydrophobicity were the physical characteristics targeting the modified HSA to SRs.

### 1.4.1.2.1 Scavenger Receptor-Class A (SR-A)

SR-A are largely expressed by macrophages and are found in numerous tissues including liver, lung, brain, lymph nodes and placenta (Matsumoto et al., 1990; Teupser et al., 1999;
Introduction

Elshourbagy et al., 2000). Additionally, oxidative stress may induce the expression of SR-A in smooth muscle cells that normally do not express them (Mietus-Snyder et al., 2000). The expression of type I class A SRs (SR-AI) is significantly upregulated during monocyte differentiation to macrophages, however there is little increase in the expression of type II class A SRs (SR-AII) under the same conditions (Geng et al., 1994). SR-A are trimeric membrane proteins with fibrous glycosylated coiled-coil domains and a collagen-like domain that is responsible for ligand binding (Kodama et al., 1990; Matsumoto et al., 1990; Rohrer et al., 1990; Acton et al., 1993). SR-A types I, II and III are formed by alternative splicing of the same gene (Emi et al., 1993). With the exception of SR-AII, SR-A (including macrophage receptor with collagenous structure; MARCO) contain a SR cysteine-rich domain (SRCR), which is highly conserved across many other proteins (Freeman et al., 1990; Resnick et al., 1994).

SR-A bind numerous ligands including oxidized LDL and advanced glycation end products (AGE), however, although uptake of these ligands is diminished in SR-A knock-out mice, they are still partially cleared from the circulation (Ling et al., 1997; Lougheed et al., 1997; Yamada et al., 1998). In animal models of atherosclerosis, SR-A knock-out significantly decreases the size of atherosclerotic lesions (Suzuki et al., 1997; Sakaguchi et al., 1998). SR-AI and SR-AII are also implicated in the phagocytosis of apoptotic cells (Platt et al., 1996; Terpstra et al., 1997) and cell adhesion (Fraser et al., 1993). All SR-A are known to bind bacteria and are believed to play an important role in fighting microbial infection (Dunne et al., 1994; Elomaa et al., 1995).

1.4.1.2 Scavenger Receptor-Class B (SR-B)

Two members of class B SRs (SR-B) have been identified, namely CD36 and SR class B type I (SR-BI). Structurally they are quite different to SR-A. SR-B are anchored by two transmembrane domains and have fatty acylated domains at both the N and C termini (Tao et al., 1996; Muaro et al., 1997). These terminal domains localise CD36 and SR-BI to caveolae, a plasma membrane microdomain important in clathrin-independent endocytosis (Lisanti et al., 1994; Babitt et al., 1997). CD36 is also known to have an immunodominant domain, which is an important ligand binding motif (Navazo et al., 1996). SR-B are expressed by monocytes and macrophages, however, CD36 is
upregulated during differentiation whereas SR-BI is downregulated (Armesilla and Vega, 1994; Armesilla et al., 1996; Muaro et al., 1997). Other sites of expression include vascular endothelial cells, adipose tissue, heart, mammary gland, muscle, platelet and red blood cells for CD36 (Armesilla and Vega, 1994; Calvo et al., 1998) and the adrenal glands, ovary and liver for SR-BI. In addition to binding modified forms of LDL, SR-BI is a receptor for native LDL, high density lipoprotein (HDL) and very low density lipoprotein (VLDL) suggesting that it has a very important role in lipid metabolism (Calvo et al., 1998).

### 1.4.1.2.3 Other Scavenger Receptors

In addition to SR-A and SR-B, Drosophila SR class C type I (dSR-CI), macrosialin/CD68, LOX-1 and SREC-1 are known members of SR classes C, D, E and F, respectively. Although classed independently, dSR-C1 and macrosialin are structurally more similar to each other than other SRs due to the presence of a mucin-like domain on both receptors which is believed to play a role in oxLDL binding (Ramprasad et al., 1996; Yamada et al., 1998). LOX-1, a member of the LDL receptor superfamily, is also considered a SR due to its high affinity for oxidized but not native LDL (Yamada et al., 1998; Baljinder and Steinbrecher, 1999). Both LOX-1 and SREC-1 are widely expressed on endothelial cells and contain structural elements consistent with playing a role in signal transduction (Adachi et al., 1997; Sawamura et al., 1997; Baljinder and Steinbrecher, 1999; Ishii et al., 2002). Other uncategorized SRs include: SR for phosphatidylserine and oxidized low density lipoprotein (SR-PSOX), receptor for AGE (RAGE), fasciclin EFG-like, laminin-type EFG-like, and link domain-containing SR type 1 (FEEL-1) and type 2 (FEEL-2). SR-PSOX is a newly described macrophage SR with a similar ligand specificity to that of LOX-1 (Shimaoka et al., 2000). The expression of SR-PSOX in lipid-laden macrophages in atherosclerotic plaques suggests that this receptor may play an important role in foam cell generation and lesion formation (Minami et al., 2001). FEEL-1, FEEL-2 and RAGE are largely known as receptors for AGE (Tamura et al., 2003).
1.5 Objectives

The focus of this study centres on a proposed model for the quality control of extracellular protein folding which involves receptor-mediated endocytosis of chaperone-stressed protein complexes (Figure 1.6). While it is predicted that all three known extracellular chaperones may participate in similar systems for the disposal of stressed proteins, the general aim here was to examine the hypothesis that CLU participates in the disposal of stressed proteins, by stabilizing them in soluble complexes during times of stress. Furthermore, it was hypothesized that these CLU-stressed protein complexes are targeted to endocytic receptors for internalization and subsequent intracellular degradation. Specific experimental objectives were to:

1) Identify physiologically relevant conditions suitable to generate HMW CLU-stressed protein complexes \textit{in vitro}.

2) Characterize the physical properties of HMW CLU-stressed protein complexes.

3) Identify potential endogenous human plasma client proteins for CLU.

4) Identify receptors for HMW CLU-stressed protein complexes.

5) Using a suitable cell model, characterize the processes of internalisation and degradation of HMW CLU-stressed protein complexes.

6) Investigate the clearance and resulting biodistribution of blood borne HMW CLU-stressed protein complexes in a rat model.

Extracellular protein folding quality control has been a largely neglected area of research. The discovery of the extracellular chaperones, including CLU, and the continued characterization of their roles are an important first step towards understanding how stressed extracellular proteins are recognized and dealt with. The demonstration of a targeted system for the disposal of stressed extracellular proteins will be of fundamental biological significance. Moreover, given the large number of extracellular PDDs, a better understanding of extracellular protein folding quality control is needed and will hopefully contribute to the future development of strategies to treat these diseases (Wyatt \textit{et al.}, 2009a; Appendix 1.2).
2 GENERAL MATERIALS AND METHODS

2.1 Materials

G7 and 41D monoclonal antibodies were obtained as previously described (Wilson and Easterbrook-Smith, 1992a). The suppliers of antibodies used in this study are listed in Table 2.1. Buffer salts and H₂O₂ were obtained from Crown Scientific (Melbourne, Australia). 4,4’-Bis(1-anilino-8-naphthalene sulfonate) (bisANS), bovine serum albumin (BSA), citrate synthase (CS), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), collagenase IV (COL), lysozyme (LYS), o-phenylenediamine dihydrochloride (OPD), propidium iodide (PI), superoxide dismutase (SOD), dithiothreitol (DTT), guanidine hydrochloride (GdHCl), 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), casein, sodium azide (Az), glutathione, bicinehoninic acid, N,N,N’,N’-tetramethylethylenediamine (TMED), sodium dodecyl sulphate (SDS), thimerosal, glycerol, bromophenol blue, deoxymethylsulfoxide (DMSO), ethylene diamine tetraacetic acid (EDTA), streptavidin (SA)-horseradish peroxidase (HRP) and 2-mercaptoethanol were all obtained from Sigma-Aldrich (St. Louis, USA). IgG was purified from human plasma using a HiTrap® Protein G cartridge fitted to an Äkta Explorer chromatography system (GE Healthcare, Chalfont St Giles, UK). Complete™ Protease Inhibitor Cocktail, DNase I and Triton X-100 were from Roche Diagnostics Australia (Castle Hill, Australia).
Table 2.1 Antibodies used in this study and their respective suppliers.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host Species</th>
<th>Form</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione-S-transferase (GST)</td>
<td>Rabbit</td>
<td>Polyclonal IgG</td>
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<tr>
<td>CS</td>
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<td>Abcam (Cambridge, UK)</td>
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<tr>
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<tr>
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<td>Abcam</td>
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2.2 CLU purification from human plasma

Whole blood kindly donated from Wollongong Hospital Pathology Department (Wollongong, Australia) containing 20 μM sodium citrate (Na₃C₆H₅O₇) was centrifuged at 1,020 × g for 30 min to pellet the cells. The plasma was collected and filtered through a GF/C microfibre glass filter (Whatman, Maidstone, UK). Using an Econo Pump (Bio-Rad), the filtered plasma was pumped at 0.5 mL/min over tandem G7 and 41D monoclonal anti-CLU immunoaffinity columns (with an approximate total bed volume of 20 mL) as described by Wilson and Easterbrook-Smith (1992). The columns were consecutively washed with several column volumes of (i) phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.2 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.4) containing 0.1% (w/v) Az (PBS/Az), and then (ii) 0.1% (v/v) Triton X-100 in PBS before being re-equilibrated in PBS/Az. The columns were next washed with several column volumes of 200 mM sodium acetate in 500 mM NaCl, pH 5, before the bound protein was eluted using 2 M GdHCl in PBS. The eluent was dialyzed against 20 mM TRIS/Az, pH 8.0, and loaded onto a 4 mL Q Sepharose™ Fast Flow column (GE Healthcare) at 1 mL/min using an Äkta fast protein liquid chromatography (FPLC) system (GE Healthcare). Using the same flow rate, the column was washed with 20 mL of 20 mM TRIS/Az, pH 8.0, before the bound protein was collected in 2 mL fractions using a continuous 0-0.7 M NaCl gradient delivered over 80 min. The purity of the fractions collected was tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (see Sections 2.4 and 2.5, respectively). Purified CLU was dialyzed against PBS or PBS/Az for storage at -20°C or 4°C, respectively (Wilson and Easterbrook-Smith, 1992a).

2.3 Estimation of protein concentration

Protein estimations were performed by measuring the absorbance at 280 nm (A280 nm) in a quartz cuvette in a WPA Biowave S2100 Diode Array Spectrophotometer (Biochrom, Cambridge, UK) and using the appropriate extinction coefficient for each respective protein. Alternatively, protein estimations were carried out by bicinchoninic acid (BCA) assay using methods previously described (Smith et al., 1985).
2.4 SDS-PAGE

SDS-PAGE was performed using standard techniques. The appropriate percentage of acrylamide:bisacrylamide (30:0.8) (Amresco Inc., Solon, USA) (i.e. from 8-15%) was made up in a solution containing 375 mM TRIS, pH 8.8, 0.001% (w/v) SDS, 0.001% (w/v) ammonium persulfate ((NH₄)₂S₂O₈) and 0.0006% (v/v) TMED. To form the stacking gel, 5% acrylamide:bisacrylamide (30:0.8) in 125 mM TRIS, pH 6.8, 0.001% (w/v) SDS, 0.001% (w/v) ammonium persulfate and 0.001% (v/v) TMED was used. Samples were prepared using 1X sample buffer (60 mM TRIS, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue with or without 100 mM DTT and/or 1 mM 2-mercaptoethanol) and placed in a boiling water bath for 5 min prior to loading onto the gel. The gel was poured and run in a Hoefer™ SE 250/260 SDS-PAGE system (GE Healthcare) at 100-140 V until sufficient separation was obtained. The gels were stained in either Coomassie blue stain (0.2% (w/v) Coomassie Blue R250 (Sigma-Aldrich), 40% (v/v) methanol, 10% (v/v) glacial acetic acid in distilled water (dH₂O)) or Imperial™ Protein Stain (Quantum Scientific, Sydney, Australia), and destained in Destain Solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid and 50% dH₂O) or MilliQ water, respectively. Molecular weight markers used were unstained or prestained PageRuler™ protein ladder (Fermentas, Burlington, Canada) or unstained or dual colour Precision Plus Protein™ Standards (Bio-Rad).

2.5 Western blot

After separation by SDS-PAGE, gels were equilibrated in transfer buffer (26 mM TRIS, 192 mM glycine, 20% (v/v) methanol, pH 8.3) and proteins were transferred to nitrocellulose membrane using a Mini Trans-Blot Cell Western blotting apparatus (Bio-Rad) at 100 V for 1 h at 4°C. Afterwards the membrane was blocked in PBS containing 1% (w/v) heat-denatured casein and 0.01% thimerosal (HDC/PBS) for 1 h at 37°C or overnight at 4°C. Primary antibodies at the manufacturer's recommended dilution were incubated with the membrane in HDC/PBS for 1 h at 37°C. After washing in PBS, the membrane was incubated with an appropriate HRP-conjugated secondary antibody in HDC/PBS for 1 h at 37°C. The membrane was subsequently washed in 0.1% (v/v) Triton X-100 in PBS followed by PBS alone. Enhanced
General materials and methods

Chemiluminescence (ECL) detection was performed using Supersignal Western Pico substrate (Pierce Biotechnology, Rockford, USA) working solution following the manufacturer's protocols. Amersham Hyperfilm™ ECL (GE Healthcare) was placed over the membrane in a Kodak X-Omatic cassette (Kodak, Rochester, New York, USA) to detect chemiluminescence. Once exposed, the film was removed from the cassette and developed using Kodak Developer and Fixer.

2.6 Protein precipitation assays

Precipitation assays were performed in 384 well plates (Greiner Bio-one, Kremsmunster, Austria) using a FLUOstar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany). Changes in turbidity were monitored by measuring the absorbance at 360 nm (A360 nm) in the presence or absence of CLU or a relevant control protein (BSA for heat stress or SOD for oxidative stress). Immediately before being placed in the microplate reader the samples were covered using SealPlate® (EXCEL Scientific Inc., Victorville, USA). Typically, experiments were performed in triplicate 100 μL aliquots at between 0.5–2 mg/mL client protein in PBS. Heat stress was induced using temperatures between 40–60°C for GST, CPK, LDH, COL, CS, FGN and IgG and oxidative stress was induced using 50 μM Cu₂SO₄ and 2 mM H₂O₂ in PBS at 37°C for LYS and IgG.

2.7 Preparation of residual stressed control proteins

Residual heated control proteins were prepared by heating the native client protein or CLU using the same heat treatment that was necessary to generate the respective HMW CLU-stressed protein complexes. This heat-treatment caused much of the protein to precipitate out of solution (except in the case of CLU and BSA) and the treatment was stopped when the solution reached maximum turbidity as measured using the A360 nm. Residual oxidized protein was generated by incubating proteins in the oxidizing buffer using the same conditions necessary to generate the respective oxidized HMW CLU-stressed protein complexes. Again, residual oxidized control proteins were collected after the solution reached maximum turbidity. After the removal of precipitated protein by filtration using a 0.45 μm Ultrafree®-MC centrifugal filter device
General materials and methods

(Millipore), the protein that remained soluble after heat or oxidizing stress was quantified by A280 nm or BCA assay. In figures, "*" denotes residual heated control protein and the prefix "ox" denotes residual oxidized controls.

2.8 Size exclusion chromatography (SEC)

SEC was carried out using a Superose™ 6 10/300 column (exclusion limit 4 x 10⁷ Da, 22 mL bed volume; GE Healthcare) with a flow rate of 0.5 mL/min operated using an Äkta FPLC system. The mobile phase was 0.45 μm filtered PBS or PBS/Az unless otherwise stated. All samples were dialyzed against PBS or PBS/Az and 0.45 μm filtered where necessary. Proteins provided in the High Molecular Weight Gel Filtration Calibration Kit (GE Healthcare) were prepared according to the manufacturer’s instructions for size estimation. HMW CLU-stressed protein complex eluted at the exclusion limit of the column; the presence of both CLU and the client protein in these fractions was verified using enzyme linked immunosorbent assay (ELISA; see Section 2.9). SEC purified HMW CLU-stressed protein complexes were periodically reanalyzed by SEC to verify that the complexes were still intact. HMW CLU-GST, HMW CLU-FGN and HMW CLU-CS were generated at 60°C, 45°C and 41°C, respectively (unless otherwise stated).

2.9 ELISA to detect HMW complexes formed in vitro

The wells of a 96 well ELISA plate (Greiner Bio-one) were coated with purified G7 anti-CLU antibody by adding 50 μL of PBS containing the antibody at 10 μg/mL to each well. After incubation the wells were washed thoroughly with PBS and blocked using 1% (w/v) BSA, 0.01% (w/v) thimerosal in PBS at 4°C overnight. After washing in PBS, protein eluting at the exclusion limit (4 x 10⁷ Da) of a Superose™ 6 10/300 column (after CLU was incubated with one of several stressed client proteins; see Section 2.8) was added to triplicate wells at 50 μg/mL in 50 μL of the blocking solution and incubated. After thorough washing with PBS, a primary antibody (or antiserum) against the stressed client protein was added to the wells at the manufacturer’s recommended dilution in the blocking solution and incubated. The plate was washed again before adding 50 μL of an appropriate horseradish peroxidase (HRP) conjugated
secondary antibody at the manufacturer’s recommended dilution in the blocking solution and incubated. All incubations were carried out for 1 h at 37°C with shaking. The plate was washed three times in PBS followed by three 5 min washes in 0.1% (v/v) Triton X-100 in PBS with shaking. After a final wash in PBS, OPD at 2.5 mg/mL in 50 mM citric acid, 100 mM Na₂HPO₄, pH 5, was added. When sufficient colour development was observed the reaction was stopped by the addition of 50 μL/well 1 M HCL and the absorbance at 490 nm (A490 nm) was measured using a SpectraMax Plus microplate reader (Molecular Devices). Non-specific binding was assessed using wells coated with G7, blocked with BSA and subsequently incubated with a species-matched polyclonal antibody or species-matched serum of irrelevant specificity and the appropriate secondary antibody.

2.10 Biotinylation

CLU was purified as in Section 2.2 and residual heated controls were prepared as in Section 2.7. HMW CLU-stressed protein complexes were prepared as in Section 2.8, however, the unfractionated heat stressed solutions were biotinylated before the HMW CLU-stressed protein complexes was purified by SEC. Proteins were prepared in 0.1 M Na₂CO₃, pH 8.5 at between 1-3 mg/mL. To each solution, 0.25 mg of EZ-Link™ NHS-LC-Biotin (Perbio, England; freshly prepared at 40 mg/mL in deoxymethylsulfoxide (DMSO)) was added per mg of protein. The solutions were incubated on a rocker for a minimum of 2 hours at room temperature. Unconjugated biotin was removed by overnight dialysis against PBS or PBS/Az and the dialysed solutions stored at -20°C for the duration of this study.

2.11 Mass spectrometry

SDS-PAGE gels were placed on a clean sheet of glass and bathed in milliQ water to prevent their dehydration. Bands of interest were carefully excised using a clean scalpel blade. The blade was rinsed in 100% methanol and dried between bands to prevent cross-contamination. Gel pieces were transferred to 1.5 mL Eppendorf tubes and covered with 100-200 μL of Mass Spectrometry Destain Buffer (30 mM NH₄CO₃, pH 7.8, 40% (v/v) acetonitrile). Following complete destaining, the gel pieces were dried in
a vacuum desiccator to facilitate subsequent trypsin absorption. Protein in the gel was digested using Sequencing Grade Modified Trypsin (Promega, USA) according to the manufacturer's instructions. Excess trypsin was removed and the digested protein was released from the gel by shaking the pieces in 50 mM NH₄CO₃, pH 7.8 at 4°C overnight. Prior to mass spectrometry the digested peptides were stored at -20°C. Immediately preceding analysis the peptides were prepared in 8 mg/mL α-cyano-4-hydroxycinnamic acid in 70% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid. Samples were analyzed at the Save Sight Institute, University of Sydney, in a Shimadzu TOF MS (MALDI-TOF/TOF instrument) in reflectron mode over the mass range 100-5,000 m/z.

2.12 Circular dichroism (CD)

CD analysis was performed using a Jasco J-810 spectropolarimeter linked to a CDF-426S/L Peltier system (Jasco, Victoria, Canada). Samples between 0.100-0.164 μg/mL in 10 mM Na₂HPO₄, pH 7.4, in a 1.0 mm cell were examined in the far-UV region (190-250 nm) at 25°C (unless otherwise specified). The data was acquired at a step resolution of 0.1 nm with a bandwidth of 1.0 nm and at 100 millidegree sensitivity. Estimates of secondary structure were obtained using the program CDSSTR (Johnson, 1999).
3 FORMATION OF CLU-STRESSED PROTEIN COMPLEXES AT PHYSIOLOGICALLY RELEVANT TEMPERATURES

3.1 Introduction

Temperatures substantially above those possible in the human body are often used to induce client protein unfolding in chaperone studies. The complexity of protein thermostability is detailed in Section 1.1.2. In brief, the summation of all interactions contributing to the native conformation of a protein determines its inherent thermostability. This will be unique for each protein, thus it can be expected that the conditions required to induce unfolding will be different for each protein. Ideally, chaperone activity would be studied at physiologically relevant temperatures, however, under these relatively mild conditions proteins rarely unfold at an experimentally convenient rate. While the process of protein unfolding is the same regardless of the incubation temperature, the rate at which unfolding can occur is highly influenced by the amount of heat supplied (Day et al., 2002). Therefore, it has been common to investigate the chaperone activity of CLU at temperatures as high as 60°C (Humphreys et al., 1999; Poon et al., 2000; Poon et al., 2002b). However, if we are to discuss the relevance of CLU in a global, physiological context it is important to study the chaperone using model substrates that can be induced to unfold at temperatures close to that at which CLU functions in vivo.

While human body temperature is typically 37 ± 1°C a number of conditions can temporarily increase human body temperature (Table 1.1). Given that the upper limit of whole body temperature consistent with survival is 42°C, this was considered the highest temperature desirable to induce CLU client protein unfolding in this study. This chapter describes a systematic approach to the development of methods to purify CLU-stressed protein complexes formed using physiologically relevant heat stress. For the purposes of convenience and comparison with complexes formed at lower temperatures, HMW CLU-GST complexes (formed at 60°C) were also examined. A major objective was to identify at least one CLU-stressed protein complex that could be generated at physiologically relevant temperatures and purified for further experiments detailed in the subsequent chapters of this thesis.
3.2 Materials and methods

3.2.1 Purification of recombinant GST

BL21 Star™ Escherichia coli (Invitrogen, Carlsbad, USA) were transformed using the plasmid pGEX-2T (GE Healthcare) containing the sequence for Schistosoma japonicum GST by electroporation. The DNA solution (1 μL) was added to 40 μL of cells in an electroporation cuvette (Invitrogen) and pulsed in a Bio-Rad Gene Pulser™ at 200 Ω resistance, 25 μFD capacitance and 2.5 kV. Once electroporated, the cells were resuspended in 500 μL of Luria-Bertani (LB) broth (10 g/L Bacto™ tryptone (Becton, Dickinson and Company (BD), Franklin Lakes, USA), 5 g/L Bacto™ yeast extract (BD) and 170 mM NaCl) and shaken for 1-1.5 h at 37°C. The cell suspension was plated onto LB agar (LB broth with 15 g/L agar (BD)) containing 100 μg/mL ampicillin in the media to select for transformed cells. After 18-24 h at 37°C colonies were selected from the plates for the inoculation of 200 mL LB broth starter cultures also containing 100 μg/mL ampicillin. Starter cultures were incubated at 37°C overnight with shaking at 225 rpm. The next day starter cultures were used to inoculate a further 4-6 L LB broth and the culture density monitored using a WPA Biowave S2100 Diode Array Spectrophotometer until the absorbance at 600 nm reached 0.6-1.0. At this point 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) was added and the culture left to grow for a further 3 h. The cells were pelleted by centrifugation at 4,000 x g at 4°C using a J2-MC centrifuge (Beckman Coulter, Fullerton, USA) with a JA-10 rotor and sterile JA-10 centrifuge tubes. The pelleted cells were stored at -20°C overnight before being resuspended in bacterial cell lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1 mg/mL LYS, Complete™ Protease Inhibitor Cocktail, 10 mM MgCl₂, 10 U/mL DNase I and 0.1% v/v Triton X-100). The solution was subjected to repeated freeze-thawing using liquid nitrogen and a 30°C waterbath. The lysate was clarified by centrifugation as before except at 10,000 x g followed by filtration through a 0.2 μm syringe filter (Millipore, Billerica, USA). The clarified lysate was loaded on to a Glutathione Sepharose™ Fast Flow column (GE Healthcare) at a flow rate of 1 mL/min using an Econo Pump (Bio-Rad, Sydney). Afterwards the column was washed with four column volumes of PBS before the protein was eluted using 10 mM glutathione in 50 mM TRIS, pH 8. The purity of the protein obtained was
verified by SDS-PAGE and Western blot analysis (see Sections 2.4 and 2.5, respectively). Purified GST was dialyzed against PBS/Az for storage at 4°C.

3.2.2 Protein precipitation assays

Protein precipitation assays were performed as described in Section 2.6. Heat stress was 60°C for GST and temperatures between 40-43°C for CPK, LDH, COL (all at 0.5-1.0 mg/mL in PBS) in the presence or absence of CLU (0.1-1.0 mg/mL in PBS). Assays were terminated if the A360 nm was not seen to increase by at least 0.2 after incubation for 24 h at a given temperature. If precipitation was observed, then the assay was stopped when no further increase in absorbance was measured.

3.2.3 Preparation of residual stressed controls

Residual heated controls were prepared as described in Section 2.7. Table 3.1 describes the heat-treatment for each protein unless otherwise stated. CLU was heated using each of these different treatments depending on the experiment. In figures, the prefix * denotes residual heated controls (e.g. *GST = residual heated GST).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Temperature (°C)</th>
<th>Incubation Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>60</td>
<td>50 min</td>
</tr>
<tr>
<td>CS</td>
<td>40-43</td>
<td>550 min-12 h</td>
</tr>
<tr>
<td>LDH</td>
<td>40</td>
<td>200 min</td>
</tr>
<tr>
<td>CPK</td>
<td>43</td>
<td>150 min</td>
</tr>
</tbody>
</table>

3.2.4 SEC

SEC was performed as described in Section 2.8.
3.2.5 ELISA to detect HMW CLU-stressed protein complexes formed \textit{in vitro}

After co-incubating CLU and either GST or CS at 60°C or 41°C, respectively, proteins were fractionated by SEC using a Superose™ 6 column 10/300 and the proteins eluting at the exclusion limit ($\geq 4 \times 10^7$ Da) tested in ELISA to detect HMW CLU-stressed protein complexes. Full details of the assay are described in Section 2.9.

3.2.6 Native gel electrophoresis

A 1% (w/v) agarose (Promega, Madison, USA) gel was prepared in TAE buffer (40 mM TRIS-acetate, 1 mM EDTA) by heating in a microwave and allowing to set in a Mini Sub™ DNA cell (Bio-Rad) with an appropriate lane comb. Once cast, the gel was covered with TAE and the protein samples loaded after dilution in 3X Native Gel Loading Buffer (25 mM TRIS, 190 mM glycine, 30% (v/v) glycerol, 1% (w/v) bromophenol blue, pH 6.8). The unit was run at 60 V using a Power Pack 300 (Bio-Rad). Once the proteins were separated, the gel was stained using Imperial™ Protein Stain (Pierce Biotechnology) and destained using milliQ water. The pH of the TAE buffer used for both the gel and running buffer was adjusted depending on the isoelectric points of the proteins to be analyzed.

3.2.7 Ion-exchange chromatography

Proteins to be separated were dialyzed against 20 mM TRIS/Az (pH 5-9) before being loaded onto a 1 mL Q Sepharose™ Fast Flow column (GE Healthcare) using an Äkta FPLC system. After washing with several column volumes of 20 mM TRIS/Az the protein was eluted using a 0-0.9 M NaCl gradient. The length of the gradient was varied in an attempt to optimize the separation of CLU-stressed protein complexes from uncomplexed proteins. Fractionated protein was examined by SDS-PAGE (see Section 2.4). The starting pH of the TRIS/Az and the length of the gradient were chosen depending on the proteins being separated.
3.2.8 CD analysis

CD analysis was performed as described in Section 2.12. The CD spectrum of CLU was obtained during heating in a stepwise manner with measurements taken after holding the sample for 30 min at 4°C, 20°C, 25°C, 30°C, 37°C, 38°C, 39°C, 41°C and 45°C. After heating at 45°C the sample was returned to 4°C with further measurements taken after 2 h and 12 h.
3.3 Results

3.3.1 GST as a supraphysiological heat-sensitive client for the chaperone activity of CLU

Incubation of 20 μM GST at 60°C resulted in rapid precipitation of the protein (as indicated by the increasing A360 nm) after a lag phase of approximately 25 min during which no increase in turbidity was observed (Figure 3.1). After 40 min the protein solution reached maximum turbidity and prolonged heating had no further effect on turbidity. Co-incubation of 20 μM GST with 3.2 μM CLU almost completely inhibited the precipitation of GST under the same conditions. This was in contrast to co-incubation with 3.2 μM BSA, where the protein solution exhibited a similar precipitation profile to that observed when GST was incubated alone. However, the maximum turbidity was marginally less in the presence of BSA. Both CLU and BSA were stable when incubated alone at 60°C. As first reported by Humphreys et al. (1999) the effect of CLU on protein precipitation was dose-dependent (data not shown). When GST was incubated alone, after maximum turbidity was reached, BCA assay and measurements of A280 nm indicated that there was a small amount of residual protein (less than 10% of the starting concentration) remaining in the solution (data not shown).

Figure 3.1 Inhibition of heat-induced GST precipitation by CLU.
20 μM GST was heated at 60°C for 50 min in the presence or absence of 3.2 μM CLU or BSA. The stability of CLU and BSA was assessed by heating the proteins alone under the same conditions. All samples were prepared in PBS. This figure shows the average A360 nm (n = 3) of the protein solutions indicated in the key.
Co-incubation of GST and CLU at 60°C for 50 min resulted in the generation of HMW species which eluted at the size exclusion limit ($V_\text{o} \geq 4 \times 10^7$ Da) of a Superose™ 6 10/300 gel filtration column (Figure 3.2; uppermost trace). Similar associations were not apparent when the native proteins were incubated for the same period of time at room temperature. Under these latter conditions, the profile observed matched the combined size exclusion profiles of monomeric GST and the major oligomeric forms of CLU (Figure 3.2). The residual GST remaining in solution after heating at 60°C for 50 min did not exhibit increased self-association. This was also apparent for CLU after undergoing the same heat treatment. When residual heated GST and heated CLU were co-incubated at room temperature, the size exclusion profile observed was similar to that of the native proteins although there was slightly less monomeric GST (eluting at around 20 mL) present. There were, however, negligible HMW species formed by the residual heated proteins under these conditions. Sandwich ELISA in which plate bound monoclonal anti-CLU antibody (G7) and a rabbit polyclonal antibody against GST were used, indicated that after co-incubation of GST and CLU at 60°C for 50 min the species eluting at $V_\text{o} \geq 4 \times 10^7$ Da (Figure 3.2; uppermost trace) contained CLU complexed to GST (Figure 3.3). Compared to SEC purified HMW CLU-GST, control samples analyzed in the same assay showed negligible absorbances ($F(4,10) = 114.98; p \leq 0.001$ and Tukey honestly significant differences (HSD) $p \leq 0.01$).
Formation of CLU-stressed protein complexes at physiologically relevant temperatures

Figure 3.2 SEC of heat stressed or native CLU and GST.
20 μM GST and 3.2 μM CLU were incubated together at 60°C for 50 min ((GST + CLU) 60°C). The resulting size exclusion profile was compared to the profiles of the corresponding native proteins or residual heated proteins (pre-heated at 60°C for 50 min and denoted by *) incubated together or alone at ambient temperature for 50 min. All samples were prepared and analyzed in PBS. The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit ($V_o$) ≥ 4 x 10⁷ Da.

Figure 3.3 Detection of HMW CLU-GST complexes by sandwich ELISA.
The following samples were tested in a sandwich ELISA using plate bound anti-CLU antibody (G7): (i) protein eluting at ≥ 4 x 10⁷ Da from a Superose™ 6 column after 20 μM GST and 3.2 μM CLU was co-incubated at 60°C for 50 min in PBS (HMW CLU-GST), (ii) *CLU, (iii) GST, (iv) *GST, and (v) *GST and *CLU co-incubated at ambient temperature for 50 min in PBS (*GST + *CLU). 50 μg/mL total protein was added to the wells followed by rabbit polyclonal anti-GST and anti-rabbit IgG-HRP. The results shown are the average A490 nm (n = 3 ± standard error) and were adjusted for non-specific binding by subtracting the absorbance produced by a species-matched primary control antibody of irrelevant specificity followed by the relevant secondary antibody. * Denotes the use of residual heated controls. + Indicates significantly greater A490 nm compared to all controls (Tukey HSD, p ≤ 0.01).
Formation of CLU-stressed protein complexes at physiologically relevant temperatures

3.3.2 Developing models for the chaperone activity of CLU at physiologically relevant temperatures.

3.3.2.1 Investigations of protein thermostability

A range of proteins was tested to identify which of them could act in vitro as chaperone client proteins for CLU at physiologically relevant temperatures. In all cases the amount of precipitation (measured as an increase in A360 nm) and the rate at which precipitation occurred was increased by increasing the incubation temperature. Precipitation followed a short lag phase, where no increase in turbidity was observed, and prolonged heating at the same temperature had no effect after the maximum turbidity was reached. An example of this is shown for the protein CPK at 60°C, 50°C and 43°C (Figure 3.4). With each protein at 0.5-1.0 mg/mL in PBS, the minimum temperature required to produce an increase in A360 nm of greater than 0.2 in less than 24 h was determined (Table 3.2). In all cases a small amount of residual protein (less than 10% of the starting concentration) remained in solution after maximum turbidity was reached (data not shown).
Formation of CLU-stressed protein complexes at physiologically relevant temperatures

Figure 3.4 Heat-induced precipitation of CPK at 60°C, 50°C and 43°C. The precipitation of 6.3 μM CPK in PBS was monitored during incubation at 60°C, 50°C and 43°C. Data points are mean A360 nm of triplicate determinations.

Table 3.2 Minimum temperatures required to induce the precipitation of COL, LDH, CPK and CS within a 24 h period. COL, LDH, CPK and CS were monitored for precipitation at concentrations between 0.5 and 1.0 mg/mL. The lowest temperature for which an increase in A360 nm ≥ 0.2 was observed within a 24 h period is reported for each protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (mg/mL)</th>
<th>Temperature (°C)</th>
<th>Time to Complete Precipitation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>1.0</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>LDH</td>
<td>1.0</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>CPK</td>
<td>0.5</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td>CS</td>
<td>0.5</td>
<td>40</td>
<td>11</td>
</tr>
</tbody>
</table>
3.3.2.2 The effect of CLU on heat-induced protein precipitation

Co-incubation of LDH, CPK or CS with CLU during heating inhibited their precipitation (Figure 3.5; panels A-C). This response was dose-dependent as previously described for other client proteins (Humphreys et al., 1999) (data not shown). The concentrations of CLU required to completely inhibit the heat-induced precipitation of the stressed client proteins (at the lowest temperature at which the client proteins could be induced to precipitate) are shown (Figure 3.5; panels A-C). CS appeared to remain stable in solution when a near equimolar concentration of CLU was present (Figure 3.5; panel A). For LDH and CPK, the ability of CLU to prevent protein precipitation appeared to be less efficient and close to a 2-fold molar excess of CLU was necessary to completely inhibit the precipitation of these stressed client proteins (Figure 3.5; panels B & C). In all cases, co-incubation of the client protein with the control protein BSA had little effect compared to co-incubation with CLU under the same conditions. Co-incubation of COL with CLU or BSA had minimal effect on the precipitation of the protein even at relatively high concentrations of the chaperone (Figure 3.5; panel D). CLU and BSA (alone) did not precipitate under any of the heating conditions used to induce the precipitation of the client proteins.
Figure 3.5 The effect of CLU on the heat-induced precipitation of CS, LDH, CPK and COL.
(A) 6.0 μM CS was heated at 40°C ± 6.6 μM CLU or BSA; (B) 7.3 μM LDH was heated at 40°C ± 13 μM CLU or BSA; (C) 6.3 μM CPK was heated at 43°C ± 13 μM CLU or BSA; (D) 9.5 μM COL was heated at 40°C ± 13 μM CLU or BSA. The stability of CLU and BSA was independently assessed by heating the proteins alone under the same conditions. This figure shows the average A360 nm (n = 3) of the protein solutions (all prepared in PBS).
Formation of CLU-stressed protein complexes at physiologically relevant temperatures

SDS-PAGE analysis showed that co-incubating COL with CLU resulted in digestion of CLU (indicated by the marked reduction of the major band for CLU migrating at approximately 85 kDa; lane 2). Furthermore, treatment with 10 mM EDTA was not sufficient to significantly reduce the enzyme activity (Figure 3.6; lane 3). Despite the low temperatures (40-43°C) at which COL could be induced to precipitate, the level of proteolytic activity against CLU under these conditions rendered it unsuitable for use as a stressed client protein in these studies.

![Figure 3.6 SDS-PAGE of COL and CLU incubated at 43°C in the presence or absence of 10 mM EDTA.](image)

A solution containing 9.5 μM COL and 13 μM CLU was heated at 43°C for 30 min in the presence or absence of 10 mM EDTA in PBS. 10 μg total protein was examined using 12% SDS-PAGE. The lanes contained (1) PageRulerTM unstained protein ladder; (2) COL and CLU in PBS; (3) COL and CLU in PBS with 10 mM EDTA; (4) COL; (5) CLU.

When heated alone CS, LDH and CPK did not self-associate and the size exclusion profiles indicated that residual heated proteins did not significantly associate with CLU when they were subsequently co-incubated at room temperature (data not shown). At the lowest temperature required to induce their precipitation, none of these proteins formed HMW complexes with CLU comparable in size to that observed when GST was stressed in the presence of CLU at 60°C (≥ 4 x 10^7 Da; Figure 3.2; uppermost trace). When 6.0 μM CS and 6.6 μM CLU were incubated together at 40°C the size exclusion profile was not markedly different to that observed when the native proteins were incubated at room temperature (Figure 3.7). Increasing the incubation temperature from 40°C to 41°C resulted in the formation of some HMW species, however the bulk of the CS remaining in solution appeared to elute according to its monomeric size. Further
increasing the incubation temperature to 43°C resulted in the majority of protein eluting at $\geq 4 \times 10^7$ Da, with only a small amount of protein migrating as smaller species between 737 kDa and 460 kDa (Figure 3.7; uppermost trace). As described for GST, protein eluting at $\geq 4 \times 10^7$ Da was verified by sandwich ELISA to contain a complex incorporating both CLU and the stressed client protein CS, but residual heated CS and CLU did not significantly associate at room temperature (Figure 3.8).

**Figure 3.7 SEC of heat stressed or native CLU and CS.**
6.0 μM CS and 6.6 μM CLU were incubated together at 40°C, 41°C or 43°C for 12 h ((CS + CLU) 40°C, (CS + CLU) 41°C, (CS + CLU) 43°C, respectively). The resulting size exclusion profiles were compared to the profiles of the corresponding native proteins incubated alone or together (CS, CLU and CS + CLU, respectively) at ambient temperature for 550 min. All samples were prepared and analyzed in PBS. The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit ($V_o$) $\geq 4 \times 10^7$ Da.
Formation of CLU-stressed protein complexes at physiologically relevant temperatures

Figure 3.8 Detection of HMW CLU-CS complexes by sandwich ELISA.

The following samples were tested in a sandwich ELISA using plate bound anti-CLU antibody (G7): (i) protein eluting at $\geq 4 \times 10^7$ Da from a Superose$^\text{TM}$ 6 column after 6.0 μM CS and 6.6 μM CLU was co-incubated at 41°C for 550 min in PBS (HMW CLU-CS), (ii) *CLU, (iii) *CS, (iv) *CS and *CLU co-incubated at ambient temperature for 550 min in PBS (*CS + *CLU). 50 μg/mL total protein was added to the wells followed by sheep anti-CS and anti-sheep/goat IgG-HRP. The results shown are the average A490 nm ($n = 3 \pm$ standard error) and were adjusted for non-specific binding by subtracting the absorbance produced by a species-matched primary control antibody of irrelevant specificity and the relevant secondary antibody. * Denotes the use of residual heated controls. + Indicates significantly greater A490 nm compared to all controls (Tukey HSD, $p \leq 0.01$).

When 7.3 μM LDH and 13 μM CLU were incubated at 40°C the size exclusion profile indicated that a portion of the protein formed large species (around 600 kDa) but not species $\geq 4 \times 10^7$ Da (Figure 3.9; second uppermost trace). Species of this size were not as abundant when the native LDH and CLU were incubated at the same ratio at room temperature. Increasing the incubation temperature to 43°C had little effect on the size of species present compared to incubation at 40°C.
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Figure 3.9 SEC of heat stressed or native CLU and LDH.

7.3 μM LDH and 13 μM CLU were incubated together at 40°C or 43°C for 200 min ((LDH + CLU) 40°C, (LDH + CLU) 43°C, respectively). The resulting size exclusion profiles were compared to the profiles of the corresponding native proteins incubated alone or together at ambient temperature for 200 min (LDH, CLU and LDH + CLU, respectively). All samples were prepared and analyzed in PBS. The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit (V_o) ≥ 4 x 10^7 Da.

Incubation of native CPK and CLU at ambient room temperature resulted in the generation of a minor fraction of protein eluting as species approximately 740 kDa which would not be expected from the theoretical summation of the profiles of CPK and CLU alone (Figure 3.10). Heating of 6.3 μM CPK and 13 μM CLU at 43°C slightly increased the proportion of these larger species, however, only a relatively small amount of protein eluted in the HMW range of ≥ 4 x 10^7 Da (Figure 3.10; uppermost trace).
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Figure 3.10 SEC of heat stressed or native CLU and CPK.
6.5 μM CPK and 13 μM CLU were incubated together at 43°C for 150 min ((CPK + CLU) 43°C). The resulting size exclusion profile was compared to the profiles of the corresponding native proteins incubated alone or together at ambient temperature for 150 min (CPK, CLU and CPK + CLU, respectively). All samples were prepared and analyzed in PBS. The results are from a Superose™6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit (V₀) ≥ 4 x 10⁷ Da.

For the client proteins CPK and LDH in the presence of CLU, heating at temperatures ≤ 43°C did not generate complexes as large as those observed for CS, however, native gel electrophoresis suggested that novel species with unique isoelectric properties were formed after these two proteins were co-incubated and exposed to mild heat stress. When analyzed without heating, at pH 8, LDH (approximate pI 8.5) migrated a small distance in the opposing direction to CLU (approximate pI 5.89). In contrast, following co-incubation at 43°C, a "smear" of stained protein extending between the positions corresponding to native LDH and native CLU was obtained (Figure 3.11; lane 1). Similar results were obtained for analyses of heated mixtures of CPK and CLU (data not shown). Therefore, ion exchange chromatography was investigated as a possible method to purify CLU-stressed protein complexes. Despite trialling many different methods, ion
Formation of CLU-stressed protein complexes at physiologically relevant temperatures

exchange chromatography was unable to successfully separate the complexes from the component proteins (data not shown).

Figure 3.11 Native gel of LDH and CLU.
10 μg total protein was loaded onto a 1% agarose gel and electrophoresed at 60 V in TAE, pH 8. The lanes contain: 7.3 μM LDH and 13 μM CLU incubated together overnight at (1) 43°C, or (2) ambient temperature; in addition to (3) LDH, and (4) CLU alone.

3.3.2.3 The effect of temperature of the secondary structure of CLU

CD analysis of CLU showed that subphysiological increases in temperature from 4°C to 30°C did not significantly change the overall secondary structure of the protein (Figure 3.12; panel A). In all cases, the profile observed showed approximately equal minima at 208 nm and 222 nm, typical of proteins with high alpha helical content. Further increasing the temperature to 37°C and then up to 45°C showed a progressive decrease in the alpha helical content of the protein (Figure 3.12; panel B). After the protein was heated to 45°C, a subsequent 2 h incubation at 4°C resulted in CLU regaining most but not all of its original structure (Figure 3.12; panel C). Further incubation at 4°C up to a total of 12 h did not change the CD profile from that observed after 2 h at 4°C. CDSSTR analysis of the CD spectra indicated that there was no significant increase in disorder between 4°C and 37°C, however, incubation at 38°C and up to 45°C increased the relative amount of disordered content disorder (t(299) = 2.745 and t(354) = 5.454, respectively; p ≤ 0.01; Figure 3.13). The predicted level of disordered content for CLU heated at 45°C and then allowed to recover, returned to a similar level as that of unheated CLU with in 2 hrs at 4°C (data not shown).
Figure 3.12 CD spectra of CLU during heating between 4-45°C.
CLU was held for 30 min at the specified temperature before measurements were taken and the incubation temperature increased. The panels show the results of incubation at (A) 20-30°C, (B) 37-45°C, and (C) after recovery at 4°C, and are from the same experiment. In each case, the mean (n = 6) is shown and the reference spectrum for the unheated sample (4°C) also provided.
Figure 3.13 Predicted content of disordered structure for CLU following heating at 4-45°C. Estimates of the relative disordered content were obtained from the data in Figure 3.12 using the program CDSSTR and the mean ± standard error is shown. The number of possible solutions for each spectrum (n) ranged between 107 and 233.
**3.4 Discussion**

The *in vitro* chaperone activity of CLU has been examined for a number of client proteins, although, previously the heat stress used to induce precipitation was supraphysiological (typically as high as 60°C for substrate including GST, ovotransferrin and catalase) (Humphreys *et al.*, 1999; Poon *et al.*, 2000; Poon *et al.*, 2002b). While protein unfolding occurs in the same manner regardless of temperature (Day *et al.*, 2002), when studying the action of chaperones it is important to investigate them in a context that is physiologically relevant in order to elucidate their possible role *in vivo*. For example, while heating at up to 60°C does not appear to reduce the chaperone activity of CLU (Humphreys *et al.*, 1999; Poon *et al.*, 2002b), CD analysis suggests that at temperatures above 37°C there is a significant increase in the level of disordered CLU structure (Figure 3.12 and 3.13). It has been proposed that CLU has a dynamic molten globule-like binding site, containing natively disordered regions (Bailey *et al.*, 2001), which is likely to be important in its chaperone action. Therefore, increases in the disordered structure content of CLU during incubation at physiologically relevant temperatures above 37°C may be linked to a conformation that favours chaperone activity, however, this has yet to be confirmed.

*In vitro* protein aggregation requires conditions (such as increased temperature) which partially disrupt native structure but which do not completely inhibit interactions such as hydrogen-bonding. In this study, it was observed that both the rate and amount of protein precipitation could be increased by increasing the temperature at which the proteins were incubated *in vitro* (Figure 3.4). However, the intrinsic stability of globular mammalian proteins means that, at 37°C, none of the purified client proteins examined in this study were found to unfold and aggregate at an experimentally convenient rate under these conditions. There are obvious practical limitations to producing CLU-stressed protein complexes *in vitro* to mimic those generated *in vivo*. If we are to consider extracellular proteins in an *in vivo* context then it is important to remember that additional stresses including ROS and shear stress will be present and contribute to the unfolding of these proteins. Moreover, macromolecular crowding such as that present in complex biological fluids would favor protein aggregation *in vivo* compared to buffered solutions containing relatively low concentrations of the purified proteins. *In vitro*, it is difficult to accurately replicate the *in vivo* effects of shear stress, especially when it is
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Experimentally inconvenient to wait for prolonged incubations spanning many days to weeks. Therefore, slightly higher than normal body temperatures were employed to induce protein unfolding for the generation of CLU-stressed protein complexes in this study. At least in the cases of CS and LDH, CLU-inhibitable precipitation was observed at physiologically relevant temperatures ($\leq 42^\circ$C) supporting the proposition that in vivo CLU is likely to be important in preventing the deposition of insoluble unfolded proteins.

The multiple glycosylation states of CLU, and the various oligomeric forms CLU adopts in solution, complicate the purification of CLU-stressed protein complexes using standard techniques. In this study, size exclusion separation of HMW complexes ($\geq 4 \times 10^7$ Da) was used as the primary means to purify CLU-stressed protein complexes. Of the client proteins examined, at physiologically relevant temperatures, species of this size were only formed at an experimentally convenient rate when CS was incubated with CLU at a minimum of $41^\circ$C (Figure 3.7). Moreover, the size exclusion profiles of uncomplexed CS and CLU suggested that SEC using a Superose™ 6 10/300 column was suitable for the bulk preparation of purified HMW CLU-CS complexes, although this technique was more efficient when higher incubation temperatures were used. It is predicted that similar complexes could be formed at lower temperatures, however, longer incubation times would be required to induce sufficient unfolding of the stressed client protein. While the SEC profile of CLU alone suggests that a very small amount of oligomerized CLU may have also been present in HMW fractions collected, it is estimated that these very large oligomers of CLU would have contributed to less than 1% of the total protein collected and would not have significantly influenced the results of subsequent experiments.

For CPK and LDH, it is apparent that at physiological or near physiological temperatures the chaperone activity of CLU involves the formation of significantly smaller complexes. It is known that the size of complexes formed between sHsp and stressed proteins is dependent on temperature (Lee et al., 1997). Since the action of CLU appears to closely resemble that of sHsp it is possible that at higher temperatures CLU may form larger complexes with CPK and LDH. However, because it was one of this study's objectives to use physiologically relevant stresses to examine the chaperone
activity of CLU, the effect of higher temperatures on CPK and LDH was not investigated. As CLU-stressed CPK or CLU-stressed LDH complexes could not be separated by ion-exchange chromatography and methods involving harsh denaturing conditions such as immunoaffinity chromatography were known to disrupt HMW CLU-stressed protein complexes, investigations using these two client proteins were not pursued. While it was shown that CLU inhibits the precipitation of a range of proteins at near-physiological temperatures, of the client proteins examined co-incubating CS and CLU at 41ºC provided the only conditions suitable for subsequent uncomplicated purification of CLU-stressed protein complexes formed at a physiologically relevant temperature. As for HMW GST-CLU complexes (≥ 4 x 10^7 Da) formed at supraphysiological temperature (60ºC), HMW CS-CLU complexes formed at 41ºC were purified by SEC and used in a series of further experiments (Chapters 5-7). The following chapter describes the identification of FGN as a putative endogenous client for CLU in plasma during physiologically relevant stress and the formation of HMW CLU-FGN complexes at near-physiological temperature (45ºC).
4 IDENTIFICATION OF PUTATIVE ENDOGENOUS PLASMA CLIENT PROTEINS FOR CLU

4.1 Introduction
CLU is found associated with extracellular protein deposits in many serious diseases including drusen in AMD patients (Crabb et al., 2002), renal immunoglobulin deposits in kidney disease (French et al., 1992), Lewy bodies in Parkinson’s disease (Sasaki et al., 2002), prion deposits in Creutzfeldt-Jakob disease (Freixes et al., 2004) and amyloid plaques in Alzheimer’s disease (Ghiso et al., 1993; Calero et al., 2000). The presence of CLU in these pathological deposits suggests that CLU associates with unfolding proteins \textit{in vivo}. While the majority of CLU containing deposits reside extracellularly, the association of CLU with intracellular deposits may be related to recent observations that CLU may evade the secretion pathway and instead be localized in the cytosol as a result of cellular stress (Nizard et al., 2007). Pathological protein deposition may result when CLU’s chaperone capacity or that of other machinery necessary to prevent the accumulation of protein aggregates is exceeded by abnormally high levels of protein unfolding. However to date, mechanisms for the disposal of aggregated extracellular proteins are undescribed.

Many different stressed and unstressed ligands for CLU have been identified (Poon et al., 2000; Lakins et al., 2002). Typically investigations of the chaperone activity of CLU have been carried out using model proteins that can be induced to unfold at experimentally favourable rates. While the effect of CLU-depletion of stressed human plasma is known to be protein precipitation (Poon et al., 2002b), little work has focused on identifying endogenous plasma proteins which may interact with CLU during conditions of stress. This chapter describes investigations of the binding of CLU to potential endogenous client proteins in plasma during physiologically relevant stresses. Development of a method to generate and purify HMW complexes formed between CLU and a putative endogenous client protein using near physiological heat stress is also described.
4.2 Materials and methods

4.2.1 Protein purification

IgG was purified from human plasma using a HiTrap® Protein G cartridge fitted to an Äkta Explorer chromatography system, and following the manufacturer's instructions (GE Healthcare). GST-receptor-associated protein (GST-RAP) fusion protein or free receptor-associated protein (RAP) were purified using the same method as that described for GST (see Section 3.2.1) with the following exceptions. GST-RAP was expressed in *E. coli* transformed with pGEX-2T containing the full length sequence for RAP inserted at the GST fusion site (kindly donated by Dr Yonghe Li, Washington School of Medicine, St Louis, USA). After the clarified lysate from transformed *E. coli* was passed over the Glutathione Sepharose™ Fast Flow column, GST-RAP was eluted using 10 mM glutathione in 50 mM TRIS, pH 8. For purification of RAP the fusion protein was first treated on the column with thrombin (Sigma-Aldrich) according to the manufacturer’s instructions and free RAP was eluted with PBS. The column was regenerated after elution of the remaining GST with 10 mM glutathione in 50 mM TRIS, pH 8. The purity of the protein obtained was verified by SDS-PAGE and Western blot analysis (see Sections 2.4 and 2.5, respectively). Purified GST-RAP or RAP was dialyzed against PBS/Az for storage at 4°C or PBS for storage at -20°C.

4.2.2 Analysis of proteins co-purifying with CLU from untreated human plasma

CLU purification was carried out as described (see Section 2.2). During this procedure, protein eluted from anti-CLU immunoaffinity columns (after detergent and high salt/low pH washes) was subjected to ion-exchange chromatography using a Q Sepharose™ Fast Flow column. The protein was fractionated such that the leading peak (contaminant) was separated from purified CLU. The fractionated protein was dialyzed against PBS/Az for further analysis as described below.

4.2.2.1 Western blot

After separation by 12% SDS-PAGE using reducing or non-reducing conditions, CLU and co-purifying proteins were subjected to Western blot (see Section 2.5) using
anti-CLU G7 or anti-IgG (heavy chain) and the relevant HRP-conjugated secondary antibody.

4.2.2.2 ELISA

Sandwich ELISA was used to detect putative CLU-IgG complexes co-purifying with CLU from immunoaffinity purification. In this assay, G7 was bound to the plate and blocked with 1% BSA in PBS before the ion-exchange fractionated protein was added at a total protein concentration of 50 μg/mL. Goat anti- (human) IgG followed by donkey anti-goat IgG-HRP was used to detect human IgG. Non-specific binding was assessed using wells coated with G7, blocked with BSA and subsequently incubated with a species-matched antibody of irrelevant specificity and the appropriate secondary antibody. Commercial antibodies were used according to the manufacturer’s instructions. For more details of the ELISA protocol see Section 2.9.

4.2.2.3 Flow cytometry

Human leukocytes were isolated from whole blood by centrifugation at 400 x g in a Heraeus Sepatech Megafuge 1.0.R (Radiometer Pacific, Australia) to pellet the cells and incubation in Red Cell Lysis Buffer (155 mM NH₄Cl, 12 mM NaHCO₃ and 0.1 mM EDTA, pH 7.4) for 5 min at 4°C with occasional inversion. The cells were then washed in ice cold Hank’s binding buffer (HBB; 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 5 mM CaCl₂, 1 mM MgCl₂, 0.1% (w/v) BSA, 0.1% (w/v) glucose, pH 7.4) by centrifugation. At approximately 10 x 10⁵ cells/mL, the cells were incubated with 100 μg/mL biotinylated putative CLU-IgG complex ("contaminant fraction" isolated from human plasma by CLU immunoaffinity chromatography and ion exchange fractionation; see Section 4.2.2), IgG, CLU or RAP in HBB for 30 min on ice. Following two washes in HBB by centrifugation, the cells were incubated in 10 μg/mL SA-Alexa fluor® 488 (ALEXA488) (Molecular Probes, USA) in HBB for 30 min on ice and then washed twice in the same manner. ALEXA488 fluorescence (Ex 488 nm, Em 500-560 nm) of viable cells was measured for 3 samples of between 1x10³-10x10³ cells; dead cells were stained with 1 μg/mL PI immediately before analysis and electronically excluded from the analyses. Measurements were obtained using an LSR-II flow cytometer (BD). The acquired data was analyzed using FlowJo7 software (TreeStar Inc.,
The geometric mean (geomean), which is a measure of central tendency for data that changes in a relative fashion, reported in arbitrary fluorescence units (AFU), was used to summarize the findings. In order to adjust the geomean for background fluorescence, in each experiment a sample of cells was incubated on ice with SA-ALEXA488 at 10 μg/mL for 30 min and then washed twice before the fluorescence was measured. Histograms plots of ALEXA488 fluorescence were displayed to show the shifts in fluorescence in relation to the background level.

### 4.2.3 Precipitation assays

Precipitation assays (see Section 2.6) were carried out for IgG and FGN using heat stress at ≤ 42°C and ≤ 45°C, respectively. Other precipitation assays were carried out for LYS and IgG by inducing oxidative stress with oxidizing buffer (50 μM CuSO₄ and 2 mM H₂O₂ in PBS at 37°C). All assays were performed in the presence or absence of CLU or SOD. Additionally, the effect of macromolecular crowding on protein precipitation was carried out by incubating FGN, GST or ovalbumin (OVA) at 1 mg/mL with 0-140 mg/mL polyethylene glycol (PEG) 4,000 (Sigma-Aldrich) at ambient room temperature for 5 min before measuring the absorbance of the solutions at 360 nm. The putative chaperone activity of FGN was assessed using CS as a client protein at 43°C, similar to the study of Tang et al. (2009). CLU was included as a control in these experiments. The molar ratio of CS to FGN or CLU in these experiments was either 1:1 or 1:2.

### 4.2.4 Preparation of residual stressed control proteins

Residual oxidized control proteins (see Section 2.7) were collected after oxidative stress (induced using 50 μM CuSO₄ and 2 mM H₂O₂ in PBS at 37°C) of LYS for 6 h or oxidation of IgG for 4 min. CLU was also oxidized using the same two sets of conditions just described. Residual oxidized control proteins are given the prefix "ox" in figures.

### 4.2.5 SEC

SEC was performed as described in Section 2.8.
4.2.6 Plasma treatments

Plasma containing heparin (20 mg/50 mL plasma), Complete™ Protease Inhibitor Cocktail and 0.1% (w/v) Az was divided into three 20 mL aliquots, one of which was stored static at 4°C, the second held in a 100 mL Schott bottle (Crown Scientific) on a gently rotating Miniperm apparatus (approximately 5 cm in diameter rotating at 60 rpm) in a 37°C incubator and the third supplemented with 50 μM CuSO₄ and 2 mM H₂O₂ and incubated static at 37°C. After 10 days, plasma was diluted 1:10 in PBS and turbidity assessed in a SpectraMax Plus³⁸⁴ plate reader by measuring the A₃₆₀ nm.

4.2.7 Analysis of plasma protein associations with CLU in stressed human plasma

4.2.7.1 Sandwich ELISA for the identification of stressed client proteins

Sandwich ELISA was used to assess the degree of CLU association with 11 major plasma proteins after plasma was incubated as described (see Section 4.2.6). The wells of a 96 well ELISA plate were coated with purified G7 anti-CLU antibody by adding 50 μL of PBS containing the antibody at 10 μg/mL to each well. After incubation at 37°C for 1 h with shaking the wells were washed thoroughly with PBS and blocked using 1% (w/v) BSA, 0.01% (w/v) thimerosal in PBS at 4°C overnight or using 1% (w/v) gelatine in PBS at 37°C for 1 h. Plasma was added to triplicate wells in 50 μL aliquots and incubated for 1 h at 37°C with shaking. After thorough washing with PBS a primary antibody (or antiserum) reactive with one of the plasma proteins to be screened was added to the wells in the blocking solution and incubated for 1 h at 37°C with shaking. The plate was washed again before adding 50 μL of the appropriate HRP conjugated secondary antibody in the blocking solution and incubating the plate for a final 1 h at 37°C with shaking. The plate was washed three times in PBS followed by three 5 min washes in 0.1% (v/v) Triton X-100 in PBS with shaking. After a final wash in PBS, OPD at 2.5 mg/mL in 50 mM citric acid, 100 mM Na₂HPO₄, pH 5, was added. When sufficient colour development was observed after stopping with 50 μL/well 1 M HCl the A₄₉₀ nm was measured using a SpectraMax Plus³⁸⁴ microplate reader. The results presented are adjusted for non-specific binding to the wells by subtracting the absorbance measured from wells coated with control mouse IgG₁ (of irrelevant
specificity) and subsequently incubated with plasma and then primary and secondary antibodies as described. The absorbance associated with the non-specific binding to G7 coated wells of control species-matched polyclonal antibodies or antisera of irrelevant specificity (followed by the relevant secondary antibody) is also shown. All antibodies were used at the manufacturer’s recommended dilution. In some instances, in lieu of an HRP conjugated secondary antibody, a biotinylated secondary antibody and SA-HRP were used to increase the sensitivity of detection. Details of the ELISAs are summarized in Table 4.1.
Identification of putative endogenous plasma client proteins for CLU

Table 4.1 Details of sandwich ELISAs used to detect the stress-induced association of CLU with 11 major plasma proteins.

Eleven major plasma proteins (indicated in bold text) were screened for their association with CLU in control and stressed plasma. The sequential (left to right) incubation procedures used in the sandwich ELISAs are outlined. Coloured shading indicates primary antibodies for which a common species-matched control antibody of irrelevant specificity was used. The species-matched control antibodies included for the measurement of non-specific binding are shown in italics.

<table>
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<th>Coating</th>
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<th>Secondary</th>
<th>Additional</th>
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<td>anti-sheep/goat IgG-HRP</td>
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</tbody>
</table>
4.2.7.2 SEC to determine the size of putative CLU-FGN complexes in stressed plasma

The different treated plasma samples (see Section 4.2.6) were fractionated by SEC (see Section 2.8) and the separated protein blotted onto nitrocellulose membrane (Pall, East Hills, USA). After blocking overnight at 4°C in HDC/PBS, the membrane was incubated with goat anti-FGN antiserum in HDC/PBS for 30 min at 37°C. The membrane was then washed and subsequently incubated with anti-sheep/goat IgG-HRP in HDC/PBS for 30 min at 37°C before being washed in 0.1% (v/v) Triton X-100 in PBS followed by PBS alone. ECL detection was performed as described above (see Section 2.5). The antiserum and antibody were used according to the manufacturer’s instructions.

4.2.8 Anti-CLU immunoaffinity chromatography for the identification of proteins complexed with CLU.

After plasma was treated as described (see Section 4.2.6), it was filtered through a GF/C filter before being passed at 0.5 mL/min over a tandem arrangement of monoclonal anti-CLU immunoaffinity columns (the same as used for CLU purification, see Section 2.2). The columns were subsequently washed with several column volumes of PBS (in lieu of standard detergent and high salt washes used for CLU purification) before bound protein was eluted in PBS containing 2 M GdHCl, pH 7.4. The eluted proteins were immediately dialyzed against PBS/Az and stored at 4°C.

4.2.8.1 SEC

SEC was performed as described in Section 2.8 to compare the size of protein species present in fractions prepared by anti-CLU immunoaffinity chromatography from (i) control plasma, and (ii) plasma incubated for 10 days at 37°C with slow rotation. Highly purified CLU (prepared as described in Section 2.2) was also analyzed as a control.
4.2.8.2 SDS-PAGE
12% SDS-PAGE was performed as described in Section 2.4 to analyse the same protein fractions referred to in Section 4.2.8.

4.2.8.3 Mass spectrometry
Protein bands that were detected by SDS-PAGE in samples prepared from "stressed" plasma (rotated at 37°C for 10 days) but not in samples prepared from control plasma (stored at 4°C) were analyzed by mass spectrometry as described in Section 2.11.

4.2.8.4 Western blot detection of FGN co-purifying with CLU
Protein fractions prepared as described in Section 4.2.8 were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane then probed using goat anti-FGN antiserum and donkey anti-sheep/goat IgG-HRP by Western blot (see Section 2.5).

4.2.8.5 ELISA for the detection of anti-CLU immunoaffinity co-purifying CLU-FGN complexes
Protein fractions prepared as described in Section 4.2.8 were also analyzed by sandwich ELISA. Samples were first incubated in wells coated with anti-CLU G7 antibody. Subsequently, wells were incubated with goat anti-FGN antiserum and then donkey anti-sheep/goat IgG-HRP. Non-specific binding was assessed using wells coated with G7, blocked with BSA and subsequently incubated with a species-matched antiserum of irrelevant specificity and the appropriate secondary antibody. Commercial antibodies were used according to the manufacturer’s instructions. For full details see Section 2.9.
4.3 Results

4.3.1 Analysis of “contaminant” proteins co-purifying with CLU from unstressed human plasma

The idea to look for endogenous plasma clients for the chaperone activity of CLU came from the initial observation that even after stringent detergent and high salt/low pH washes, “contaminant” proteins co-purify with CLU by immunoaffinity chromatography of unstressed human plasma. It was thought that identification of these co-purifying proteins may shed light on which proteins are targeted to CLU in vivo. Q Sepharose™ ion exchange chromatography of the anti-CLU immunoaffinity column eluate from normal plasma resolved two major fractions. The first to elute in the NaCl gradient was a "contaminant" fraction that mostly contained species > 170 kDa with a smaller amount of protein present around 80 kDa and 150 kDa according to SDS-PAGE (Figure 4.1). SDS-PAGE analysis of the second fraction to elute suggested that it contained only purified CLU. This was confirmed by Western blot analysis (data not shown). Western blot analysis of the "contaminant" fraction suggested that CLU and IgG were likely to correspond to the bands visible at around 80 kDa and 150 kDa, respectively and also contributed to the species >170 kDa (Figure 4.2). In non-reduced Western blot species > 170 kDa were immunoreactive using either anti-CLU or anti-IgG (Figure 4.2; panels A and B). In addition, a smaller amount of CLU was found at the expected size of 80 kDa (Figure 4.2; panel B). After reduction the species > 170 kDa were no longer immunoreactive using either anti-CLU or anti-IgG and the reduced components of CLU and IgG were found at their expected sizes (Figure 4.2; panels C and D). IgG was a more major component compared to CLU in the “contaminant” fraction as visualized after reducing SDS-PAGE (data not shown). Sandwich ELISA suggested that the "contaminant" fraction contained CLU-IgG complexes (Figure 4.3). Given the possibility of self-oligomerization of CLU or IgG it is not possible to know the exact proportion of protein present as CLU-IgG complexes, although, SDS-PAGE suggested that the majority of CLU and IgG was present in a HMW form > 170 kDa.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.1 Ion-exchange profile and corresponding SDS-PAGE of proteins purified from unstressed human plasma by anti-CLU immunoaffinity chromatography.

Unstressed human plasma was subjected to anti-CLU immunoaffinity chromatography including stringent detergent and high salt/low pH column washes. (A) The subsequently eluted protein was dialyzed into 20 mM TRIS/Az, pH 8 and loaded onto a Q Sepharose™ column before fractionation using a 0-1 M NaCl gradient elution. (B) The eluted protein was analyzed using non-reducing 12% SDS-PAGE. The lanes are aligned such that the protein in each lane corresponds to their approximate elution position in (A), with molecular weight markers (Mr) shown on the far left.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.2 Western blot of "contaminant" protein fraction from ion-exchange chromatography of anti-CLU immunoaffinity proteins purified from human plasma. The figure shows non-reduced and reduced samples incubated with anti-heavy chain IgG (anti-IgG) or G7 (anti-CLU) as indicated on the panels. The prestained molecular weight markers (Mr) are shown in kDa.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.3 Detection of putative CLU-IgG complexes in the "contaminant" protein fraction (from CLU immunoaffinity chromatography) by sandwich ELISA.

50 μg/mL CLU, IgG or the "contaminant" protein fraction were incubated in wells coated with G7 anti-CLU antibody and subsequently incubated with anti-(human) IgG antibody and the relevant HRP conjugated secondary antibody. The results shown are the average A490 nm (n = 3 ± standard error) and were adjusted for non-specific binding by subtracting the absorbance produced in G7-coated wells by a species-matched primary control antibody of irrelevant specificity followed by the relevant HRP-conjugated secondary antibody. + Indicates significantly increased A490 nm compared to CLU or IgG (Tukey HSD, p ≤ 0.01).

Given that “contaminant” protein (leading peak in Figure 4.1) co-purifying with CLU appeared to contain both CLU and IgG, including CLU-IgG complexes, this fraction was designated CLU/IgG. The binding of the CLU/IgG fraction to peripheral blood human leukocytes was examined by flow cytometry (Figure 4.4). There was little difference between the background fluorescence and the fluorescence of lymphocytes after incubation with 100 μg/mL CLU/IgG, CLU, IgG or the pan-specific LDL receptor family ligand RAP (Figure 4.4; panel A). In contrast, all ligands appeared to bind to monocytes, with similar binding measured for IgG and CLU and comparatively greater binding measured for RAP and CLU/IgG (Figure 4.4; panel B). Only low levels of RAP and CLU binding to granulocytes was measured, with slightly greater binding for IgG, however, CLU/IgG bound to a much greater extent than all three other ligands (Figure 4.4; panel C).
Identification of putative endogenous plasma client proteins for CLU

4.3.2 Purification of CLU-stressed protein complexes formed under oxidative conditions

4.3.2.1 Lysozyme (LYS) as a model client protein for the chaperone activity of CLU during oxidative stress

LYS is one of the most extensively studied models of protein unfolding (Booth et al., 1997; Chang and Li, 2002; Merlini and Bellotti, 2005; Trexler and Nilsson, 2007). Several studies have used reductive stress to examine LYS as a stressed client for CLU chaperone activity (Poon et al., 2000; Poon et al., 2002a; Poon et al., 2002b). The
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Unfolding of LYS is followed by aggregation, however, the mechanisms for when induced by reductive or oxidative stress appear different and less is known regarding LYS aggregation induced by oxidative means (Goldberg et al., 1991; Radford et al., 1992; van den Berg et al., 1999; Minton, 2000b). In this study, oxidative conditions were optimized for inducing LYS precipitation in CLU chaperone assays.

Incubation of 70 μM LYS in oxidizing buffer (PBS, supplemented with 50 μM CuSO₄ and 2 mM H₂O₂) at 37°C resulted in precipitation of the protein after an initial lag phase of approximately 40 min (Figure 4.5). Under these conditions no further precipitation was apparent beyond 360 min. Co-incubation of 70 μM LYS with 13 μM CLU significantly reduced but did not abolish precipitation. In contrast, co-incubation with 13 μM SOD had no effect. In the same oxidizing buffer, when incubated alone, CLU and SOD remained soluble. A slight difference between the SEC profiles of native and oxidized CLU was apparent (Figure 4.6). Oxidized CLU appeared to favor smaller oligomeric forms (around 200 kDa) compared to native CLU, although, both small and large oligomers were present in addition to monomeric CLU (Figure 4.6; lower two traces). A small amount of protein, slightly larger than monomeric LYS was present after LYS was incubated alone in the oxidizing buffer. However, compared to the amount of total protein this fraction of soluble aggregates was less than 10%.

Incubation of native or pre-oxidized proteins in PBS indicated that LYS and CLU do not interact under these conditions. However, co-incubation of the proteins together in oxidizing buffer resulted in the generation of HMW species (≥ 4 x 10⁷ Da) similar to that observed for mixtures of CLU and heat stressed client proteins (Figure 4.6; uppermost trace). Moreover, the protein eluting at ≥ 4 x 10⁷ Da was verified by sandwich ELISA to contain CLU-LYS complexes (Figure 4.7).
Identification of putative endogenous plasma client proteins for CLU

Figure 4.5 Inhibition of oxidative stress-induced LYS precipitation by CLU.
70 μM LYS was incubated at 37°C in the presence or absence of 13 μM CLU or SOD in oxidizing buffer for 6 h. The stability of CLU and SOD was assessed by incubating the proteins alone under the same conditions. This figure shows the average A360 nm (n = 3) of the protein solutions.

Figure 4.6 SEC of native or oxidized CLU and LYS.
70 μM LYS and 13 μM CLU were incubated together in oxidizing buffer for 6 h at 37°C (ox(LYS + CLU)). The resulting size exclusion profile was compared to the profiles of the corresponding native proteins or pre-oxidized proteins (given the prefix ox) incubated alone or together at ambient temperature in PBS for 6 h. The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit (V₀) ≥ 4 x 10⁷ Da.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.7 Detection of CLU-LYS complexes by sandwich ELISA.

Samples analyzed in a sandwich ELISA using plate bound anti-CLOT antibody (G7) were: (i) Protein eluting at $\geq 4 \times 10^7$ Da from a Superose$^{TM}$ 6 column after 70 μM LYS and 13 μM CLU was co-incubated at 37°C for 360 min in oxidizing buffer (HMW CLU-LYS), (ii) CLU, (iii) LYS, and (iv) a mixture of 70 μM LYS and 13 μM CLU co-incubated at ambient temperature for 360 min in PBS. 50 μg/mL total protein was added to the wells followed by rabbit polyclonal anti-LYS and then anti-rabbit IgG-HRP. The results shown are the average A490 (n = 3 ± standard error) and were adjusted for non-specific binding by subtracting the absorbance produced by a species-matched control primary antibody of irrelevant specificity followed by the secondary antibody in G7 coated wells. + Indicates significantly increased A490 nm compared to all other controls (Tukey HSD, p ≤ 0.01).

4.3.2.2 In vitro methods for the formation of oxidized CLU-IgG complexes

High temperature instability of IgG has been reported (Vermeer and Norde, 2000), however in the absence of other stresses, precipitation was not observed when IgG was incubated at physiologically relevant temperatures ($\leq 41°C$) for 1-2 days (data not shown). When exposed to oxidizing buffer at 37°C, IgG exhibited rapid CLU-inhibitable precipitation (9.8 μM CLU completely inhibited the precipitation of 6.7 μM IgG; Figure 4.8). Co-incubation with the control protein SOD had no effect at the same concentration and both CLU and SOD were stable when incubated alone under these conditions. Examination of oxidized IgG by SEC showed that incubation in oxidizing buffer at 37°C for 4 min caused oligomerization of the protein and the formation of soluble aggregates (Figure 4.9). The majority of IgG eluted at a position approximately twice the size of the native protein (150 kDa) with a small shoulder of higher order aggregates eluting from the column between 730 kDa and 460 kDa. Despite being able to almost completely inhibit the precipitation of 6.7 μM IgG, co-incubation with 9.8 μM CLU did not appear to form species larger than those formed when IgG was incubated alone. Similarly, co-incubation of pre-oxidized IgG and CLU did not result in the formation of HMW species.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.8 Inhibition of oxidative stress-induced precipitation of IgG by CLU.
6.7 μM IgG was incubated at 37°C in the presence or absence of 9.8 μM CLU or SOD in oxidizing buffer for 4 min. The stability of CLU and SOD was assessed by incubating the proteins alone under the same conditions. This figure shows the average A360 nm (n = 3) of the protein solutions.

Figure 4.9 SEC of native or oxidized CLU and IgG.
6.7 μM IgG and 9.8 μM CLU were incubated together in oxidizing buffer for 5 min at 37°C (ox(IgG + CLU)). The resulting size exclusion profile was compared to the profiles of the corresponding native proteins or pre-oxidized proteins (given the prefix ox) incubated alone or together at ambient temperature in PBS for 5 min. The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit (Vₑ) ≥ 4 x 10⁷ Da.
4.3.3 Analysis of plasma protein associations with CLU in stressed human plasma

After 10 days at 37°C with gentle rotation, plasma was visibly more turbid than static plasma stored at 4°C. The difference according to the A360 nm was approximately 2-fold when comparing the treated plasma samples diluted 1:10 in PBS (Figure 4.10; \( t(4) = 12.04; p \leq 0.01 \)). When 11 major plasma proteins were screened for their relative CLU-association in these two plasma samples it was found that incubation at 37°C with gentle rotation or oxidative stress significantly increased the association of many abundant plasma proteins with CLU (Figure 4.11). Specifically, compared to static storage at 4°C, incubation at 37°C with gentle rotation resulted in greater CLU association with FGN (\( t(4) = 5.341; p \leq 0.02 \)), ACID GLY (\( t(4) = 8.44; p \leq 0.02 \)), \( \alpha_2 \)M (\( t(4) = 5.021; p \leq 0.02 \)), Hp (\( t(4) = 4.107; p \leq 0.02 \)) and IgM (\( t(4) = 17.45; p \leq 0.02 \)). In an additional plasma sample that had been subjected to oxidative stress for the same 10 day period it was observed that oxidative stress also resulted in greater CLU association to FGN (\( t(4) = 7.042; p \leq 0.02 \)), TRANS (\( t(4) = 4.431; p \leq 0.02 \)), ACID GLY (\( t(4) = 4.098; p \leq 0.02 \)) and IgM (\( t(4) = 5.261; p \leq 0.02 \)) compared to static storage at 4°C. Although both incubation at 37°C with gentle rotation and oxidative stress appeared to increase the association of HSA with CLU (\( t(4) = 7.692 \) and \( t(4) = 13.31 \), respectively; \( p \leq 0.02 \)), given that absorbance measured was comparable to that measured for normal rabbit serum (a species-matched control for anti-HSA) it is likely that the absorbance measured is largely the result of non-specific binding (Figure 4.11; panel D).
Figure 4.10 Relative turbidity of plasma stored static at 4°C compared to plasma gently rotated at 37°C for 10 days.

Plasma containing 20 μM sodium citrate, protease inhibitors and 0.1% (w/v) Az was stored static at 4°C or kept gently rotating at 37°C for 10 days. After this period the plasma samples were diluted 1:10 in PBS and the A360 nm measured as an indicator of relative turbidity. The figure shows the average A360 nm (n = 3 ± standard error) for each plasma sample. + Denotes significantly increased turbidity according to Student’s t-test, p ≤ 0.01.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.11 Sandwich ELISAs measuring the relative association of major plasma proteins with endogenous plasma CLU after various treatments.

Plasma was incubated for 10 days either static at 4°C, rotating at 37°C, or subjected to oxidative stress at 37°C. The plasma was incubated in wells coated with anti-CLU (G7) followed by primary antibodies (A) goat-antisera reactive with FGN, TRANS, ACID GLY, or control goat serum; (B) rabbit IgG fractions reactive with α2M, Hp, TRYP, or control normal rabbit IgG; (C) goat antibody-HRP conjugates reactive with IgG, IgA, IgM, or control goat-anti-biotin-HRP; (D) rabbit anti-sera reactive with ApoAI, CS, HSA or control rabbit serum. Species-matched controls of irrelevant specificity for the primary antibodies are labelled in grey text and are shown to the right of the corresponding results. The average A490 nm (n = 3 ± standard error) were adjusted for non-specific binding by subtracting the absorbance generated in wells coated with mouse IgG, isotype and species-matched control for anti-CLU G7). * Denotes increased association relative to static storage at 4°C (Student’s t-test, p ≤ 0.02).
4.3.4 Analysis of proteins co-purifying with CLU from stressed plasma

When plasma, stored static at 4°C or kept gently rotating at 37°C, was subjected to anti-CLU immunoaffinity purification with the omission of normally used detergent and low pH/high salt washes, distinctly different SEC profiles were observed between the two plasma treatments (Figure 4.12). These profiles were also different to that of purified CLU (obtained by anti-CLU immunoaffinity purification using stringent detergent and low pH/high salt washes), which eluted in several broad peaks representing CLU α-β heterodimers (80 kDa) and earlier eluting higher order oligomers (Figure 4.12; lower trace). In the case of plasma stored static at 4°C, in addition to a peak corresponding to 80 kDa CLU α-β, a small peak eluting at the size exclusion limit of the column (≥ 4 x 10^7 Da) and a more substantial asymmetric peak with a maximum at about 400 kDa were detected (Figure 4.12; middle trace). SEC analysis of protein purified from plasma that had been rotated for 10 days at 37°C using the same procedure (i.e. lacking the detergent/salt washes) showed a clear increase in the proportion of HMW material. The majority of proteins in this sample eluted in a broad peak centred at about 700 kDa (Figure 4.12; upper trace). The larger apparent size of protein species in the shear/heat-treated sample suggested that CLU may have formed chaperone-stressed protein complexes under these conditions.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.12 SEC of CLU and proteins co-purifying with CLU after incubation of plasma with gentle rotation at 37°C or static storage at 4°C for 10 days.

Samples analyzed were: (i) CLU purified from human plasma as described in Section 2.2 (lower trace), and all proteins bound to an anti-CLU column from human plasma that had for 10 days been (ii) stored at 4°C or (iii) rotated at 37°C (in both (ii) and (iii), the protein was eluted without first washing the column with detergent or low pH/high salt buffers). The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit (V_o) \( \geq 4 \times 10^7 \) Da.

When proteins co-purifying with CLU from plasma stored static at 4°C or gently rotated at 37°C (both for 10 days) was examined by SDS-PAGE, several additional bands were apparent in the latter sample (Figure 4.13). The most prominent of these bands migrated to sizes of approximately 107, 82.9, 73.0, 61.2 and 45.9 kDa and were excised for mass spectrometry analysis. From these analyses only two bands were identified. When the peptide mass fingerprints were analyzed the bands migrating at about 73.0 kDa and 61.2 kDa matched vitronectin precursor protein which exists in plasma as a single 75 kDa chain and also as a 65 kDa cleaved form (data not shown). Using a similar approach (but treating human plasma at 43°C for 3 days), preliminary experiments performed by C. McKay and J. A. Aquilina (University of Wollongong, Australia) identified FGN as a presumptive endogenous substrate for CLU (unpublished data). For this reason immunoblotting analysis was carried out to investigate whether FGN co-purified with CLU after plasma is stressed. Using this technique, it was found that FGN co-purified with CLU from human plasma rotated for 10 days at 37°C, but not
from plasma stored at 4°C (Figure 4.14). Bands corresponding to the α, β and γ chains of FGN were apparent in this sample, migrating to positions corresponding to approximately 60 kDa, 56 kDa and approximately 42 kDa, respectively. The formation of CLU-FGN complexes was confirmed by sandwich ELISA using plate bound G7 anti-CLU antibody and goat anti-FGN antiserum (Figure 4.15; t(4) = 11.46; p ≤ 0.01).

**Figure 4.13** SDS-PAGE of protein co-purifying with CLU from human plasma that was for 10 days stored static at 4°C or gently rotated at 37°C.
Proteins were separated using 12% SDS-PAGE under reducing conditions. The figure shows (i) molecular weight markers (Mr), and all proteins bound to the column from human plasma (ii) stored at 4°C, or (iii) gently rotated at 37°C. The identity of the samples is indicated above the corresponding lanes. Arrows indicate unique bands that were selected for analysis by mass spectrometry.

**Figure 4.14** Anti-FGN immunoblot of proteins co-purifying with CLU from human plasma that was for 10 days stored static at 4°C or gently rotated at 37°C.
After separation of equivalent amounts of protein by reducing SDS-PAGE, proteins were transferred to nitrocellulose membrane and probed with anti-FGN antiserum. The figure shows (i) molecular weight markers (Mr), and all proteins bound to the column from human plasma (ii) stored at 4°C or (iii) gently rotated at 37°C. The identity of the samples is indicated above the corresponding lanes and bands corresponding to the approximate molecular weight of the α, β and γ chains of FGN are indicated.
Identification of putative endogenous plasma client proteins for CLU

Figure 4.15 Detection of putative CLU-FGN complexes in human plasma by sandwich ELISA.

Human plasma was gently rotated at 37°C for 10 days, or stored static at 4°C for the same time period, and subsequently passed over an anti-CLU immunoaffinity column. In each case, 50 μL of a solution containing the proteins eluting from the column was incubated in anti-CLU G7 coated wells followed by goat anti-FGN antiserum and then anti-sheep/goat IgG-HRP. The results shown are the average A490 nm (n = 3 ± standard error) and were adjusted for non-specific binding by subtracting the absorbance produced by a species-matched primary control antibody of irrelevant specificity followed by the secondary antibody in the G7 coated wells. + Indicates significantly greater A490 nm by Student’s t-test, p ≤ 0.01.

4.3.5 An in vitro method to form and purify HMW CLU-FGN complexes

When whole plasma was fractionated by Superose™ 6 SEC, FGN and CLU were only detected in fractions corresponding to ≥ 4 x10⁷ Da when the plasma had been rotated for 10 days at 37°C, but not if it had been stored static at 4°C. This suggested that under these conditions endogenous plasma CLU and FGN formed complexes comparable in size to HMW CLU-GST or HMW CLU-CS protein complexes formed during heat stress in vitro (Figure 4.16).
Identification of putative endogenous plasma client proteins for CLU

Figure 4.16 Anti-FGN and anti-CLU dot blot analysis of SEC fractionated plasma stored static at 4°C for 10 days or incubated with gentle rotation at 37°C. After 10 days incubation with gentle rotation at 37°C or static storage at 4°C, plasma was fractionated using a Superose™ 6 column ($V_0$ ≥ $4 \times 10^7$ Da at approximately 8 mL). 20 μL from each fraction was spotted onto nitrocellulose membrane. The blocked membranes were incubated in either goat anti-FGN antiserum followed by anti-sheep/goat IgG-HRP or a cocktail of monoclonal anti-CLU hybridoma culture supernatants (G7 + 41D) and then sheep-anti-mouse Ig-HRP prior to development by ECL. The identity of the sample is indicated to the left of the corresponding blot.

Incubation of 6 μM FGN in PBS at temperatures between 37-43°C for 72 h did not produce a measurable increase in turbidity without additional stressors such as co-incubation with the macromolecular crowding agent PEG 4,000 (data not shown). However, compared with GST and OVA, FGN was highly susceptible to precipitation induced by PEG 4,000-mediated macromolecular (Figure 4.17); this effect was dose-dependent ($F(2, 6) = 462.99; p \leq 0.0001; $ Tukey HSD, $p \leq 0.01$ in all cases). While co-incubation with CLU inhibited the PEG 4,000-induced precipitation of FGN, CLU and FGN did not form HMW complexes under these conditions (data not shown). Given that HMW complex formation was necessary to separate CLU-stressed protein complexes from free CLU or residual stressed client protein using SEC, the precipitation of FGN at higher temperatures was investigated.
Figure 4.17 The effect of macromolecular crowding on precipitation of FGN.

FGN, OVA or GST were incubated at 1 mg/mL in PBS containing PEG 4,000 at 0, 70 or 140 mg/mL. The mean turbidity (n = 3 ± standard deviation) as measured using the A360 nm is shown for the three proteins and PEG 4,000 in PBS in the absence of protein. * Denotes significantly increased turbidity compared to 0 mg/mL PEG 4,000 (Tukey HSD, p ≤ 0.01).

Incubation of FGN at 45°C in the absence of crowding agents caused rapid precipitation of the protein after an initial lag phase of approximately 200 min; only minor increases in turbidity occurred after about 600 min (Figure 4.18). Co-incubation of 6.0 μM FGN with 6.5 μM CLU completely abolished any increase in turbidity under the same conditions. Co-incubation of 6.0 μM FGN with 6.5 μM BSA had a negligible effect. BCA confirmed that there was a residual amount of protein (less than 10% of the original amount) left remaining in solution after heat treatment of FGN (data not shown).
Identification of putative endogenous plasma client proteins for CLU

Figure 4.18 Inhibition of heat-induced precipitation of FGN by CLU.
6.0 μM FGN was heated at 45°C for 1,000 min in the presence or absence of 6.5 μM CLU or BSA. The stability of CLU and BSA was also assessed by heating the proteins alone under the same conditions. All samples were prepared in PBS. The average (n = 3) A360 of the protein solutions is plotted.

Co-incubation of FGN with CLU at 45°C resulted in the generation of HMW species at the exclusion limit of a Superose™ 6 column (Figure 4.19; uppermost trace). There was no observable difference in the SEC profiles of native FGN and the residual heated FGN remaining in solution after heat-induced precipitation. In both instances the majority of FGN was seen to elute in a peak corresponding to approximately twice the normal molecular weight of monomeric FGN (340 kDa). Previous studies have reported that FGN exists mainly as a dimer in solution (Fowler et al., 1981; Rivas et al., 1999). When residual heated FGN and CLU were allowed to mix at room temperature there was a small increase in larger species eluting at the exclusion limit of the column, however this was much less than that observed after co-incubation during heat stress (Figure 4.19). As observed for other stressed client proteins, species eluting at $V_o \geq 4 \times 10^7$ Da (Figure 4.19; uppermost trace) were verified to contain CLU-FGN complexes by sandwich ELISA (Figure 4.20).
Identification of putative endogenous plasma client proteins for CLU

Figure 4.19 SEC of heat stressed or native CLU and FGN.
6.0 μM FGN and 6.5 μM CLU were incubated together at 45°C for 1,000 min in PBS ((FGN + CLU) 45°C). The size exclusion profile of these proteins was compared to that of the corresponding native proteins or residual heated proteins (denoted by *) incubated alone or together at ambient temperature for 50 min in PBS. The results are from a Superose™ 6 column running at 0.5 mL/min and the positions of molecular weight markers are indicated by labelled arrows; the exclusion limit (V_o) ≥ 4 × 10⁷ Da.

Figure 4.20 Detection of CLU-FGN complexes by sandwich ELISA.
Samples analyzed in a sandwich ELISA using plate bound anti-CLU antibody (G7) were: (i) Protein eluting at ≥ 4 × 10⁷ Da from a Superose™ 6 column after 6.0 μM FGN and 6.5 μM CLU was co-incubated at 45°C for 1,000 min in PBS (HMW CLU-FGN), (ii) CLU (iii) FGN (iv) *FGN or (v) *FGN and *CLU after co-incubation at ambient temperature for 50 min in PBS (*FGN + *CLU). 50 μg/mL total protein was added to the wells followed by goat anti-FGN antiserum and anti-sheep/goat IgG-HRP. The results shown are the average A490 nm (n = 3 ± standard error) and were adjusted for non-specific binding by subtracting the absorbance produced by a species-matched control primary antibody of irrelevant specificity followed by the secondary antibody in G7 coated wells. * Denotes the use of residual heated controls. + Indicates significantly greater A490 nm compared to all controls (Tukey HSD, p ≤ 0.01).
4.3.6 Investigations of the chaperone activity of FGN compared to CLU

After it was recently reported that FGN has chaperone-like activity (Tang et al., 2009), using a method similar to that of the original study, the chaperone activity of FGN was examined (Figure 4.21). At a molar ratio of 1:1, FGN appeared to slow the precipitation of CS, however, at the conclusion of the experiment there was little difference between the final turbidity of the solution containing CS:FGN at 1:1 or a solution containing CS alone. In contrast, a solution containing CS:CLU at 1:1 exhibited markedly less turbidity. When CS and FGN were coincubated at a molar ratio of 1:2 after initially slowing the precipitation of CS, the presence of FGN appeared to increase the overall turbidity of the solution after prolonged heating. However, when CLU was present at twice the molar concentration as CS, any increase in precipitation was abolished. Instability of FGN at 43°C is suggested by a slight increase in turbidity in the solution containing FGN alone.

Figure 4.21 Inhibition of heat-induced CS precipitation by CLU or FGN.
3 μM CS was heated at 43°C for 500 min in the presence or absence of CLU or FGN at a molar ratio of 1:1 or 1:2. The stability of FGN was assessed by heating the proteins alone under the same conditions. All samples were prepared in PBS. This figure shows the average A360 nm (n = 3) of the protein solutions indicated in the key.
4.4 Discussion

The idea to search for endogenous human plasma client proteins for CLU arose from the observation that a fraction of protein collected after anti-CLU immunoaffinity chromatography, even after washing with detergent, is "contaminant" (Figure 4.1). When this contaminant protein was separated from purified CLU by ion-exchange chromatography, SDS-PAGE and Western blot analysis showed that it contained predominantly species > 170 kDa that, when reduced, migrated during electrophoresis to the approximate size of free CLU and IgG (Figure 4.2). Moreover, sandwich ELISA indicated that IgG was complexed to CLU in the fraction containing contaminant species > 170 kDa (Figure 4.3). Interaction between CLU and immunoglobulins has previously been documented (Wilson and Easterbrook-Smith, 1992b). Therefore, it is highly feasible that CLU-IgG complexes exist in vivo. Evidence that IgG may be an endogenous client protein for CLU includes the co-localization of CLU in immunoglobulin deposits in vivo and the development of progressive glomerulopathy in CLU knock-out mice (French et al., 1992; Rosenberg et al., 2002). It is unknown whether the association of IgG with CLU in this contaminating fraction is the result of the chaperone activity of CLU acting on damaged IgG or an association between CLU and native IgG. However, the result of SEC in this study suggests that there is minimal association of CLU and native IgG in solution (Figure 4.9). Given that both heat and oxidative stress-induced precipitation of IgG can be inhibited by CLU in vitro, taken together with the above observations in vivo, it appears likely that stressed IgG is an endogenous in vivo client protein for CLU.

Although complete characterization of the putative CLU-IgG complex reported in this study was not carried out, an experiment was performed to examine the binding of the "contaminant" CLU/IgG fraction (containing an unknown amount of putative CLU-IgG complexes) to human leukocytes. The rationale for undertaking this investigation came from reports that stimulation of neutrophils by aggregated IgG results in a self-perpetuating cycle of free radical generation by respiratory burst and protein aggregation (Lunec et al., 1985). The protein from the CLU/IgG fraction bound to both monocytes and granulocytes, however, the difference between the binding of uncomplexed CLU or IgG and the protein from the CLU/IgG fraction was much greater for granulocytes compared to monocytes, suggesting that granulocytes preferentially bind the putative CLU-IgG complexes. Interestingly, co-incubation with
excess uncomplexed CLU or IgG could only inhibit less than one third of the binding of the protein from the CLU/IgG fraction to granulocytes (data not shown). This suggests that the binding of the protein from the CLU/IgG fraction to granulocytes is via a unique binding site, although further work is required to confirm this. As the results suggest that the association of CLU with IgG promotes binding of the latter to granulocytes, it is recommended that further investigations are carried out to gauge the effect of CLU-association with IgG on the stimulation of granulocytes and respiratory burst.

Oxidative stress is implicated in many disease states including atherosclerosis, arthritis, muscular dystrophy, cataract, pulmonary dysfunction, certain neurological disorders and also has importance in the normal biology of aging (Stadtman and Oliver, 1991; Berlett and Stadtman, 1997). Proteins are constantly exposed to potentially damaging ROS which are generated from numerous endogenous and exogenous sources. Damage to proteins by oxidation may result in side chain oxidation, protein-protein cross-linking or oxidation of the protein backbone allowing cleavage by diamide or $\alpha$-amidation pathways (Berlett and Stadtman, 1997). Fortunately, antioxidant defenses including proteins (e.g. SOD and catalase) and small molecules (e.g. vitamin C, vitamin E, vitamin A and bilirubin) exist to protect us from these damaging ROS. Antioxidants extend to a range of trace elements including zinc, magnesium and manganese and the proteins ceruloplasmin and transferrin which are all found extracellularly (Taysi et al., 2002; Yilmaz et al., 2005). Instinctively, disease states arise when there is an imbalance between antioxidants and oxidants, however, the free-radical theory of aging suggests that there is progressive overwhelming of antioxidant defenses during life (Harman, 1957; Harman, 1981). This highlights the relevance of studying chaperones in an oxidizing system. In plasma, hydrogen peroxide and overall ROS concentrations in vivo are much less than those used to induce protein aggregation in this study (Hammadeh et al., 2008; Tsukimori et al., 2008). As previously stated it is very difficult to accurately mimic in vivo conditions in vitro and the degree of stress required to induce the precipitation of low levels of purified proteins in buffered solutions will undoubtedly be different to the cumulative stresses experienced in physiological fluids. This is particularly true of oxidative stress since it is the cumulative effect of numerous different reactive species. Localized concentrations of ROS may change quite dramatically during normal physiological processes such as respiratory burst (Bostan et al., 2001; Ferretti et al., 2006).
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The copper catalyzed hydrogen peroxide reaction used in this study is routinely used to elicit protein oxidation in a manner that is comparable to that which may occur in vivo (Ramirez et al., 2005). Using the model protein LYS, it was observed that oxidative stress-induced precipitation was inhibited by CLU (Figure 4.5). Interestingly, HMW CLU-LYS formation occurred when the proteins were co-incubated in the oxidizing buffer, but not if oxidized separately and then incubated. This suggests that transient stress-induced changes in both CLU and the stressed client protein are required for their interaction. Complex formation was similar to that observed for GST heated in the presence of CLU at 60°C - the species formed were ≥ 4 x 10^7 Da in size and could be purified by SEC. Given that heat-induced precipitation of IgG could not be achieved at physiologically relevant temperatures and aggregated IgG is implicated in the pathology of numerous inflammatory conditions, the purification of oxidized CLU-IgG complexes was attempted. Despite inhibiting the precipitation of oxidized IgG, HMW CLU-IgG complexes were not formed (Figure 4.9). Given the inherent difficulties in purifying CLU-stressed protein complexes that are not in the HMW range, further experiments were designed to identify other potential endogenous clients for CLU (see Chapter 3).

In an attempt to model physiologically relevant stresses in a biological fluid a series of experiments using human plasma were carried out. In the first instance human plasma was incubated at normal human body temperature (37°C) with gentle rotation to simulate shear stress of plasma being pumped around the body. Considering that arterial shear stress can reach levels >15 dyne/cm² and rotational devices such as cone-plate viscometers would require fluid to move at speeds more than 15 times greater than those used here to exert a similar force, the conditions used in this study are likely to represent a "stress" scenario significantly less harsh than that which can be encountered in vivo. Nevertheless, even under these mild conditions a difference was seen between shear stressed plasma at 37°C and plasma stored static at 4°C for 10 days. Stressed plasma was significantly more turbid compared to plasma stored stationary at 4°C, suggesting that the stress involved was sufficient to induce protein unfolding (Figure 4.10). It is important to note that although plasma stored static at 4°C is a condition considered to pose minimal stress, cold denaturation of globular proteins may result after incubation at low temperatures (Privalov et al., 1986; Gursky and Atkinson, 1996). Therefore, the difference in turbidity between the two plasma treatments is likely to be
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an underestimate compared to the difference between plasma gently rotated at 37°C and a true negative control in which minimal protein unfolding would occur.

While it is expected that many thousands of different proteins may be detected in human plasma, on a mass basis, a relatively small number of proteins make up the overwhelming majority of the total protein content. Eleven of the most abundant plasma proteins were screened for their association with CLU after shear or oxidative stress at 37°C. This was carried out using a sandwich ELISA in which CLU and any plasma proteins that were bound to the chaperone were captured using G7 anti-CLU antibody and the CLU-associated plasma proteins detected using relevant primary and secondary antibodies. Stringent controls were included to account for non-specific binding of plasma proteins to plate bound G7 and non-specific binding of the primary and secondary antibodies. This approach indicated that several abundant plasma proteins displayed increased association with CLU after 10 days of shear or oxidative stress at 37°C compared with static storage at 4°C (Figure 4.11). The increased association of the two other known extracellular chaperones (α2M and Hp) with CLU after shear stress may be due to shared recognition of stressed plasma proteins resulting in the formation of complexes containing the stressed proteins and more than one type of chaperone. Of the remaining proteins, FGN, ACID GLY and IgM showed increased association with CLU as a result of shear or oxidative stress, while the association of TRANS with CLU was only increased after oxidative stress. It is likely that proteins displaying increased CLU association were relatively unstable under the experimentally imposed stresses compared to other plasma proteins under the same conditions. Stress-dependent CLU association suggests that these proteins are putative endogenous clients for the chaperone. This may be particularly significant in the case of proteins displaying increased CLU association after shear/heat stress, as the experimental stress was much less severe than proteins in vivo are expected to encounter.

SEC analyses of protein fractions prepared by anti-CLU immunoaffinity chromatography of plasma that had been rotated at 37°C for 10 days or stored at 4°C for the same period indicated that the molecular species contained in the former fraction were biased towards those of very high mass (Figure 4.12). This result suggests that the experimental conditions of shear stress at 37°C was sufficient to partially unfold the client proteins and target them to CLU, and that these large species are complexes
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formed between CLU the client proteins. However, CLU binds to aggregated immunoglobulin in non-stressed plasma (see Section 4.3.1) and is also found associated with apoAI-containing HDL particles (Jenne et al., 1991). In the absence of the detergent and low pH/salt washes, these species are likely to co-purify with CLU from unstressed plasma and probably account for some of the larger species present in both stressed and unstressed plasma. It is predicted that chaperone-stressed protein complexes formed \textit{in vivo} are rapidly cleared from circulation and degraded, however, a small amount may be found in freshly isolated plasma (see Section 1.4). When comparing the SEC profiles of the protein fraction recovered by immunoaffinity chromatography of plasma that had been rotated for 10 days at 37°C to that of HMW CLU-stressed protein complexes generated \textit{in vitro}, it is apparent that only a small fraction of the former eluted at the same position as the latter (i.e. at the exclusion limit of the column). Possible reasons for this difference include partial disruption of complexes during elution from the anti-CLU column with 2 M GdHCl or that harsher conditions than rotation for 10 days at 37°C are required to induce unfolding to the degree necessary to form HMW CLU-stressed protein complexes with many plasma proteins. Given that the conditions used to elute proteins from the immunoaffinity column may have led to at least partial disruption of some of the complexes it is not possible to accurately estimate the size of CLU-client protein complexes generated \textit{in situ} in human plasma. However SEC suggests they are likely to be at least 500 kDa and may reach \(4 \times 10^7\) Da (Figure 4.16).

FGN was the most abundant plasma protein that consistently showed increased association with CLU in plasma after exposure to stresses. The fact that FGN is one of the most abundant plasma proteins probably facilitated its identification compared to other less abundant plasma proteins. However, the results of ELISA and mass spectrometric analyses of proteins co-purifying with CLU from stressed plasma suggested other endogenous client proteins for CLU are likely to exist. Proteins exhibiting increased association with CLU after plasma was stressed have already been discussed. In addition, vitronectin precursor protein and several other unidentified proteins co-purified with CLU from plasma that had been incubated at 37°C with slow rotation (Figure 4.13). More than 10,000 different proteins may be normally present in plasma at low levels. Given that the chaperone activity of CLU has been demonstrated for a wide variety of proteins \textit{in vitro} it is feasible that there are many endogenous client proteins for this and other extracellular chaperones. The identification of FGN as a
Identification of putative endogenous plasma client proteins for CLU

putative endogenous client protein for CLU may have relevance to a variety of diseases. Both CLU and FGN are found in deposits known as drusen in AMD (Crabb et al., 2002). It has been suggested that compositional similarities between drusen in AMD and other extracellular deposits found in atherosclerosis, elastosis, amyloidosis, and dense deposit disease may be the result of a shared cause (Mullins et al., 2000). The deposition of aggregated FGN is also reported in breast cancer, (Costantini et al., 1991), mesothelioma (Wojtukiewicz et al., 1989a), colon cancer (Wojtukiewicz et al., 1989b) and lymphoma (Costantini et al., 1992). Recently it has been reported that FGN has chaperone-like activity (Tang et al., 2009). However, attempts to reproduce the findings of that study using CS as a substrate for FGN at 43ºC (as reported) showed that FGN does not possess a potent chaperone activity (Figure 4.21). While in the early stages of heat stress, co-incubation of CS with FGN had some limited inhibitory effect on precipitation, after prolonged heating at a molar ratio of 1:1 there was no difference in the final turbidity compared to CS alone, and at a molar ratio of 1:2 the final turbidity was actually increased. This may be due to the higher concentration of FGN having macromolecular crowding effect on CS, or a combined effect of macromolecular crowding and destabilization of FGN via interaction with hydrophobic regions on unfolding CS. From this it appears that FGN is not an extracellular chaperone. Moreover, compared to the three known extracellular chaperones, FGN displays relatively low thermostability.

Attempts to form HMW CLU-FGN complexes at physiologically relevant temperatures (≤ 42ºC) were unsuccessful and the elevated temperature of 45ºC was required to induce the precipitation of FGN within an experimentally convenient timeframe. As previously discussed, HMW complex formation was a requirement for purification by SEC since other methods for the bulk purification of CLU-stressed protein complexes, such as ion-exchange chromatography, were not possible (see Chapter 3). In the case of FGN, dot blot analysis of SEC fractionated human plasma suggests that a portion of CLU-FGN complexes formed in plasma after incubation under conditions of physiologically relevant stress are comparable in size to the HMW complexes (≥ 4 x 10^7 Da) formed by purified proteins in buffered solutions (Figure 4.16). It is difficult to replicate \textit{in vitro} the conditions found in complex biological fluids \textit{in vivo}. This may have particular relevance to inducing FGN precipitation, since FGN is highly susceptible to unfolding under conditions of macromolecular crowding (Figure 4.17).
For example, at room temperature, an increase in PEG 4,000 concentration from 0 mg/mL to 70 mg/mL (the approximate total protein concentration of plasma) resulted in a significant increase in turbidity of a solution also containing FGN. However, under these conditions, co-incubation with PEG 4,000 prevented the formation of easily-purified HMW CLU-FGN complexes. Therefore, a slightly elevated temperature (45°C) in the absence of macromolecular crowding agents was used to form HMW CLU-FGN complexes.

The following chapters describe further experiments using HMW CLU-FGN complexes in addition to HMW CLU-GST formed using supraphysiological temperature (60°C) and HMW CLU-CS complexes formed at a physiologically relevant temperature (41°C).
5 CHARACTERIZATION OF HMW CLU-STRESSED PROTEIN COMPLEXES

5.1 Introduction

In vitro the chaperone activity of CLU is similar to that of sHsps and involves the formation of soluble HMW complexes with stressed (partially unfolded) client proteins (Humphreys et al., 1999; Poon et al., 2000; Wilson and Easterbrook-Smith, 2000; Poon et al., 2002b). However, currently little is known about the physical characteristics of the complexes CLU forms with stressed client proteins. Understanding the physical characteristics of HMW CLU-stressed protein complexes is necessary to provide further insight into the mechanism of CLU’s chaperone action. Additionally, description of the physical characteristics of HMW CLU-stressed protein complexes may help identify their receptors since many physical factors are known to affect the affinity of ligands for receptors. For example the fructose receptor displays a 3,000-fold increase in affinity for glycoproteins with diameters of 15 nm compared to glycoproteins with diameters closer to 5 nm (Biessen et al., 1994). Also, factors such as exposed hydrophobicity such as might be observed when the native conformation of a protein is disrupted, and polyanionic charge are common characteristics of ligands for scavenger receptors.

The work described in this chapter is focussed upon characterizing the physical properties of HMW CLU-stressed protein complexes. The experiments were performed using a range of client proteins in order to establish whether the characteristics of HMW CLU-stressed protein complexes are largely similar or differ depending on the client protein. Most of the work was carried out using SEC purified HMW CLU-GST formed at a supraphysiological temperature (60°C; see Section 3.3.1), HMW CLU-CS formed at a physiologically relevant temperature (41°C; see Section 3.3.2.2) and HMW CLU-FGN formed using a potential endogenous plasma client protein at a near physiological temperature (45°C; see Section 4.3.5); limited data is also presented for HMW CLU-LYS (oxidative stress; see Section 4.3.2.1). Much of this work outlined in this chapter has now been published (Wyatt et al., 2009b; Appendix 1.3).
5.2 Materials and methods

5.2.1 Purification of HMW CLU-stressed protein complexes

Client proteins were stressed in vitro using heat or oxidative stress, in the presence of CLU, to generate HMW CLU-stressed protein complexes (see Section 2.6). Stressed protein mixtures were fractionated by SEC using a Superose™ 6 10/300 column (see Section 2.8) and protein eluting at the exclusion limit (≥ 4 x 10^7 Da) retained and designated HMW CLU-stressed protein complex. HMW CLU-stressed protein complexes were stored in PBS/Az at 4°C and were routinely re-injected back on to the Superose™ 6 10/300 column before their use in experiments to ensure that breakdown of the complex had not occurred. HMW CLU-GST, HMW CLU-FGN and HMW CLU-CS were generated at 60°C, 45°C and 41°C, respectively (unless otherwise stated).

5.2.2 Preparation of residual stressed protein controls

Residual (soluble) heated GST, CS, FGN and CLU were prepared as described in Section 2.7 and residual (soluble) oxidized LYS or CLU were prepared as described in Section 4.2.4. Below and in the figures, residual heated control proteins are denoted by * (e.g. *GST) and residual oxidized controls are given the prefix ox (e.g. oxLYS). In instances where only one result is shown for *CLU, incubation was carried out at 60°C for 50 min unless otherwise described.

5.2.3 Transmission electron microscopy (TEM)

Carbon-coated nickel grids (3 mm nickel coated 400 mesh; kindly donated by Dr Sarah Meehan, Cambridge University, Cambridge, UK) were pseudo-glow discharged by exposure to ultraviolet (UV) lamp for 10 min. 2 μL samples at 0.2-0.5 mg/mL were loaded on the grids and diluted with 10 μL of 0.22 μm filtered milliQ water and left to stand for 2 min. The grids were dried using blotting paper by capillary action and washed three times by applying a further 10 μL of 0.22 μm filtered milliQ water and drying in the manner described. A stock of 2% (w/v) uranyl acetate (ProsciTech, Kirwan, Australia) was prepared in milliQ water and 0.22 μm filtered before 10 μL was used to stain each grid. After incubating for 1 min the uranyl acetate was removed using blotting paper and the grids were allowed to air dry in a fume cabinet for 5 min. The
Characterization of HMW CLU-stressed protein complexes

 grids were viewed under 63,000x magnification at 120 kV excitation voltage on a Phillips CM100 transmission electron microscope. The images were processed using the SIS Megaview II Image Capture System (Olympus, Hamburg, Germany). TEM was kindly performed by Dr Sarah Meehan (University of Cambridge, Cambridge, UK).

5.2.4 Dynamic light scattering (DLS)

Solutions of GST, *GST (residual protein after 50 min at 60°C), FGN, *FGN (residual protein after 12 h at 45°C), CS and *CS (residual protein after 9 h at 41°C), CLU, *CLU (residual protein after 50 min at 60°C), SEC purified HMW CLU-GST, HMW CLU-CS and HMW CLU-FGN at 0.3 mg/mL, 0.4 mg/mL and 0.5 mg/mL in 0.22 μm filtered PBS were analyzed in low volume plastic cuvettes using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). Particle diameters were recorded as a frequency distribution curve and the average diameter and range (average peak and width of 9 normally distributed curves) reported.

5.2.5 Densitometry

Purified CLU, FGN, CS and GST were dialyzed against several changes of distilled water. The proteins were then freeze dried, weighed using a XS205 Dual Range analytical balance (Mettler Toledo, Columbus, USA) and redissolved in PBS. The absorbance of the solutions was measured to calculate the extinction co-efficient at 280 nm using Beer’s law $A = \varepsilon \cdot c \cdot d$. Volumes equivalent to 5 μg, 2 μg, 1 μg and 0.5 μg were loaded in triplicate on a 12% SDS-PAGE gel following reduction using 100 mM DTT and 1 mM 2-mercaptoethanol. SEC purified HMW CLU-GST, HMW CLU-CS and HMW CLU-FGN were also reduced and loaded onto the gel. After staining and destaining, standard curves were constructed for each protein using a GS 800 calibrated densitometer (Bio-Rad) and Quantity One software (Bio-Rad). The average optical density/mm² of the major bands was used to construct a standard curve for each protein. Using these standard curves, the relative amounts of CLU and FGN, CS or GST present in the HMW fractions were calculated. Where reduction resulted in multiple bands the most prominent bands were selected for the construction of standard curves.
5.2.6 BisANS fluorescence

Heated and unheated proteins were added to give a final concentration of 50 μg/mL in solutions containing 0-32 μg/mL bisANS. These samples were prepared in triplicate in a black 96 well plate (Greiner Bio-one) to achieve a final volume of 100 μL/well. The plate was incubated at 37°C with shaking for 20 min before the fluorescence (Ex 360 nm, Em 520 nm) was measured using a FLUOstar Optima spectrophotometer (BMG Labtechnologies). Samples included GST, *GST, FGN, *FGN, CLU, *CLU (after 50 min at 60°C or 12 h at 45°C), SEC purified HMW CLU-GST and HMW CLU-FGN. In these assays, HMW CLU-stressed protein complexes were compared with mixtures of residual heated or native CLU or client proteins present at the same final concentrations and same mass ratio as in the complexes.

5.2.7 Thioflavin T fluorescence

A final concentration of 62.5 μM thioflavin T (Sigma-Aldrich) was added to protein solutions and the fluorescence measured using a FLUOstar Optima microplate reader (BMG Labtechnologies) with an excitation wavelength of 440 nm and an emission wavelength of 490 nm (slit-width 10 nm). SEC purified HMW CLU-FGN and HMW CLU-GST complexes were analyzed at 50 μg/mL in PBS. LYS amyloid (50 μg/mL in PBS), formed as in Yerbury et al. (2007), was used as a positive control. Samples included GST, *GST, FGN, *FGN, CLU, *CLU (after 50 min at 60°C or 12 h at 45°C), SEC purified HMW CLU-GST and HMW CLU-FGN. In these assays, HMW CLU-stressed protein complexes were compared with mixtures of residual heated or untreated individual proteins present at the same final concentrations and same mass ratio as in the complexes.

5.2.8 CD analysis

CD analysis was performed as described in Section 2.12. Samples included GST, *GST (after 50 min at 60°C), FGN, *FGN (after 12 h at 45°C), CLU, *CLU (after 50 min at 60°C or 12 h at 45°C), SEC purified HMW CLU-GST and HMW CLU-FGN. HMW CLU-stressed protein complexes were compared with mixtures of residual heated or untreated individual proteins present at the same final concentrations and same mass ratio as in the complexes.
5.3 Results

5.3.1 Quality control analysis of SEC purified HMW CLU-stressed protein complexes

Quality control was routinely performed before experiments to assess the stability of SEC purified HMW CLU-stressed protein complexes by re-injection of the Superose\textsuperscript{TM} 6 10/300 column fractionated protein onto the same column. It was found that none of the HMW CLU-stressed protein complexes remained stable when freeze dried and reconstituted and only HMW CLU-GST was able to withstand repeated freeze (-20°C) thawing (data not shown). In contrast, all HMW CLU-stressed protein complexes were stable for months when stored at 4°C in PBS/Az - under these conditions, there was no evidence of a time-dependent dissociation of any of the complexes (Figure 5.1).

![SEC analysis of HMW CLU-stressed protein complexes stored at 4°C for 3 months.](image)

The SEC purified HMW CLU-stressed protein complexes (collected at the exclusion limit $V_e \geq 4 \times 10^7$ Da from a Superose\textsuperscript{TM} 6 column) were stored in PBS/Az at 4°C for 3 months before they were re-injected onto the same column. The resulting size exclusion profiles of the stored HMW CLU-stressed protein complexes are shown.
5.3.2 TEM

TEM showed a clear difference between mixtures of (i) CLU and (ii) GST, CS or LYS incubated in the presence or absence of heat or oxidative stress (Figure 5.2). While individual native molecules are barely discernable (Figure 5.2; panels A, C and E), at the same magnification species in the size range of 10-100’s nm are clearly visible on grids containing the oxidized or heat stressed proteins (Figure 5.2; panels B, D and F). Of the 3 client proteins examined, it appeared that GST formed slightly smaller complexes with CLU (mostly 20-50 nm) compared to LYS or CS (mostly 50-100 nm). Although some of the largest species may be an artifact of the drying down process, the images obtained strongly suggest that amorphous aggregates result from the co-incubation of these client proteins with CLU during heat or oxidative stress.
Figure 5.2 TEM images of native and stressed LYS, GST and CS in the presence of CLU.
70 μM LYS was incubated overnight at 37°C in the presence of 13 μM CLU in (A) PBS, or (B) oxidizing buffer. 20 μM GST was incubated for 50 min in the presence of 3.2 μM CLU in PBS at (C) room temperature or (D) 60°C. 6.0 μM CS was incubated for 550 min in the presence of 6.6 μM CLU in PBS at (E) room temperature or (F) 43°C.

5.3.3 DLS

The results of DLS were consistent between 0.1 and 1 mg/mL; representative results obtained at 0.5 mg/mL are shown (Figure 5.3). The limitations of DLS in resolving polydisperse solutions are well known (Pusey and Tough, 1985). In contrast to SEC,
DLS was unable to resolve different sized oligomers of CLU in solution; DLS analysis of CLU samples indicated a normally distributed particle size (approximately 10 nm in diameter). Also, there was no apparent difference in size between native and heated control proteins, however, this does correspond with the results from SEC. Incubation of CLU and *FGN or *GST resulted in a peak corresponding to an intermediate size compared to CLU or the stressed client protein alone. Again this suggested that the instrument was limited in its ability to resolve particles of similar size. DLS indicated that SEC purified HMW CLU-FGN, HMW CLU-CS and HMW CLU-GST complexes had average diameters (± range) of 108.57 ± 18.09 nm, 51.06 ± 6.87 nm and 52.61 ± 7.71 nm, respectively.

Figure 5.3 DLS estimates of the mean diameters of HMW CLU-stressed protein complexes and uncomplexed native and residual heated control proteins. Samples analyzed were SEC purified HMW CLU-FGN, HMW CLU-CS and HMW CLU-GST complexes, FGN, CS, GST, CLU, *FGN, *CS, *GST and *CLU, and equivalent amounts of residual heated proteins, co-incubated at ambient temperature for 20 min (*FGN + *CLU, *CS + *CLU and *GST + *CLU). For *CLU, the incubation was 50 min at 60°C. Histograms represent mean diameter ± range of 9 normally distributed curves.
5.3.4 Estimates of stoichiometry

To estimate the stoichiometry of HMW CLU-stressed protein complexes, complexes were separated by SDS-PAGE under reducing conditions. The intensity of individual protein bands were quantified by densitometry after staining the gels with Coomassie blue. Figure 5.4 shows an image of a representative gel for the HMW CLU-GST complex. After standard curves were generated for each protein, the approximate mass and molar ratios of proteins in each complex were calculated. The mass ratio of CLU to stressed client protein was similar for FGN, CS and GST complexes (about 1:2 in each case). However, the molar ratios were very different (Table 5.1). These estimated CLU:client protein ratios were employed when selecting controls for subsequent structural studies of the HMW CLU-stressed protein complexes. An additional degree of error is unaccounted for since in some cases not all protein was fully reduced. In the example provided this is evident from a faint band around 100 kDa. However in all cases, the protein not fully reduced was estimated to be less than 1% of the total protein and as such would not significantly affect the results.

![Image of a reducing SDS-PAGE gel of HMW CLU-GST.](image)

SEC purified HMW CLU-GST (≥ 4 x 10^7 Da) was analyzed under reducing conditions on a 12% SDS PAGE gel. The lanes contain (1) Page Ruler™ prestained protein ladder and (2) reduced HMW CLU-GST. The positions of reduced monomeric CLU and GST are indicated to the right of the figure.
Characterization of HMW CLU-stressed protein complexes

Table 5.1 Approximate ratio of CLU to stressed client protein in SEC purified HMW CLU-FGN, HMW CLU-CS and HMW CLU-GST complexes.

SEC purified HMW CLU-FGN, HMW CLU-CS and HMW CLU-GST complexes were separated by reducing SDS-PAGE, stained with Coomasie blue, and analyzed using a GS800 calibrated densitometer and Quantity One software. Using standard curves plotting the optical density versus the amount of each protein, the average ratios (CLU:client protein) ± standard error were calculated.

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<tbody>
<tr>
<td>CLU-FGN</td>
<td>12 h, 45°C</td>
<td>1 : 1.88</td>
<td>0.146</td>
<td>1 : 0.34</td>
<td>0.03</td>
</tr>
<tr>
<td>CLU-CS</td>
<td>9 h, 41°C</td>
<td>1 : 1.81</td>
<td>0.027</td>
<td>1 : 1.17</td>
<td>0.01</td>
</tr>
<tr>
<td>CLU-GST</td>
<td>50 min, 60°C</td>
<td>1 : 2.11</td>
<td>0.168</td>
<td>1 : 5.14</td>
<td>0.41</td>
</tr>
</tbody>
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5.3.5 Estimates of exposed hydrophobicity

In this study bisANS binding was used as a measure of exposed hydrophobicity. The results suggested that there was little difference in the level of exposed hydrophobicity between native CLU and CLU heated at 60°C for 50 min (Figure 5.5; panel A). Of the stressed client proteins, bisANS binding was greater for *GST heated at 60°C for 50 min compared to native GST and this was reflected in terms of both the $F_{\text{max}}$ and $K_d$ (Figure 5.5; panel B and Table 5.2). However there was little difference between the bisANS binding of residual FGN, heated at 45°C for 9 h, compared to native FGN (Figure 5.5; panel C). HMW CLU-GST bound less bisANS than a mixture of CLU and *GST at the same final concentrations - this was reflected in terms of both the $F_{\text{max}}$ and $K_d$ (Figure 5.5; panel D and Table 5.2). In contrast, there was little difference between the bisANS binding of HMW CLU-FGN versus a mixture of CLU and *FGN (at the same final concentrations) (Figure 5.5; panel E).
Characterization of HMW CLU-stressed protein complexes

Figure 5.5 Plots showing concentration dependence of bisANS fluorescence for HMW CLU-stressed protein complexes and native or heated client proteins. Each panel represents a single experiment. Samples analyzed were (A) CLU and *CLU (50 min at 60°C); (B) GST and *GST; (C) FGN and *FGN; (D) HMW CLU-GST complexes and a mixture of *CLU and *GST (preheated for 50 min at 60°C; present at the mass ratio 1:2.11); (E) HMW CLU-FGN complexes, and a mixture of *CLU and *FGN (preheated for 45 min at 60°C; present at the mass ratio 1:1.88). Data points shown represent the mean fluorescence (n = 3 ± standard error) in AFU.
Table 5.2 Binding parameters for the binding of bisANS to HMW CLU-stressed protein complexes and heated or native client proteins.

Estimated values of binding affinity ($K_d$) and maximum fluorescence ($F_{\text{max}}$; in arbitrary fluorescence units, AFU) for the binding of bisANS to a variety of proteins and complexes are shown. *CLU was heated at 60°C for 50 min, except when used as a control for HMW CLU-FGN where *CLU was heated at 45°C for 12 h. The results shown are mean values ($n = 3 \pm$ standard error).

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>$K_d$ (μM)</th>
<th>$F_{\text{max}}$ (AFU)</th>
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<tr>
<td>GST</td>
<td>21.34 ± 1.335</td>
<td>51649 ± 1368</td>
</tr>
<tr>
<td>*GST</td>
<td>38.46 ± 3.820</td>
<td>84586 ± 4302</td>
</tr>
<tr>
<td>FGN</td>
<td>17.90 ± 2.649</td>
<td>66832 ± 3453</td>
</tr>
<tr>
<td>*FGN</td>
<td>21.44 ± 2.277</td>
<td>64763 ± 2584</td>
</tr>
<tr>
<td>CLU</td>
<td>4.332 ± 0.296</td>
<td>58260 ± 1278</td>
</tr>
<tr>
<td>*CLU</td>
<td>3.303 ± 0.253</td>
<td>53664 ± 1217</td>
</tr>
<tr>
<td>*CLU: *GST (1:2.11)</td>
<td>8.860 ± 1.232</td>
<td>53744 ± 2721</td>
</tr>
<tr>
<td>HMW CLU-GST</td>
<td>3.232 ± 0.702</td>
<td>30727 ± 1910</td>
</tr>
<tr>
<td>*CLU: *FGN (1:1.88)</td>
<td>16.27 ± 1.676</td>
<td>55077 ± 2166</td>
</tr>
<tr>
<td>HMW CLU-FGN</td>
<td>18.04 ± 3.951</td>
<td>63697 ± 5497</td>
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5.3.6 Thioflavin T analysis

CLU is known to form soluble complexes with intermediate species on the amyloid-forming pathway (Yerbury et al., 2007). Thioflavin T is a dye that displays enhanced fluorescence after binding to amyloid fibrils and was used to determine if SEC purified HMW CLU-stressed protein complexes possessed any amyloid-like characteristics (i.e. contained beta-sheet rich structures). SEC purified HMW CLU-FGN and HMW CLU-GST complexes and the relevant native and heated protein controls all produced less than 8% of the fluorescence arising from a sample of LYS amyloid present at the same mass concentration (Figure 5.6).
Characterization of HMW CLU-stressed protein complexes

Figure 5.6 Thioflavin T fluorescence of HMW CLU-GST, HMW CLU-FGN and relevant native and heated control proteins compared to a sample of LYS amyloid.

Thioflavin T (62.5 μM) was added to 50 μg/mL of LYS amyloid or an equivalent concentration of the proteins or complexes indicated (generated as described in Section 5.2.7). The figure shows the mean fluorescence at 490 nm (n = 3 ± standard error) in AFU.

5.3.7 CD analysis

The CD spectra of native and heated CLU suggests that heating at 45°C for 12 h or 60°C for 50 min disrupts the native secondary structure of CLU to a similar extent (Figure 5.7; panel A). CDSSTR analysis of this data suggested this involved a loss of almost half the native CLU α-helical content and an increase in the content of β-sheet structure and disordered regions (Figure 5.8; panel A). The CD spectra of GST heated at 60°C for 50 min or FGN heated at 45°C for 12 h were different to those of the respective native proteins (Figure 5.7; panels B and C, respectively). CDSSTR analysis of this data suggested that the differences were largely attributable to a loss of α-helical content for both proteins and in the case of heated FGN also a loss of β-sheet content (Figure 5.8; panels B and C, respectively). Collectively, the CD spectra suggest that CLU retains more native secondary structure after heating than GST or FGN. There was very little difference between the CD spectra of HMW CLU-FGN complexes and a mixture
of native CLU and FGN (present at the same final mass concentrations as that found in the complex) (Figure 5.7; panel E and Figure 5.8; panel E). However, HMW CLU-FGN complexes had a very different CD spectrum to a corresponding mixture of heated *CLU and *FGN. The typical shape of CD spectra for proteins with high α-helical content (minima at 222 nm and 208 nm) was not evident in the mixture of heated *CLU and *FGN and this was supported by CDSSTR analysis which predicted a loss in α-helical content and a corresponding increase in β-sheet structure. In contrast, when comparing the CD spectrum for SEC purified HMW CLU-GST complexes with that of a corresponding mixture of native CLU and GST, HMW CLU-GST appeared to retain some but not all of the native secondary structure. CDSSTR analysis predicted that the complexes had an increased α-helical content and a large increase in β-sheet structure. This apparent change was not as severe as that experienced after heating of the proteins separately (Figure 5.7; panel D and Figure 5.8; panel D). As for the mixture of *CLU and *FGN (heated individually at 45°C), CDSSTR predicted that the mixture of *CLU and *GST (heated individually at 60°C) contained much lower α-helical content, and higher β-sheet and unordered structure content compared to the native proteins.
Figure 5.7 Near UV CD spectra for HMW CLU-client protein complexes and other protein samples.
Identity of samples analyzed are indicated in the panels. The data shown are means of six scans. NB: *CLU in panel (D) was heated using the conditions to form HMW CLU-GST (60°C for 50 min) and *CLU in panel (E) was heated using the conditions to form HMW CLU-FGN (45°C for 12 h)
Characterization of HMW CLU-stressed protein complexes

Figure 5.8 CDSSTR predictions from near UV CD data of HMW CLU-stressed protein complexes and other protein samples. Identity of samples analyzed are indicated in the panels. The data shown are the predicted percentages (± standard error) of secondary structural content for the samples. NB: *CLU in panel (D) was heated using the conditions to form HMW CLU-GST (60ºC for 50 min) and *CLU in panel (E) was heated using the conditions to form HMW CLU-FGN (45ºC for 12 h).
5.4 Discussion

In complex biological fluids found _in vivo_, CLU-stressed protein complexes are likely to contain more than one different stressed protein client. Moreover, the work described in this thesis suggests that CLU-stressed protein complexes may exist in the range of \( \geq 4 \times 10^7 \text{ Da} \), however, much smaller complexes may also be formed (see Section 4.3.4). Despite the potential differences between complexes formed _in vivo_ and those formed _in vitro_, the latter are likely to give important insights into the biophysical characteristics of CLU-stressed protein complexes and thereby a better mechanistic understanding of the chaperone action of CLU. 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). However, at physiologically relevant temperatures most proteins do not unfold at experimentally convenient rates when present at low concentrations in buffered solution. As demonstrated for FGN using the synthetic crowding agent PEG 4,000, the effect of macromolecular crowding (such as may be encountered in complex biological solutions such as plasma) promotes protein unfolding _in vivo_ (see Section 4.3.5). However, attempts to form HMW CLU-FGN protein complexes _in vitro_ at physiologically possible temperatures with the aid of PEG 4,000 were unsuccessful. Therefore, as in many other studies of chaperone action, supraphysiological temperatures were used as an empirical necessity to induce FGN and GST to unfold and form HMW complexes with CLU _in vitro_. In the case of CS, it was possible to generate HMW complexes with CLU at a physiologically relevant temperature.

In an attempt to visualise HMW CLU-stressed protein complexes, both atomic force microscopy and TEM were used. Due to the limitations of the tapping apparatus and the large size of the complexes, useful atomic force microscopy images were not obtained. Some information regarding the complexes was gained from TEM. At 63,000x magnification, individual molecules were hardly discernable when LYS, GST and CS were incubated with CLU in the absence of stress. However, when LYS was incubated with CLU in the presence of oxidative stress the negative staining suggests that a range of much larger amorphous species were present (Figure 5.2). Similar results were obtained when GST or CS were incubated with CLU during heat stress. It is expected that some of the very large species (> 100 nm) present on the grid containing HMW CLU-CS (Figure 5.2; panel F) may be aggregates of individual HMW CLU-stressed
Characterization of HMW CLU-stressed protein complexes

protein complexes resulting from the sample preparation process. The electron transmission microscopy images suggest that for a single stressed client protein, HMW complexes formed with CLU are amorphous and are not uniform in size. Moreover, the size of the complexes depends on the identity of the stressed client protein. As TEM is a relatively crude method for accurate size estimation, DLS was also used. It should be noted that the smaller particle size measured for HMW CLU-CS by DLS (51.06 ± 6.87 nm) versus TEM (50-100 nm) may relate to the slightly different conditions used to generate the complexes in each case (generated at 41°C for DLS but at 43°C for TEM). It is seen that CLU-stressed CS complex formation is dependent on temperature within this range (Figure 3.7). DLS suggested the particle sizes were normally distributed with less variation than was suggested by TEM. Since the limitations of the machine were such that molecules of clearly different sizes were not discernable when mixed in solution, the range of particles present in the HMW CLU-stressed protein fractions may have been more variable than apparent from the measurements obtained. DLS measurements suggested that HMW CLU-FGN complexes had a diameter approximately twice that of HMW CLU-GST or HMW CLU-CS complexes (approximately 100 versus 50 nm; Figure 5.3). At this scale, the soluble CLU-client protein complexes are very large indeed, being of a similar size to virus particles.

Given that the crystal structure of CLU is yet to be deciphered, this complicates investigations of CLU’s chaperone activity. Much more is known about other “holding-type” chaperones such as the sHsp family. Both CLU and sHsps have a tendency to self-associate forming very large oligomers (Humphreys et al., 1999). The chaperone activity of sHsp appears to involve the dynamic heat-dependent dissociation of these large oligomers to yield smaller units with patches of surface exposed hydrophobicity that can interact with hydrophobic regions on unstable, stressed client proteins and then re-associate to form HMW sHsp-stressed protein complexes (Ehrnsperger et al., 1997; Lee et al., 1997; van Montfort et al., 2001; Treweek et al., 2003). A similar mechanism has been proposed for CLU after it was observed that pH-dependent dissociation of CLU oligomers enhances chaperone activity (Poon et al., 2002a; Carver et al., 2003). BisANS binding suggests heat denatured proteins exhibit a transient increase in exposed hydrophobicity, however, the residual amount of protein able to withstand this heating and remain soluble does not necessarily display enhanced exposed hydrophobicity
Characterization of HMW CLU-stressed protein complexes

(Wyatt et al., 2009b). Comparatively, when incubated with CLU, stressed proteins do not display increased bisANS binding during heat stress (Wyatt et al., 2009b). This is also observed for other HMW complexes forming chaperones (Abderrahim et al., 2008). These results suggest that, whatever dynamic changes occur, the packaging of hydrophobic regions of unfolding proteins by CLU (and other chaperones) that allows the stressed proteins to remain soluble as very large aggregates is rapid and efficient.

Although FGN, CS and GST are very different proteins with a large discrepancy in mass (approximately 340, 85 and 26 kDa, respectively), and the complexes were formed at very different temperatures, in all cases the estimated mass stoichiometry of CLU:client protein in the complexes was about 1:2 (Table 5.1). This indicates that CLU is able to form soluble complexes in which it "carries" about twice its own mass in the form of client protein. Since the molar ratio of CLU:client protein was quite different between the complexes (Table 5.1), the structure of the complexes appears to be limited by the relative total masses of CLU and client protein (not the molar ratio). The reasons for consistent stoichiometry on a mass basis are unknown, however, they are likely to involve the ability of CLU to shield a certain mass of protein from the aqueous solution through hydrophobic interactions with other CLU molecules and stressed client proteins.

Measurements of bisANS fluorescence indicated that residual heated GST but not FGN or CLU exposed relatively more hydrophobicity to solution compared to the native proteins at the end-point of their respective heat treatments (Figure 5.5). However, transient exposure of hydrophobic regions on GST and FGN during their unfolding is likely given that in both cases heating resulted in aggregation and precipitation of the proteins. The binding of bisANS to HMW CLU-GST complexes had a lower $F_{\text{max}}$ than that of a corresponding mixture of *CLU and *GST (Figure 5.5; panel C and Table 5.2). This suggests that the molecular interactions involved in the formation of HMW CLU-GST complexes, either shield otherwise exposed hydrophobic regions and/or prevent certain structural changes associated with the unfolding of GST. This agrees with the suggestion that the action of CLU involves binding to regions of exposed hydrophobicity (Poon et al., 2002a). In contrast, the $K_d$ and $F_{\text{max}}$ for the binding of bisANS to HMW CLU-FGN complexes were not significantly different to those determined for the corresponding mixture of *CLU and *FGN (Figure 5.5; panel D and
Table 5.2). Heat treatment did not affect the $K_d$ or $F_{\text{max}}$ for the binding of bisANS to FGN (Table 5.22). However, while *FGN (remaining in solution after prolonged heat treatment) does not expose significantly more hydrophobicity to solution than native FGN, other results (not shown here) indicate that there is a transient increase in exposed hydrophobicity during heat-treatment of FGN (J. Yerbury unpublished data) - this may be the point at which CLU binds to the stressed protein.

Remarkably, the CD spectra indicated that HMW CLU-FGN complexes had a predicted overall content of the various secondary structures indistinguishable from that of a mixture of unheated CLU and FGN at the same concentrations (Figure 5.7; panel E and Figure 5.8; panel E). The most likely interpretation of this result is that under the conditions tested the interaction between CLU and FGN resulted in a mutual stabilization of secondary structures. Interestingly, the situation was quite different when examining the interaction between CLU and GST, which was carried out under much harsher conditions ($60^\circ$C). In this case, the apparent loss of overall $\alpha$-helical structure content was quantitatively very similar for the complexes and the corresponding mixture of *CLU and *GST. However, the complexes had more $\beta$-sheet content and less unordered structure than the corresponding mixture of residual heated proteins (Figure 5.7; panel D and Figure 5.7; panel D). The differences between the amounts of secondary structure retained upon formation of CLU-stressed protein complexes may be due to the conformational changes necessary to target an individual client protein for CLU intervention. It is possible that individual proteins traverse quite different partially unfolded states during unfolding and that intermediates with greater exposed hydrophobicity, which target proteins to CLU, are not necessarily conformations with the least secondary structure. It has previously been reported that unfolding intermediate states include conformations where disruption of native tertiary contacts expose the hydrophobic core and the native secondary structure may be preserved to varying degrees depending on the intermediate state (Li and Daggett, 1994; Zaidi et al., 1997; Balasubramanian et al., 2000).

Protein unfolding and the accumulation of insoluble protein aggregates is implicated in a variety of diseases (Kelly, 1996; Carrell and Lomas, 1997; Hamidi et al., 1997; Carrell and Gooptu, 1999; Thomas et al., 1999; Soto, 2001). In vivo, the sequestration of stressed
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proteins into large, soluble complexes with extracellular chaperones including CLU is likely to be the first step in preventing them from forming toxic or otherwise pathological aggregates. On the basis of evidence reviewed in Chapter 1 of this thesis, it is proposed that these soluble complexes are cleared from the body by receptor-mediated endocytosis and subsequent lysosomal degradation. While it is unknown how closely HMW CLU stressed-protein complexes formed under controlled experimental conditions resemble CLU-stressed protein complexes formed in vivo, it is possible that the shared characteristics of the model HMW CLU-stressed protein complexes examined in this study also extend to CLU-stressed protein complexes in vivo. Of the HMW CLU-stressed protein complexes examined here, there was a remarkable consistency in the mass ratio of CLU:stressed protein client, however, the physical dimensions of the complexes examined appeared to vary from 50 to 100 nm. In vivo mass presentation of unfolding protein may occur in disease states, however, it is predicted that under normal conditions complex formation may not necessarily result in maximum loading of CLU. Very large complexes (4 x 10^7 Da), in the range of those generated experimentally, appear be present in stressed plasma (Figure 4.16). These complexes may have physical dimensions close to those measured here although smaller CLU-stressed protein complexes may also exist (see chapters 3 and 4). CLU-client protein complexes appear to have an amorphous structure and do not exhibit increased thioflavin T binding compared to the native constituent proteins. CLU is known to be associated with amorphous protein deposits in vivo (Crabb et al., 2002) and the results of this study support that CLU may also be found associated with stressed protein in soluble complexes (see Chapter 4). The following chapters describe the work carried out to identify whether HMW CLU-stressed protein complexes are recognized and disposed of by endocytic receptors.
6 POTENTIAL RECEPTORS FOR HMW CLU-STRESSED PROTEIN COMPLEXES

6.1 Introduction

It has been reported that CLU is a ligand of certain LDL superfamily members (Kounnas et al., 1995; Zlokovic et al., 1996; Hammad et al., 1997; Calero et al., 1999; Mahon et al., 1999; Bartl et al., 2001; Kristiansen et al., 2001; Lakins et al., 2002; Bajari et al., 2003). Specifically this includes the two most ligand-promiscuous LDL family members megalin (Kounnas et al., 1995; Bartl et al., 2001) and LRP (Bartl et al., 2001), in addition to the smaller and more specialized ApoER2 and VLDLR (Bajari et al., 2003). Although the precise binding site on CLU for megalin is unknown, megalin was considered a strong candidate for the CLU-dependent clearance of stressed client proteins since separate binding sites on CLU for stressed ligands and megalin have previously been suggested (Lakins et al., 2002). Prior studies have reported the CLU-dependent clearance of Aβ at the blood-brain or blood-cerebrospinal barrier via megalin (Zlokovic et al., 1996; Hammad et al., 1997). Moreover, the report of CLU-dependent clearance of cellular debris via megalin on non-professional phagocytes, supports that CLU may act as a carrier for the disposal of proteins (Bartl et al., 2001). However, at the commencement of this study it was unknown how the interaction of CLU with stressed proteins involved in the formation of complexes would affect the binding of CLU to megalin or other receptors.

If CLU-stressed protein complexes are recognized by cell surface receptors it is feasible that their binding may be dependent on a specific recognition site on CLU or some other inherent characteristic of the complexes. In this context, a prime candidate for the recognition of CLU-stressed protein complexes are the scavenger receptors which preferentially bind damaged or modified ligands. The binding of a diverse but restricted array of polyanionic ligands and denatured and modified lipoproteins to class A SRs is mediated in part by several positively charged lysine residues in the collagen domain of the receptor (Doi et al., 1993). SR-A recognize denatured collagen type I and type III, but not the native forms, which is important for mediating macrophage homing in inflammatory conditions (Gowen et al., 2001). Moreover, many SRs recognize oxLDL.
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but not the native form. There is some evidence to suggest that SRs are implicated in the pathology of Alzheimer’s disease. Specifically, there is strong expression of SR-A on microglia associated with amyloid plaques (Christie et al., 1996). Furthermore, SR-A and SR-B have been shown to bind and internalize native and aggregated Aβ_{1-42} (Paresce et al., 1996).

The work described in this chapter was aimed at identifying potential receptors for HMW CLU-stressed protein complexes and characterizing the complex-receptor interaction. Specifically, demonstration of internalization and degradation of the complexes would strongly support the proposed model for CLU’s involvement in the disposal of stressed client proteins (Figure 1.6).
6.2 Materials and methods

6.2.1 Maleylation of BSA

A stock solution of 300 mg/mL maleic anhydride (Sigma-Aldrich) in acetonitrile was prepared and slowly added to BSA in 0.1 M Na\textsubscript{2}CO\textsubscript{3} pH 9 at a molar ratio of 15:1 (maleic anhydride: protein amine). The solution was incubated for 1 h at room temperature in the dark on a rotating wheel. Extensive dialysis at 4°C against PBS was used to remove any free maleic anhydride from the maleylated BSA (mBSA).

6.2.2 Cell culture

A Brown Norway rat yolk sac teratocarcinoma cell line (BN) was kindly donated by Dr. M. Paz Marzolo of Pontificia University Catolica, Chile. A human leukaemic monocyte lymphoma cell line (U937), a human promyelocytic leukaemia cell line (HL60), a lymphoblastic leukaemic cell line (Jurkat) and a choriocarcinoma cell line (Jeg3) were obtained from ATCC (Manassas, USA). All cell lines were routinely cultured in Dulbecco’s modified eagle medium (DMEM):F-12 (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) (Thermotrace, Melbourne, Australia) and maintained in an incubator at 37°C with humidified air containing 5% (v/v) CO\textsubscript{2}. BN and Jeg3 cells were routinely passaged using trypsin/EDTA (1:250, pH 7.0) (Thermotrace, USA) for 5 min at 37°C. Prior to all binding assays, BN and Jeg3 cells were cultured for approximately 48 h without a change of media before they were detached using 5 mM EDTA in PBS (without trypsin). U937, HL60 and Jurkat cells were passaged by decanting the cells and diluting them with the culture medium to a suitable density.

For long term storage, cells were transferred to freeze mix (50% (v/v) FCS, 10% (v/v) DMSO and 40% (v/v) DMEM: F-12) to a final cell density of 5 x 10\textsuperscript{6} cells/mL. Aliquots of this cell suspension (1 mL) were put in sterile labelled cryovials, placed in a Cryo 1°C Freezing Container (Nalgene, Sydney), and stored in a -80°C freezer overnight to achieve a -1°C/min rate of cooling. The following day the cells were transferred to liquid nitrogen for long term storage.
6.2.3 Isolation of peripheral human leukocytes

Human blood was obtained from a donor and diluted 1:3 in PBS. The cells were pelleted by centrifugation at 1,020 x g for 30 min. The cells were pooled in a small volume of PBS and then diluted 1:10 in PharmLyse™ red blood cell lysis buffer (BD) and incubated at room temperature for 15 min. The remaining cells were pelleted by centrifugation at 700 x g for 5 min at 4°C and washed with HBB. A small sample of cells was separated based on their side scatter and forward scatter using a BD LSR II flow cytometer to verify the success of red blood cell lysis and the ability to separate populations of lymphocytes, monocytes and granulocytes based on these parameters. PI staining was used to estimate the viability of leukocytes.

6.2.4 Isolation of hepatocytes and non-parenchymal rat liver cells

Livers were obtained from Sprague Dawley rats immediately post-sacrifice. The tissue was trimmed of fat and cut into fine slices (approximately 3 mm thick) using a scalpel blade. The tissue was then washed for 1 min by gentle shaking at 37°C in 50 mL of wash buffer (37 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.1% (w/v) BSA, 0.5 mM EGTA, pH 7.4). The tissue was then drained and the process repeated twice. Ca²⁺ and Mg²⁺ were returned to the tissue by washing three times with digestion buffer (37 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.3% (w/v) BSA, 5 mM CaCl₂, 1 mM MgCl₂ and 0.1 mg/mL DNAse I (Roche Diagnostics Australia), pH 7.4). After the final wash the tissue was drained and covered with 5 mL digestion buffer containing 0.6 mg/mL collagenase type 1A (Sigma-Aldrich) and incubated with shaking at 37°C for 10 min. The digestion solution was decanted through 80 μm wire mesh, diluted in HBB and held on ice while another 5 mL of digestion buffer (containing 0.6 mg/mL collagenase type 1A) was added to the remaining tissue. The tissue was incubated with shaking at 37°C for 10 min and the entire process was repeated once more. After the third collagenase incubation the remaining tissue was gently squeezed through cheese cloth and then passed through 80 μm wire mesh and diluted with HBB. A hepatocyte enriched cell pellet was obtained by low speed centrifugation for 10 min at 70 x g in a Labofuge centrifuge (Heraeus, Thermo Scientific, Waltham, USA). The supernatant and pellet were separated and the process was repeated until no (or few) cells were seen to pellet at 70 x g. A non-parenchymal enriched cell pellet was obtained by centrifugation at 700 x g in a
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Labofuge centrifuge. The cell pellets were washed once with HBB and resuspended in small volume of the same buffer before being diluted 1:10 in PharmLyse™ and incubated at 37°C for 1 min. The remaining cells were pelleted by centrifugation at 700 x g at 4°C and washed with HBB. A small sample of cells was separated based on their side scatter and forward scatter using a BD LSR II flow cytometer to verify the success of red blood cell lysis and stained with PI to estimate the viability of the cell preparations.

6.2.5 Isolation of rat splenocytes

Spleens were obtained from Sprague Dawley rats immediately post-sacrifice. The cells were gently dissociated by mechanical disruption using forceps and a scalpel blade. Dissociated cells were washed through 80 μm wire mesh using HBB at room temperature. The cells were washed once by centrifugation at 700 x g for 5 min in a Labofuge centrifuge. The cells were resuspended in a small volume of HBB and then diluted 1:10 in PharmLyse™ red blood cell lysis buffer and incubated at 37°C for 1 min. The remaining cells were pelleted by centrifugation at 700 x g for 5 min at 4°C and washed with HBB. A small sample of cells was separated based on their side scatter and forward scatter using a BD LSR II flow cytometer to verify the success of red blood cell lysis and stained with PI to estimate the viability of the cell preparation.

6.2.6 Flow cytometry

6.2.6.1 Receptor screening

Cells were washed free of medium in ice cold HBB by centrifugation at 700 x g for 5 min at 4°C in a Heraeus Sepatech Megafuge 1.0.R (Heraeus) and then approximately 5 x 10⁴ cells/well were aliquoted into the wells of a 96 well plate. The cells were pelleted by centrifugation and then incubated with mouse anti-LRP or goat anti-megalin (Santa Cruz Biotechnology) antibody in HBB for 30 min on ice according to the manufacturer’s instructions. The cells were washed twice in HBB by centrifugation and then incubated with anti-mouse IgG-fluorescein isothiocyanate (FITC) or anti-sheep/goat IgG-FITC, respectively for 30 min on ice. The expression of LDL superfamily receptors was measured after incubating cells with 50-100 μg/mL
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biotinylated RAP or GST-RAP, followed by incubation with 10 μg/mL SA-ALEXA488, with washing and incubation conditions as previously described. After being washed twice the cells were resuspended in HBB containing 1 μg/mL PI immediately before analysis using an LSR-II flow cytometer. Viable cells were gated based on PI exclusion and discrete cell populations were gated based on their forward scatter and side scatter properties. The acquired data was analyzed using FlowJo7 software (TreeStar Inc., USA). Background fluorescence was measured using cells incubated with a species-matched control antibody of irrelevant specificity and the relevant FITC labelled secondary antibody or using cells incubated with 10 μg/mL SA-ALEXA488 only.

6.2.6.2 Binding assays

Cells were washed free of medium in ice cold HBB by centrifugation at 700 x g for 5 min at 4°C in a Heraeus Sepatech Megafuge 1.0.R (Heraeus) and then approximately 5 x 10^4 cells/well were aliquoted into the wells of a 96 well plate. The cells were pelleted by centrifugation and then incubated at approximately 1 x 10^6 cells/mL in HBB on ice for 30 min with biotinylated protein (HMW CLU-stressed protein complexes, CLU or the relevant uncomplexed control proteins; see Section 2.10). After washing in HBB the cells were resuspended in HBB containing 10 μg/mL SA-ALEXA488 or SA-ALEXA633 (Molecular Probes, USA) and again incubated on ice for 30 min. After this period the cells were washed twice in HBB and resuspended in HBB containing 1 μg/mL PI immediately before analysis by flow cytometry as described above (see Section 6.2.6.1). Viable cells were gated based on PI exclusion and discrete cell populations were gated based on their forward scatter and side scatter properties. In the case of peripheral leukocytes, CD14+ monocytes were also selected for analysis on the basis of green fluorescence using anti-CD14-FITC (Chemicon). The acquired data was analyzed using FlowJo7 software (TreeStar Inc., USA). The geomean, which is a measure of the central tendency for data that changes in a relative fashion, was used to summarize the findings. All results are adjusted for the background fluorescence by subtracting the fluorescence of cells incubated with SA-ALEXA488 or SA-ALEXA633 at 10 μg/mL for 30 min on ice as above. When appropriate, comparisons of the fluorescence of different samples were made using the Student’s t-test.
Non-specific binding was evaluated using a number of methods including co-incubation of the cells with 25-100 μg/mL of the biotinylated protein and 1 mg/mL of the unlabelled protein. In other experiments dose-dependent binding was measured over a large concentration range. For BN cells, additional experiments using cells pre-treated with trypsin/EDTA for 10 min at 37°C to remove cell surface proteins were also carried out. Additionally, comparisons of ligand binding in PBS or PBS supplemented with 0.5 mM EDTA (in place of HBB) were performed to gauge the effect of metal-ion co-factors in ligand binding.

### 6.2.6.3 Inhibition of binding to cell surface receptors

Specific binding was assessed using a variety of receptor antagonists. The methods used are as described in Section 6.2.6.2 but with co-incubation of the cells with the inhibitor and the biotinylated protein, or the additional step of pre-incubation (for 30 min on ice) with the inhibitor before washing and incubating with the biotinylated protein. The concentration of inhibitors used were as follows: 25 μg/mL inhibitory anti-LRP antibody, kindly donated by Soren Moestrup (University of Aarhus, Aarhus C, Denmark); 250-500 μg/mL RAP or GST-RAP (pan-specific inhibitors of LDL superfamily receptors); 500 μg/mL galactose or asialofetuin (ASF) (inhibitors of the asialoglycoprotein receptor) or mBSA or fucoidin (SR inhibitors) at 250-500 μg/mL. In the case of mBSA and fucoidin, after incubation of cells with the inhibitor for 30 min on ice, the cells were co-incubated with the inhibitor and the biotinylated protein for a further 30 min on ice without an intermediate washing step.

### 6.2.7 Surface plasmon resonance

The binding of HMW CLU-stressed protein complexes to megalin or LRP was assessed with surface plasmon resonance using a Biacore 3,000 instrument (Biacore, Uppsala, Sweden). Biacore sensor chips (CM5) were activated with N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccimide in water according the manufacturer. Megalin or LRP (purified as described previously by Moestrup et al., (1993)) was immobilized on the chip at 10 μg/mL in 10 mM sodium acetate pH 4.5 prior to blocking with 1 M ethanolamine pH 8.5. A control flow cell was generated by activating and blocking a chip without immobilized protein. Samples were
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prepared to the appropriate concentration in the running buffer (10 mM HEPES, 150 mM NaCl, 3.0 mM CaCl₂, 1 mM EGTA, pH 7.4, 0.005% (v/v) Tween 20®). Sensor chips were regenerated with 1.6 M glycine-HCl buffer pH 3.0. The relative response units (RU; i.e. the difference in response between protein and control flow channel) resulting from binding of HMW CLU-stressed protein complexes, CLU or the uncomplexed client protein at 40 μg/mL are presented. K_d values were determined by BIAevaluation 4.1 software using a Langmuir 1:1 binding model. The experiments and kinetic analyses were performed by Christian Jacobsen at the University of Aarhus, Denmark.

6.2.8 Confocal microscopy

HMW CLU-stressed protein complexes were labelled with ALEXA488 according to the manufacturer’s instructions in order to visualize their cellular location using confocal microscopy. BN cells or isolated hepatocytes were prepared as previously described (see Sections 6.2.2 and 6.2.4, respectively). The cells were incubated in HBB containing ALEXA488 labelled HMW CLU-stressed protein complexes at 100-250 μg/mL or 200 nM Lysotracker Red DND-99 (Molecular Probes) or both. Samples were either held on ice or incubated in a 37°C waterbath for 1-2 h. After being washed the cells were mounted on glass slides for examination by confocal microscopy.

The cells were excited using an Argon-ion laser (Ex 488 nm) for ALEXA488 (Em 490-520 nm) or a Helium/Neon laser for Lysotracker Red DND-99 (Em 590-680 nm) and were examined using a Leica TCS SP system (Leica, Germany). Fluorescence and transmission images were collected using TCS NT software version 1.6.587 (Leica, Germany). Gain settings were adjusted such that there was no detectable leakage of fluorescence emissions between the channels for ALEXA488 and Lysotracker Red DND-99.

6.2.9 Protein degradation assays

Hepatocytes were isolated as previously described in Section 6.2.4 and incubated with 250 μg/mL biotinylated HMW CLU-stressed protein complex in HBB at 37°C (see Section 2.10). A separate aliquot of the cells was incubated with the biotinylated HMW
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CLU-stressed protein complex in the presence of 100 μM chloroquine (Sigma-Aldrich). At given intervals (between 1-3 h) the cells were washed twice in HBB by centrifugation at 700 x g. Washed pellets were resuspended in reducing SDS-PAGE buffer containing 100 mM DTT and the total cell lysate was separated on a 12% gel (see Section 2.4). SEC purified biotinylated HMW CLU-stressed protein complexes were analyzed alongside the cell lysates to show the migration of the intact component proteins. In addition, control cell lysate (not incubated with the biotinylated HMW CLU-stressed protein complex) was also run in one lane of the gel. After separation by SDS-PAGE, gels were equilibrated in transfer buffer and the proteins were transferred to nitrocellulose membrane using a Mini Trans-Blot Cell Western blotting apparatus at 100 V for 1 h at 4°C. Afterwards the membrane was blocked in HDC/PBS for 1 h at 37°C or overnight at 4°C. The membrane was incubated for 30 min at 37°C with a mixture of SA (Sigma-Aldrich) and biotinylated-HRP (Pierce Biotechnology), both at 10 μg/mL in HDC/PBS. After washing in 0.1% (v/v) Triton X-100 in PBS followed by several washes in PBS alone, ECL detection was performed (see Section 2.5).

6.2.10 Identification of CLU receptors from plasma membrane protein preparations

6.2.10.1 Liver plasma membrane protein isolation

Bovine liver, kindly donated by Wollondilly Abattoir (Tahmoor, Australia) was finely minced using a scalpel blade and homogenized in ice cold PBS containing 5 mM EDTA and Complete™ Protease Inhibitor Cocktail using a domestic hand-held electric blender. The homogenate was centrifuged at 535 x g for 5 min in a Megafuge 1.0 centrifuge (Heraeus) to pellet intact cells, nuclei and large cell debris. The supernatant was then centrifuged at 27,000 x g for 30 min in a TL-100 ultracentrifuge (Beckman, USA) to pellet total membrane. The total membrane pellet was resuspended in Separation buffer (10 mM HEPES, 2 mM EDTA and Complete™ Protease Inhibitor Cocktail, pH 7.2). Following the method of Maeda et al. (1983) 1 mL of the membrane suspension was layered on top of 2 mL of the separation buffer containing 41% (w/v) sucrose and centrifuged at 100,000 x g for 1 h at 4°C in a TL-100 ultracentrifuge. After centrifugation the resulting milky layer at the sucrose-buffer interface was collected and washed twice in separation buffer by ultracentrifugation at 100,000 x g for 20 min at
4°C. The pelleted membrane was resuspended in a small volume of membrane lysis buffer (1% (v/v) NP40, 2 mM EDTA and Complete™ Protease Inhibitor Cocktail in PBS) and incubated on ice for 30 min to solubilize the membrane.

### 6.2.10.2 CLU affinity chromatography of isolated membrane

Approximately 12 mg of CLU was purified from human plasma as previously described (see Section 2.2). The purified protein was conjugated to a 4 mL HiTrap™ NHS-activated HP column (GE Healthcare) according to the manufacturer’s instructions. After filtration through a 0.45 μm filter, the plasma membrane lysate was recirculated through the pre-prepared CLU affinity column several times at approximately 0.5 mL/min. The column was washed with several volumes of PBS containing 1% NP40 before the bound protein was eluted by passing 20 mL of 100 mM glycine, 1% NP40, pH 2.3 over the column. The fractions collected were neutralized by the addition of a few drops of 3M TRIS-HCl, pH 8. The eluted protein was reduced by boiling in SDS-PAGE loading buffer containing 100 mM DTT and supplemented with additional SDS to a final concentration of 10% (w/v) before separation by SDS-PAGE. Individual bands were excised for mass spectrometry analysis (see Section 2.11).
6.3 Results

6.3.1 Cell binding assays involving uncomplexed CLU

A number of cell lines were examined for their ability to bind CLU. These cell lines were selected based on their reported expression of LDL receptors megalin or LRP, which reportedly interact with CLU (Kounnas et al., 1995; Bartl et al., 2001). In the case of HepG2 and Jurkat cells the binding of CLU to carbohydrate receptors was also examined on the basis that CLU is heavily glycosylated.

6.3.1.1 BN cells

BN cells are reported to express megalin (Sousa et al., 2000; Gonzalez-Villalobos et al., 2006). However, in flow cytometry when BN cells were incubated with anti-megalin antibody the resulting fluorescence was not greater than a species-matched control for this antibody suggesting that either megalin was not being expressed by the BN cells or that the antibody was not functional at the time of these experiments (Figure 6.1).

Figure 6.1 Surface expression of megalin by BN cells, assessed by flow cytometry.

BN cells were incubated with polyclonal goat anti-megalin antibody or a species-matched polyclonal control antibody of irrelevant specificity in HBB. Binding of the primary antibody to the cell surface was detected using donkey anti-sheep/goat Ig-FITC. The figure shows the relative FITC (515/20-A) fluorescence for each treatment in addition to the background fluorescence of cells incubated with the secondary antibody only.
The binding of biotinylated RAP and CLU to BN cells was assessed by flow cytometry. At 15 μg/mL, similar levels of binding to the cells were detected for both proteins, however, at 50 μg/mL relatively more binding was observed for CLU compared to RAP (Figure 6.2; t(4) = 3.881, p ≤ 0.022). Pre-incubation of the cells with 500 μg/mL unlabelled GST-RAP significantly reduced the binding of CLU to the BN cells only when the cells were incubated with 15 μg/mL CLU (t(4) = 4.616 p ≤ 0.011). In contrast, pre-incubation with 500 μg/mL unlabelled GST-RAP reduced the binding of RAP regardless of whether the cells were incubated with the latter at either 15 or 50 μg/mL (t(4) = 5.465 p ≤ 0.005807 and t(4) = 10.45 p ≤ 0.0004746, respectively). Moreover, when biotinylated CLU or RAP were used at 15 μg/mL, pre-incubation with GST-RAP had a greater effect on the binding of RAP versus CLU. This suggests that a small fraction of the CLU binding may occur via LDL superfamily receptors but that the majority occurs via an alternative mechanism.

**Figure 6.2** The effect of pre-incubation with GST-RAP on the binding of CLU and RAP to BN cells, assessed by flow cytometry.

BN cells were incubated in the presence or absence of 500 μg/mL GST-RAP before incubation with 15 or 50 μg/mL biotinylated CLU or RAP. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in arbitrary fluorescence units (AFU). * Denotes significant inhibition of CLU or RAP binding according to Student’s t-test, p ≤ 0.05.
The binding of biotinylated CLU to BN cells appeared to occur in a dose-dependent manner and suggests that the measured binding was not solely due to non-specific mechanisms (Figure 6.3). There was a roughly linear dependence of CLU binding on concentration within the range 0-100 μg/mL. There was no significant further increase in binding when the concentration was increased from 100 to 200 μg/mL.

Figure 6.3 Dose-dependant binding of CLU to BN cells, assessed by flow cytometry.
BN cells were incubated with 0-200 μg/mL biotinylated CLU followed by 10 μg/mL SA-ALEXA488 prior to analysis by flow cytometry. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in AFU.

To assess the degree of non-specific binding the effect of pre-treatment of the cells with trypsin/EDTA was performed prior to CLU binding experiments. Also, to determine metal-dependent interactions the experiment was also performed in the presence of 0.5 mM EDTA in PBS (i.e. to remove Ca$^{2+}$ and Mg$^{2+}$ from the binding buffer). Regardless of the concentration of CLU tested, incubation with 0.5 mM EDTA or pre-treatment with trypsin dramatically reduced binding of CLU to BN cells (Figure 6.4; $F(2,6) = 120.4$, $F(2,6) = 781.8$, $F(2,6) = 19.95$, respectively $p \leq 0.0001$; Tukey HSD, $p \leq 0.01$ all cases). Similarly, treatment with EDTA or trypsin also significantly inhibited the binding of RAP ($F(2,6) = 12.08$, $F(2,6) = 314.3$, $F(2,6) = 535.7$, respectively $p \leq 0.0001$; Tukey HSD, $p \leq 0.01$ in all cases except at 15 μg/mL RAP where the difference between EDTA and HBB was $p \leq 0.05$). For CLU, incubation with EDTA or pre-treatment with trypsin inhibited binding to a similar extent (Figure 6.4; panel A). In contrast, trypsin pre-treatment inhibited the binding of RAP to a greater extent than EDTA, although both treatments significantly inhibited the binding compared to incubation in HBB (Figure 6.4; panel B; Tukey HSD, $p \leq 0.01$ in all cases).
Figure 6.4 The effect of EDTA or pre-treatment with trypsin on the binding of CLU and RAP to BN cells, assessed by flow cytometry. BN cells were incubated with biotinylated (A) CLU or (B) RAP at the indicated concentrations in HBB or 0.5 mM EDTA in PBS. In some cases, cells were pre-treated with trypsin/EDTA for 10 min at 37°C and subsequently incubated with the ligands in PBS followed by SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in AFU.

6.3.1.1.2 HepG2 cells

HepG2 cells were verified by flow cytometry as expressing LRP, however, the level of fluorescence was only marginally higher than that produced by a species-matched control (primary) antibody of irrelevant specificity suggesting that the expression of the receptor was low or the antibody was poorly active (Figure 6.5). The expression of LRP by HepG2 cells is reportedly variable and dependent on cell lineage and long-term culture conditions (Grimsley et al., 1997). The expression of the asialoglycoprotein receptor by HepG2 cells has been reported elsewhere but was not quantified in this study (Collins et al., 1988; Nakaya et al., 1994).
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Figure 6.5 Surface expression of LRP by HepG2 cells, assessed by flow cytometry.
HepG2 cells were incubated with rabbit anti-LRP or a species-matched control antibody of irrelevant specificity in HBB. Binding of the primary antibody to the cell surface was detected using sheep anti-rabbit Ig-FITC. The figure shows the relative fluorescence of 10,000 viable cells for each treatment.

The binding of CLU to HepG2 cells occurred in a biphasic manner over the concentration range 0-200 μg/mL (Figure 6.6). In the range 0-50 μg/mL, the binding appeared to show evidence of saturation, however, at higher concentrations (50-200 μg/mL) the binding appeared to show a largely linear dependence upon concentration, suggesting that the majority of the binding measured in this range was non-specific.

Figure 6.6 Dose-dependant binding of CLU to HepG2 cells, assessed by flow cytometry.
HepG2 cells were incubated with 0-200 μg/mL biotinylated CLU followed by 10 μg/mL SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 2 ± range) in AFU.
Pre-incubation with RAP or the asialoglycoprotein receptor inhibitor ASF had no effect on the subsequent binding of biotinylated CLU (at 15 μg/mL or 100 μg/mL; Figure 6.7). The effect of excess unlabelled CLU on the binding of biotinylated CLU to HepG2 cells was next examined. The concentrations of biotinylated CLU tested were chosen to correspond to the “saturating” and “linear” phases of the binding curve (Figure 6.6). At both 25 and 100 μg/mL biotinylated CLU, excess unlabelled CLU did not significantly inhibit the binding of the labelled ligand suggesting that the binding of biotinylated CLU at either concentration was largely non-specific (Figure 6.8).

Figure 6.7 The effect of pre-incubation with ASF or RAP on the binding of biotinylated CLU to HepG2 cells, assessed by flow cytometry. HepG2 cells were incubated in the presence or absence of 500 μg/mL ASF or RAP prior to incubation with biotinylated CLU at the concentrations indicated and then SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard deviation) in AFU.

Figure 6.8 The effect of excess unlabelled CLU on the binding of biotinylated CLU to HepG2 cells, assessed by flow cytometry. HepG2 cells were incubated with biotinylated CLU at the concentrations indicated in the presence or absence of 1 mg/mL unlabelled CLU. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard deviation) in AFU.
6.3.1.1.3 JEG3 cells

The expression of LRP by JEG3 cells was verified by flow cytometry using an anti-LRP antibody (Figure 6.9). After incubation with 50 μg/mL biotinylated CLU a small amount of binding (equivalent to ~ 5 AFU) above the background level was observed. At this level, this is probably the result of a low level of non-specific interaction with the cell surface. Moreover, pre-incubation with RAP or an inhibitory anti-LRP antibody did not significantly reduce the binding of biotinylated CLU (Figure 6.10).

![Figure 6.9](image1.png)

**Figure 6.9** Surface expression of LRP by JEG3 cells, assessed by flow cytometry.

JEG3 cells were incubated with rabbit anti-LRP antibody or a species-matched control antibody of irrelevant specificity in HBB. Binding of the primary antibody to the cell surface was detected using sheep anti-rabbit Ig-FITC. The figure shows the relative fluorescence for each treatment.

![Figure 6.10](image2.png)

**Figure 6.10** The effect of pre-incubation with RAP or an inhibitory anti-LRP antibody on the binding of biotinylated CLU to JEG3 cells, assessed by flow cytometry.

JEG3 cells were incubated with 50 μg/mL biotinylated CLU, in the presence or absence of 500 μg/mL RAP or 25 μg/mL inhibitory anti-LRP antibody, and then 10 μg/mL SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard deviation) in AFU.
6.3.1.4 Jurkat cells

Jurkat cells are known to express the asialoglycoprotein receptor (Park et al., 2006). Flow cytometry showed that the binding of biotinylated CLU to Jurkat cells is partially inhibited by pre-incubation with the asialoglycoprotein inhibitor galactose (Figure 6.11).

![Figure 6.11 The effect of pre-incubation with galactose on the binding of CLU to Jurkat cells, assessed by flow cytometry.](image)

Jurkat cells were incubated with 500 μg/mL galactose or held on ice in HBB prior to incubation with 50 μg/mL biotinylated CLU and then 10 μg/mL SA-ALEXA488. The figure shows the relative FITC fluorescence for each treatment in addition to the background fluorescence of cells incubated with the secondary antibody only.

6.3.1.2 Surface plasmon resonance

Binding to megalin and LRP was examined for CLU, three different HMW CLU-stressed protein complexes and the native client proteins by surface plasmon resonance (Figure 6.12 and Table 6.1). Compared to CLU, all HMW CLU-stressed protein complexes displayed lower binding to megalin at the same mass concentration (Figure 6.12; panels B, D, F and G). Of these HMW CLU-stressed protein complexes, the response measured from HMW CLU-CS and HMW CLU-LYS suggested that binding to megalin was negligible, however, a greater response was measured for HMW CLU-GST. The response measured for HMW CLU-GST was also comparatively greater than that for GST alone suggesting that this complex binds to the receptor. Given that the precise molecular mass of HMW CLU-GST is not known, kinetic analysis was not possible. While negligible binding was measured for CS, binding of LYS to megalin was detected. This suggests that although both native CLU and LYS bind to megalin, after co-incubation of LYS and CLU under oxidative stress the species formed do not contain available megalin binding sites. For CLU and the HMW CLU-stressed protein complexes, negligible binding was observed to LRP, therefore, kinetic analysis was not carried out.
Figure 6.12 Surface plasmon resonance measurements of binding to megalin and LRP.
This figure shows sensorgrams for (A) GST, (B) HMW CLU-GST, (C) CS, (D) HMW CLU-CS, (E) LYS, (F) HMW CLU-LYS, and (G) CLU binding to immobilized megalin (blue) or LRP (green). Protein samples were injected over the flow cells at 20 μL/min. Where appropriate, the sensorgram data was then used to estimate kinetic parameters shown in Table 6.1.
Table 6.1 Kinetic data for the binding of CLU and HMW CLU-stressed protein complexes to megalin.
This table indicates, where appropriate, estimated $K_d$ values for the binding of CLU, HMW CLU-stressed protein complexes and the relevant uncomplexed client proteins to megalin, as determined by surface plasmon resonance. $K_d$ values were determined by BIAevaluation 4.1 software using a Langmuir 1:1 binding model.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Binding to Megalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>Native</td>
<td>$K_d \sim 0.5 \mu M$</td>
</tr>
<tr>
<td>HMW CLU-GST</td>
<td>Heat stressed 60°C</td>
<td>Unable to calculate $K_d$, but binding measured.</td>
</tr>
<tr>
<td>CS</td>
<td>Native</td>
<td>Negligible binding</td>
</tr>
<tr>
<td>HMW CLU-CS</td>
<td>Heat stressed 43°C</td>
<td>Negligible binding</td>
</tr>
<tr>
<td>LYS</td>
<td>Native</td>
<td>$K_d \sim 6 \mu M$</td>
</tr>
<tr>
<td>HMW CLU-LYS</td>
<td>Oxidized</td>
<td>Negligible binding</td>
</tr>
<tr>
<td>CLU</td>
<td>Native</td>
<td>$K_d \sim 150 \text{nM}$</td>
</tr>
</tbody>
</table>

6.3.2 Binding interactions involving HMW CLU-stressed protein complexes

6.3.2.1 Cell binding assays

6.3.2.1.1 BN cells

When BN cell were incubated with 50 μg/mL biotinylated CLU, FGN or HMW CLU-FGN, the greatest level of cell-associated fluorescence was obtained for FGN (Figure 6.13). Pre-incubation with GST-RAP did not significantly inhibit the binding of any of the labelled proteins.
Potential receptors for HMW CLU-stressed protein complexes

Figure 6.13 The effect of pre-incubation with GST-RAP on the binding of biotinylated CLU, FGN and HMW CLU-FGN to BN cells, assessed by flow cytometry.

BN cells were incubated with or without 250 μg/mL GST-RAP before incubating with 50 μg/mL biotinylated CLU, FGN or HMW CLU-FGN and then 10 μg/mL SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in AFU.

Fucoidin (a SR inhibitor) dramatically inhibited the binding of both biotinylated HMW CLU-FGN and biotinylated HMW CLU-GST (t(4) = 6.912 and t(4) = 7.438, respectively; p ≤ 0.01) to BN cells (Figure 6.14). For the uncomplexed control proteins, fucoidin had no effect on the binding of biotinylated CLU (t(4) = 0.811), however, significant inhibition was measured for the binding of native biotinylated FGN and GST (t(4) = 5.85 and t(4) = 6.14, respectively p ≤ 0.01). Maleylated BSA (mBSA; another SR inhibitor) inhibited the binding of HMW CLU-FGN to BN cells (t(4) = 5.17; p ≤ 0.01) but had no effect on the binding of CLU (Figure 6.15; t(4) = 1.13; p ≤ 0.01).
Potential receptors for HMW CLU-stressed protein complexes

Figure 6.14 The effect of fucoidin on the binding of biotinylated HMW CLU-stressed protein complexes and control proteins to BN cells, assessed by flow cytometry.

BN cells were incubated with or without 500 μg/mL fucoidin before incubating with 50 μg/mL biotinylated A) CLU, FGN or HMW CLU-FGN or B) GST or HMW CLU-GST and then 10 μg/mL SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in AFU. + Indicates significant inhibition by fucoidin (Student’s t-test, p ≤ 0.05).
6.3.2.1.2 Monocytes

The binding of CLU or HMW CLU-stressed protein complexes to the cultured human leukocyte cell lines U937 or HL60 was minimal and was not enhanced by phorbol 12-myristate 13-acetate (PMA) or DMSO treatment used to induce the differentiation of these cells into macrophage-like or neutrophil-like cell types, respectively (data not shown). However, compared to CLU and the control proteins, HMW CLU-FGN bound preferentially to peripheral CD14+ monocytes (freshly isolated from human blood) (Figure 6.16; panel A; \( F(2,6) = 43.03 \), Tukey HSD, \( p \leq 0.01 \)). Similar results were obtained when the binding of GST and HMW CLU-GST was compared (Figure 6.16; panel B; \( t(4) = 15.75, p \leq 0.0001 \)). Comparatively low binding to other human blood cell types (granulocytes and lymphocytes) was measured.
Figure 6.16 Binding of HMW CLU-stressed protein complexes, CLU and uncomplexed client proteins to isolated human leukocytes, assessed by flow cytometry.

Freshly isolated human leukocytes were incubated with 100 μg/mL biotinylated A) CLU, FGN or HMW CLU-FGN or B) GST or HMW CLU-GST and then SA-ALEXA633. Granulocyte and lymphocyte populations were gated based on their separation according to size and granularity. Incubation with anti-CD14 antibody and a relevant FITC conjugated secondary antibody was used to separately gate monocytes. The results are the geometric mean of the ALEXA633 fluorescence (n = 3 ± standard deviation) in AFU. * Denotes significantly higher binding of HMW CLU-stressed protein complexes compared across the cell types and also compared to the binding of uncomplexed control proteins to all cell types (as shown on the same panel; Tukey HSD, and Student’s t-test, p ≤ 0.01).

Fucoidin significantly inhibited the binding of biotinylated HMW CLU-FGN and HMW CLU-GST to peripheral blood monocytes (Figure 6.17; t(4) = 81.32 and t(4) = 15.05, respectively; p ≤ 0.05). Although uncomplexed biotinylated CLU, FGN and GST bound...
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less to the monocytes, fucoidin also inhibited this binding ($t(4) = 4.61$, $t(4) = 11.93$ and $t(4) = 10.17$, respectively; $p \leq 0.05$)

![Image](image.png)

**Figure 6.17** The effect of fucoidin on the binding of HMW CLU-stressed protein complexes and control proteins to peripheral blood CD14+ monocytes, assessed by flow cytometry.

Freshly isolated monocytes were incubated with or without 250 μg/mL fucoidin before incubating with 100 μg/mL biotinylated A) CLU, FGN or HMW CLU-FGN or B) GST or HMW CLU-GST and then SA-ALEXA633. The results are the geometric mean of the ALEXA633 fluorescence ($n = 3 \pm$ standard deviation) in AFU. * Denotes significant inhibition by fucoidin (Student’s t-test, $p \leq 0.05$).

6.3.2.1.3 Splenocytes

The results of biodistribution studies in a rat model suggested that HMW CLU-stressed protein complexes were targeted primarily to the liver and to a lesser extent the spleen (see Chapter 7). For this reason the binding of HMW CLU-stressed protein complexes was examined using a crude preparation of Sprague Dawley rat splenocytes. Only low levels of HMW CLU-stressed protein complexes bound to splenocytes and pre-treatment with fucoidin did not inhibit this (Figure 6.18). This binding was greater than that of CLU or the uncomplexed client proteins (either native or heat-treated), however at this low level the measured binding is likely to be non-specific.
Potential receptors for HMW CLU-stressed protein complexes

Figure 6.18 The effect of fucoidin on the binding of biotinylated HMW CLU-stressed protein complexes and control proteins to rat splenocytes, assessed by flow cytometry.

Freshly isolated splenocytes were incubated with or without 250 μg/mL fucoidin before incubating with 50 μg/mL biotinylated CLU, native FGN, *FGN, HMW CLU-FGN, GST, *GST or HMW CLU-GST and then SA-ALEXA488. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in AFU.

6.3.2.1.4 Isolated rat liver cells

HMW CLU-stressed protein complexes bound to a greater extent to isolated rat hepatocytes compared to the non-parenchymal cells (Figure 6.19). Greater binding was measured for HMW CLU-FGN and HMW CLU-GST to hepatocytes compared to the relevant control proteins whether the control proteins were native or heat-treated (F(4,10) = 37.09 and F(4,10) = 82.36, respectively; p ≤ 0.0001; Tukey HSD, p ≤ 0.01 in all cases). For CLU and GST, heat treatment appeared to increase the binding of the ligands to the hepatocytes (Tukey HSD, p ≤ 0.05 in both cases). With the same level of confidence this was not true for FGN using Tukey HSD, however using student t-test, heat-treatment also significantly increased the binding of FGN to hepatocytes (t(4) = 9.37; p ≤ 0.01).
Potential receptors for HMW CLU-stressed protein complexes

Figure 6.19 The binding of biotinylated HMW CLU-stressed protein complexes and control proteins to isolated rat liver cells, assessed by flow cytometry. Enriched preparations of hepatocytes or non-parenchymal liver cells were incubated with 100 μg/mL biotinylated A) CLU, *CLU (heated at 60°C for 50 min), FGN, *FGN (heated at 45°C for 12 h), or HMW CLU-FGN or B) GST, *GST (heated at 60°C for 50 min), or HMW CLU-GST and then SA-ALEXA488. Non-parenchymal and hepatocyte populations were gated based on the size and granularity of the cells. The results are the geometric mean of the ALEXA488 fluorescence (n = 3 ± standard error) in AFU. + Denotes significantly enhanced binding of control proteins after heat-treatment and ^ indicates significantly enhanced binding of HMW CLU-stressed protein complexes compared to the heated or native uncomplexed control proteins as shown in the same panel (Tukey HSD, p ≤ 0.01 in all cases).

When hepatocytes were incubated with HMW CLU-stressed proteins or the relevant control proteins at the same final mass concentrations present in the complex (i.e. molar ratio of client:CLU 2:1) much greater binding was measured for the complexes compared to the control proteins (t(4) = 9.719 for HMW CLU-FGN and t(4) = 6.513 for HMW CLU-GST; p ≤ 0.01). This suggests that some unique characteristic of the complexes increases their affinity for hepatocytes (Figure 6.20).
Figure 6.20 The binding of HMW CLU-stressed protein complexes and control proteins to isolated rat hepatocytes, assessed by flow cytometry. An enriched fraction of hepatocytes was incubated with 60 μg/mL biotinylated HMW CLU-FGN or HMW CLU-GST, or mixtures of (i) 20 μg/mL biotinylated CLU and 40 μg/mL biotinylated FGN, or (ii) 20 μg/mL biotinylated CLU and 40 μg/mL biotinylated GST (Client:CLU mass ratio 2:1), and finally SA-ALEXA488. Hepatocytes were electronically selected for analysis based on size and granularity. The results are the geometric mean (n = 3 ± standard error) in AFU. * Indicates significantly reduced binding of the uncomplexed proteins compared to HMW CLU-stressed protein complexes (Student’s t-test, p ≤ 0.01).

When hepatocytes were treated with fucoidin the binding of biotinylated HMW CLU-FGN and HMW CLU-GST was dramatically reduced (Figure 6.21; t(4) = 26.42 and t(4) = 10.54, respectively; p ≤ 0.01). Fucoidin also inhibited the binding of heat-treated biotinylated FGN and GST (t(4) = 11.73 and t(4) = 7.73, respectively; p ≤ 0.01), however, with the same degree of confidence there was no effect on the binding of native or heat-treated CLU or FGN.
6.3.2.2 Confocal microscopy

Given that a high level of binding of HMW CLU-stressed protein complexes to BN cells was detected by flow cytometry, this cell line was selected for experiments using confocal microscopy. At 4°C, ALEXA488 labelled HMW CLU-FGN bound to the BN cell surface with a patchy distribution (Figure 6.22). Following incubation at 37°C for 1 h, the majority of the labelled complexes had moved inside the cells, with a distribution overlapping that of Lysotracker Red DND-99 labelled acidified compartments (Figure 6.23).
Figure 6.22 Confocal microscopy images of ALEXA488 labelled HMW CLU-FGN bound to the surface of a BN cell at 4°C. The image shows (A) ALEXA488 labelled HMW CLU-FGN fluorescence, and (B) the corresponding transmission image.

Figure 6.23 Confocal microscopy images of BN cells after a 1 h incubation at 37°C with 100 μg/mL ALEXA488 labelled HMW CLU-FGN and 200 nM Lysotracker Red DND-99. The image shows (A) ALEXA488 labelled HMW CLU-FGN fluorescence, (B) Lysotracker Red DND-99 fluorescence, (C) an overlay of the two fluorescence images, and (D) the corresponding transmission image.
The following experiments involving freshly isolated hepatocytes were performed using HMW CLU-FGN (formed at both 45°C and 41°C). As the results obtained were very similar, a selection of representative images is shown. Similar to the results with BN cells, confocal microscopy showed that HMW CLU-stressed protein complexes bound to the surface of freshly isolated rat hepatocytes at 4°C (Figure 6.24). Moreover, binding to the cell surface was followed by internalization when the cells were incubated at 37°C and the location of the internalized HMW CLU-stressed protein complexes overlapped with that of Lysotracker Red DND-99 labelled compartments (Figures 6.25), suggesting that the ligands were following the classical route of endocytosis towards acidified compartments.

Figure 6.24 Confocal images of ALEXA488 labelled HMW CLU-FGN bound to the surface of rat hepatocytes.
The image shows (A) ALEXA488 labelled HMW CLU-FGN fluorescence and (B) the corresponding transmission image.
Figure 6.25 Confocal images of internalized ALEXA488 labelled HMW CLU-FGN and Lysotracker Red DND-99 fluorescence in a rat hepatocyte. Hepatocytes were incubated for 2 h at 37°C with 250 μg/mL ALEXA488 labelled HMW CLU-CS and 200 nM Lysotracker Red DND-99 before analysis by confocal microscopy. The image shows (A) ALEXA488 labelled HMW CLU-FGN fluorescence, (B) Lysotracker Red DND-99 fluorescence, (C) an overlay of the two fluorescence images, and (D) the corresponding transmission image.

6.3.3 Protein degradation assays

The ability of hepatocytes to degrade extracellular HMW CLU-stressed protein complexes was investigated by probing Western blots of cell lysates for biotin labelled HMW CLU-stressed protein complexes following incubation at 37°C in the presence or absence of chloroquine (an inhibitor of lysosomal proteases). Under reducing conditions, bands smaller than those associated with intact HMW CLU-stressed protein
Potential receptors for HMW CLU-stressed protein complexes

complexes were visible after the cells were incubated at 37°C (Figure 6.26). The degree of fragmentation appeared to increase with prolonged incubation. Incubation in the presence of chloroquine abolished the fragmentation of the HMW CLU-stressed protein complex in the hepatocytes. It was apparent that in the control cell lysate there was a prominent band (around 70 kDa) containing endogenous biotinylated protein.

Figure 6.26 Western blot of cell lysates of rat hepatocytes incubated for 1-3 h at 37°C with 250 μg/mL biotinylated HMW CLU-GST in the presence or absence of 100 μM chloroquine.
Cell lysates were separated using 12% SDS-PAGE and transferred to nitrocellulose membrane for probing with SA-HRP and development with enhanced chemiluminescence. Mr: Prestained protein ladder, CON: control cell lysate (endogenous biotinylated protein only), no inhibitor after 1, 2 or 3 h, chloroquine-treated after 1, 2 or 3 h and HMW: undigested partially reduced and fully reduced components of HMW CLU-GST (as indicated on the figure).

6.3.4 CLU affinity chromatography

After isolation of plasma membrane proteins from homogenized bovine liver, CLU affinity chromatography was used in an attempt to identify potential cell surface receptors for CLU. SDS-PAGE of the eluted proteins suggested that a large number of proteins bound to the column after CLU affinity chromatography, however, their separation during electrophoresis was impaired by the presence of NP-40 detergent (present at 1% (v/v) in the samples) (Figure 6.28). When the loaded samples contained 2% (w/v) SDS, the proteins were not able to enter the resolving gel. In an attempt to overcome this problem the concentration of NP-40 in the loaded samples was reduced by dilution in detergent-free buffer, however, it was found that at concentrations of
NP-40 less than 1% (v/v) the proteins precipitated from solution. Using a lower percentage of acrylamide in the gels (to increase pore size) did not improve the ability of the proteins to enter the gel (data not shown). At 1% (v/v) NP-40, the samples required a final concentration of 10% (w/v) SDS for the proteins to enter a 12% resolving gel. Even at this high concentration of SDS the separation of the protein bands was not ideal. Nevertheless, several bands were isolated for further analysis by mass spectrometry. Of these bands the mass spectrometry profile of the band running at approximately 39 kDa was matched to bovine β-actin with high identity (data not shown). No reliable matches were found for the other excised bands. This is likely to be due to poor isolation of individual protein bands or interference of NP-40 in the mass spectrometry analysis.

Figure 6.27 12% SDS-PAGE of bovine liver membrane proteins isolated by CLU affinity chromatography.

A plasma membrane preparation from bovine liver was subjected to CLU affinity chromatography using a 4 mL HiTrap HP column to which purified human CLU had been immobilized. The lanes show 1) Prestained protein ladder, 2) CLU in 1% (v/v) NP-40 and 2% (w/v) SDS, 3) isolated plasma membrane proteins in 1% (v/v) NP-40 and 2% (w/v) SDS, 4) CLU in 1% (v/v) NP-40 and 10% (w/v) SDS and 5) isolated plasma membrane protein in 1% (v/v) NP-40 and 10% SDS. Arrows indicate proteins selected for mass spectrometry analysis and their approximate molecular mass.
6.4 Discussion

BN cells were originally selected for investigations of CLU binding due to its reported expression of megalin (Sousa et al., 2000; Gonzalez-Villalobos et al., 2006). Biotinylated CLU did bind significantly to the surface of BN cells (Figure 6.2 and Figure 6.3), however, no evidence of megalin expression by the particular line used in this study was detected (Figure 6.1). This was consistent regardless of the concentrations of fetal calf serum used to supplement the media or the state of cell confluency (data not shown). It is possible that after continued passage the cell line had lost expression of megalin. Altered protein expression is a common phenomenon amongst immortalized cell lines after prolonged culture. In particular, megalin is a very large protein and other researchers have experienced similar difficulties using cultured cell lines to study this receptor (Holthofer et al., 1991; Biemesderfer et al., 1993). Alternatively, it is possible that the antibody (acquired from Santa Cruz Biotechnology, Santa Cruz, USA) was inactive, however, at the time of these experiments we did not possess purified megalin to test the antibody’s activity. The binding of RAP to BN cells was inhibited by both EDTA and GST-RAP, suggesting that LDL superfamily receptors were being expressed. However, since the cells could not be confirmed as expressing either megalin or LRP, the precise identity of these receptors is not known. In any case, the identification of the RAP binding receptor(s) appeared to be of limited value since only a small portion of the detectable CLU binding was inhibited by GST-RAP (Figure 6.2).

The saturable dose-dependent binding of CLU to BN cells appeared to support a single saturable mechanism. However, at concentrations of biotinylated CLU between 50-200 μg/mL a significant portion of the binding appeared to be attributed to non-specific mechanisms since treatment with EDTA or trypsin only partially reduced cell surface binding (Figure 6.4). Considering that GST-RAP inhibited the binding of RAP to a much greater degree than for CLU, alternative mechanisms were likely to be responsible for the majority of CLU binding whereas the binding of RAP was predominately via LDL receptors (Figure 6.2). Binding to LDL superfamily receptors and many other receptors is dependent on metal ions, therefore binding inhibited by EDTA (a metal chelator), is likely to represent specific receptor interactions. Given that the binding of CLU was partially inhibited by EDTA this suggests that a portion of the binding was due to specific receptor interactions, however, digestion of cell surface
proteins by trypsin did not abolish binding of biotinylated CLU. The binding of CLU to trypsin-treated cells suggested that at 50 μg/mL and 200 μg/mL, approximately 20% or 50% of the binding of CLU to BN cells was non-specific, respectively (Figure 6.4). In contrast, trypsin treatment almost completely abolished the binding of RAP regardless of the RAP concentration tested suggesting that there was little non-specific binding of RAP to BN cells.

The binding of CLU to the surface of HepG2 cells (which expressed LRP) was not influenced by GST-RAP which suggests that LDL superfamily receptors are not involved. The asialoglycoprotein is one of the major carbohydrate recognition receptors expressed in the liver and is responsible for the uptake and catabolism of many plasma glycoproteins. Given that CLU is a heavily glycosylated protein it was reasonable to suspect that binding to the cell surface might be mediated by one or more carbohydrate recognition receptors. However, the binding of CLU to HepG2 cells was not affected by ASF (an inhibitor of the asialoglycoprotein receptor). Moreover, the binding of CLU was not saturable and excess unlabelled CLU had no effect on the binding of biotinylated CLU when it was present at either high or low concentrations, suggesting that cell surface binding was primarily via non-specific interactions. On untreated HepG2 cells, the binding of monoclonal anti-CLU antibodies suggests that endogenous CLU was present at the cell surface (data not shown). CLU is constitutively expressed at high levels by the liver and secretion by HepG2 cells has previously been reported (Burkey et al., 1992). It is possible that very high levels of endogenous CLU may quench receptor binding sites for the labelled ligand making this cell line unsuitable for receptor studies. JEG3 cells expressing LRP bound CLU via a mechanism that was not inhibited by either GST-RAP or an inhibitory anti-LRP antibody. This suggests that LRP does not bind CLU and while other LDL superfamily receptors may be involved in the binding of CLU at low concentrations to BN cells, alternative mechanisms make a much greater contribution to the overall cell surface binding. The involvement of carbohydrate receptors in the binding of CLU to Jurkat cells is supported by the inhibitory effect of galactose (Figure 6.11), however, at his stage the precise identity of this receptor(s) remains unknown. Taken together the results of the cell binding studies suggest that the role of LDL superfamily receptors in binding CLU was limited. Although, it was not possible to know the precise receptor expression profile of each cell type, inhibition assays using RAP (or GST-RAP) to block binding to LDL
superfamily receptors are a widely accepted method for examining the involvement of receptors including LRP and megalin in ligand binding (Hertz et al., 1991; Striekland et al., 1991). While galactose had an inhibitory effect on the binding of CLU to Jurkat cells the mechanisms for cell surface binding of CLU remain largely unknown. Furthermore, a large degree of non-specific binding of CLU to cell surfaces increased the difficulty of analyzing specific binding interaction. While all proteins are expected to exhibit some degree of non-specific binding to cell surface it is likely that high-levels of non-specific CLU cell surface binding was due to the chaperone’s propensity to bind to hydrophobic surfaces.

In accordance with the results from cell binding studies, surface plasmon resonance analysis suggested that CLU did not bind to LRP, however, it was confirmed that CLU is a ligand of megalin. Although detailed kinetic analysis could not be performed it was apparent that at the same mass concentration where significant CLU binding was measured, HMW CLU-CS or HMW CLU-LYS complexes did not bind to megalin and HMW CLU-GST showed only a low level of binding (Figure 6.12). This suggests that the binding site for megalin on CLU is either obstructed or altered after the formation of HMW CLU-stressed protein complexes. In any case the results of surface plasmon resonance did not support megalin or LRP as receptors for HMW CLU-stressed protein complexes in vivo.

As expected, GST-RAP did not inhibit the binding of HMW CLU-stressed protein complexes to BN cells, however both fucoidin and mBSA (SR inhibitors) did (Figure 6.14 and Figure 6.15). Neither fucoidin nor mBSA inhibited the binding of free CLU to BN cells. However, the control proteins (FGN and GST) also displayed fucoidin inhibitable binding to this cell line, therefore, it cannot be ruled out that binding was via specific recognition sites on these particular proteins and was not attributable to a common characteristic of the HMW CLU-stressed protein complexes. Nevertheless, a feasible explanation is that fucoidin-inhibitable SRs on BN cells may recognize non-native proteins that are stabilized in HMW CLU-stressed protein complexes. The yolk sac is a known site of high SR expression, where they are important in transporting maternal lipoproteins to the developing embryo (Hatzopoulos et al., 1998). Published studies of SRs on yolk sac cells appear limited to class B, which are not inhibited by
fucoidin (Rigotti, 2003); however, the precise SR profile of BN cell remains uncharacterized.

As mentioned previously the unknown molecular mass of the complexes prevents detailed kinetic analysis of binding, however, it is fair to assume that compared to CLU or the uncomplexed client protein, HMW CLU–stressed protein complexes have a much smaller surface area to mass ratio. Considering that the conjugation of biotin to the ligands can only occur via surface accessible lysine residues, it is also fair to assume that the intensity of fluorescence resulting from interaction with a given mass of a SA-fluor conjugate is biased towards molecules with a greater surface area to mass ratio (which would produce a higher level of biotin labelling per unit mass). This was tested by coating the wells of an ELISA plate with biotinylated HMW CLU–stressed protein complexes or an equivalent mass of biotinylated CLU or the uncomplexed client protein. A similar test was carried out by directly adsorbing the proteins onto nitrocellulose membrane. In both instances the signal from the HMW CLU–stressed protein complexes was comparatively much less that that of the equivalent mass of CLU or uncomplexed client protein after development using OPD substrate or ECL, respectively (data not shown). Therefore, the very large fluorescence measured when human peripheral blood monocytes were incubated with biotinylated HMW CLU-stressed protein complexes (Figure 6.16) indicates that monocytes preferentially bind these complexes compared to CLU and the uncomplexed control proteins. Moreover, the binding of HMW CLU-stressed protein complexes was inhibited by the scavenger receptor inhibitor fucoidin. Peripheral monocytes express low levels of SR-AI and SR-AII, both of which are inhibited by fucoidin, suggesting that these receptors may have been involved in the binding of HMW CLU-stressed protein complexes (Geng et al., 1994). However, fucoidin inhibitable binding of complexes to the cultured human monocyte cell line U937, which also reportedly express SR-A (Rice et al., 2002), was not detected (data not shown). It is important to note that fucoidin also partially inhibited the binding of free CLU and the uncomplexed control proteins (GST and FGN) to peripheral monocytes, although, their relative binding at the same mass concentration was considerably less and the inhibitory effect of fucoidin was also less than that observed for the complexes. The results of these experiments suggest that fucoidin inhibitable receptors on peripheral monocytes are important in the binding HMW CLU-GST complexes, however the precise identity of the receptor(s) remains to
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be identified. The uncomplexed native components of HMW CLU-stressed protein complexes also appeared to bind to fucoidin inhibitable receptors, but to a much lesser degree. This suggests that a shared property of the HMW CLU-FGN and HMW CLU-GST target them to fucoidin inhibitable receptors and that this characteristic is less pronounced in the native proteins.

The results of biodistribution studies in a rat model suggested that the liver and the spleen were the key organs responsible for the uptake of HMW CLU-stressed protein complexes (see Chapter 7). For this reason the binding of HMW CLU-stressed protein complexes to freshly isolated rat splenocytes and liver cells was examined. Only low binding was detected to rat splenocytes and there was no clear effect of fucoidin. In contrast, freshly isolated hepatocytes bound high levels of HMW CLU-stressed protein complexes. Using density gradient centrifugation, it was possible to prepare cell fractions enriched in hepatocytes or non-parenchymal (kupffer cell and endothelial) cells from rat liver. Since hepatocytes are much larger than non-parenchymal cells, using flow cytometry, the two populations were clearly discernable based on their forward and side scatter. A much higher level of binding of HMW CLU-stressed protein complexes to hepatocytes compared to non-parenchymal cells suggests that these are the primary cell type in the liver responsible for the uptake of the complexes (Figure 6.19). As seen for human peripheral blood monocytes, enhanced binding of HMW CLU-stressed protein complexes to isolated rat hepatocytes was measured compared to free CLU or heated or unheated control proteins. However, there was increased binding of *CLU and *GST compared to the corresponding native proteins. Moreover, the binding of *FGN and *GST to hepatocytes, but not that of the corresponding native proteins, was inhibited by fucoidin. This suggests that the heat treatment fundamentally changed the structure of the proteins such that they were subsequently recognized and bound by fucoidin inhibitable receptors. CLU is known to be relatively heat stable and retains its chaperone activity even after heating at 60°C (see Section 3.3.1). Fucoidin had a much greater inhibitory effect on the binding of HMW CLU-stressed protein complexes than that of native CLU, *CLU, *FGN or *GST (Figure 6.19). This suggests that the recognition of HMW CLU-stressed protein complexes by fucoidin inhibitable mechanisms on hepatocytes may occur as a result of structural changes in the client proteins and not via a specific site(s) on CLU. Of the known SRs, fucoidin inhibition is only reported for classes A (Platt et al., 1996), E (Oka et al., 1998) and F (Berwin et al., 2004), none of
which are reportedly expressed by hepatocytes. Class B SRs are highly expressed on hepatocytes but are not inhibited by fucoidin (Acton et al., 1994; Rigotti, 2003). However, the presence of novel hepatocyte scavenger-like receptors was previously proposed after the uptake of heparin by hepatocytes was inhibited by dextran sulphate, a class A SR ligand, but not by Ac-LDL, a known ligand of class B SRs (Yuasa and Watanabe, 2003).

Confocal microscopy and protein degradation assays suggested that binding of HMW CLU-stressed protein complexes to hepatocytes precedes their internalization via a classical route of endocytosis, which leads to them being transported to lysosomes where they are degraded. The patchy distribution of the labelled complexes at the cell surface could be the result of localization of the endocytic receptors to protein coated pits on the plasma membrane (Figure 6.22 and Figure 6.24). Using confocal imaging it appeared that BN cells internalized higher levels of HMW CLU-stressed protein complexes compared to hepatocytes. However, it should be noted that directly before binding, internalization or degradation assays, hepatocytes were isolated from liver tissue using collagenase digestion. Although 0.3% (w/v) BSA was included in the digestion buffer as a competitive substrate for collagenase, and the cells were quickly removed from conditions where digestion may continue upon their release from the tissue mass, a fraction of cell surface proteins may have been damaged during the tissue dissociation procedures. A great deal of time was spent optimizing conditions to produce hepatocyte or non-parenchymal cell enriched fractions with high viability, however, it is impossible to know the extent of unwanted digestion of cell surface proteins as a result of incubation with collagenase. This may be particularly significant in the case of SRs, many of which contain collagen-like domains (Figure 1.8). Although the results clearly show binding and internalization of HMW CLU-stressed protein complexes by hepatocytes, if the receptors involved were susceptible to collagenase digestion then the results shown are likely to be an underestimate of what may occur in vivo.

In an attempt to identify other putative cell surface receptors for CLU, CLU affinity chromatography was performed using membrane proteins isolated from bovine liver cells. During these experiments the isolated membrane proteins exhibited a tendency to aggregate and precipitate out of solution (likely due to their amphipathic nature) unless stored in high concentrations of detergent. This complicated their separation by SDS-
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PAGE and analysis. Poor solubility of membrane proteins in electrophoresis buffers leading to partial or complete exclusion from entering the resolving gel has been previously reported (Santoni et al., 2000). In the absence of 10% (w/v) SDS and 1% (v/v) NP-40 it was not possible for proteins to enter the gel even when low percentages of acrylamide were used (e.g. 6%). Solubilization of membrane protein in strong detergents is a commonly used technique for increasing their suitability for electrophoresis, however high concentrations of detergents may interfere with the migration of the proteins once they have entered the gel. Individual membrane proteins respond differently to the detergents and therefore conditions that solublize some proteins do not necessarily mean that all membrane proteins were successfully solubilized (Fountoulakis and Takacs, 2001). Despite the difficulties in working with the bovine membrane proteins several bands were resolved when 1% (v/v) NP-40 and 10% (w/v) SDS were included in SDS-PAGE loading buffer and were subsequently selected for mass spectrometric analysis (Figure 6.28). Of these bands only one was identified, a band of 39 kDa corresponding to \( \beta \)-actin. No reported interactions between \( \beta \)-actin and CLU are known and at this stage the significance of this finding is uncertain. \( \beta \)-actin is predominately an intracellular protein however, it is also found in extracellular fluid at the plasma membrane (Welch and Herman, 2002; Sheng et al., 2006). Further experiments to characterize the interaction between CLU and \( \beta \)-actin are required before any physiological importance can be implied. Although it is not known what final concentration of NP-40 may have been present in the samples analyzed, this detergent is known to interfere with mass spectrometric analysis (Link et al., 2005). Given the smeared appearance of the proteins on the gel, incomplete separation of the protein bands during SDS-PAGE is likely to have hindered the identification of individual proteins by mass spectrometry. The use of 2D-electrophoresis was not attempted, however, it is expected that this technique would provide better resolution of individual proteins for mass spectrometric analysis. Nevertheless, to use this approach the problem of solubilizing the membrane proteins in an appropriate buffer still needs to be overcome. Additionally, membrane proteins are generally alkaline and standard pH gradients are often inadequate for their complete separation on 2D gels (Wilkins et al., 1998). Therefore, the identification of receptors for CLU via affinity chromatography will require optimization of conditions for their solubilization and complete separation.
Although the precise mechanisms involved are yet to be identified, it appears likely that HMW CLU-stressed protein complexes are preferentially recognized by fucoidin-inhibitable receptors, and that their recognition is via one or more structural features they share. Taken together, this strongly implicates SRs in a global mechanism for the disposal of HMW CLU-stressed protein complexes. While the liver was identified as the primary site of uptake for blood-borne HMW CLU-stressed protein complexes, perplexingly, hepatocytes (which are not known to express fucoidin inhibitable SRs) were identified as the cell type which bound the complexes most strongly. Moreover, binding to hepatocytes was followed by internalization and proteolytic degradation, supporting the suggestion that these cells play a primary role in the disposal of complexes \textit{in vivo}. It is possible that fucoidin inhibitable SRs are expressed by hepatocytes, however, an alternative explanation is that fucoidin is a ligand of other hepatocyte receptors. Fucoidin inhibitable receptors, distinct from SRs, have been implicated in the recognition and phagocytosis of apoptotic leukocytes (Johnson \textit{et al.}, 2003). Fucoidin is also a known ligand of the adhesion molecules L-selectin (Ley \textit{et al.}, 1993) and P-selectin (Aruffo \textit{et al.}, 1991; Skinner \textit{et al.}, 1991). Given that the binding of fucoidin to selectins is mediated by their c-type lectin domains and numerous other cell surface receptors contain similar domains it is possible that fucoidin may inhibit many different receptors. However, if binding was mediated solely by carbohydrate recognition it would be expected that binding of the uncomplexed control proteins would be enhanced compared to the HMW CLU-stressed complexes due to a greater ratio of surface exposed carbohydrate moieties to mass on the former. The complex receptor profile of hepatocytes complicates their use as a model to study specific receptor interaction. If hepatocytes are to be used in future studies to identify the precise receptors involved in the binding and uptake of chaperone-stressed protein complexes, specific inhibitory antibodies will need to be used in lieu of inhibitors such as fucoidin which exhibit broad specificity. Other methods such as the use of transfected cell lines or studies involving purified receptors immobilized on surface plasmon resonance chips may also be useful.
7 CLEARANCE OF BLOOD-BORNE HMW CLU-STRESSED PROTEIN COMPLEXES IN VIVO

7.1 Introduction

Normal plasma proteins are cleared from the blood according to their individual half-lives which can be highly variable between different proteins. For example, in a rat, IgG may persist in plasma for longer than 4 days (Campbell et al., 1956; Morgan, 1968), whereas LDL is cleared in less than 5 h (Mahley et al., 1980). As a general rule, proteins that are not normally found extracellularly are cleared more rapidly than endogenous plasma proteins. In rats, when injected intravenously, many intracellular enzymes usually display half-lives of less than 30 min and may have half-lives less than 10 min such as in the case of mitochondrial malate dehydrogenase, trypsinogen and ribonuclease A (Kooistra et al., 1977; Brodrick et al., 1980; Bijsterbosch et al., 1985). The reasons for the large differences in the half lives of proteins in plasma are not clear. Two very similar proteins with varying half-lives are the LDH isoenzymes M4 and H4, which have very similar gross three-dimensional structure, but have plasma half-lives of 30 min and 8 h, respectively (Bijsterbosch et al., 1985).

Renal filtration is a major route of clearance for proteins below the glomerular filtration limit of 50 kDa. However, factors such as positive charge can favour renal clearance and positively charged proteins as large as 70 kDa are reported to be cleared via this route (Rennke et al., 1978; Brodrick et al., 1980; Bijsterbosch et al., 1981; Bijsterbosch et al., 1985). Generally proteins that are able to pass into the kidneys are not excreted via the urine, but are taken up by endocytic vesicles at the apical border of the renal tubular epithelium (Maack et al., 1979; Bouma, 1982). Proteins that are excluded by glomerular filtration may be cleared directly from plasma into other tissues. Non-specific pinocytosis contributes to the clearance of all proteins since essentially this process samples extracellular fluid and all of its contents, however, this is a relatively slow process and as such the total contribution to clearance will depend largely on the concentration of the protein in plasma. A far more efficient mechanism for plasma protein clearance is receptor-mediated endocytosis. This is governed by the specific binding of proteins to receptors that are capable of facilitating their internalization. Largely, proteins are cleared from the bloodstream by endocytic receptors expressed by
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parenchymal cells, endothelial cells and kupffer cells in the liver, however, a great number of other cell types also express endocytic receptors.

To date there are no known interactions between CLU and SRs. However, the preferential uptake of damaged or modified proteins by SRs suggests that this receptor family could be important in the clearance of unfolded protein-extracellular chaperone complexes from extracellular spaces. With a few exceptions, affinity for oxLDL is characteristic of all known SRs. SRs also display affinity for other types of damaged or modified ligands including proteins with mutations that induce conformational disruptions resulting in increased exposed surface hydrophobicity (Iwao et al., 2006). Moreover, aggregation of oxLDL increases the efficiency with which macrophage SRs can take up this potentially cytotoxic protein (Amis et al., 2005). Although little research has focussed on identifying global systems for the targeted clearance of "damaged" extracellular proteins, at least in the case of LDL it appears that there are a large number of receptors that are able to recognize damaged or aggregated forms. If damaged LDL is recognized by characteristics that are shared by stress damaged proteins, then it is feasible that unfolded protein-extracellular chaperone complexes may also be recognized by the same receptors.

A number of proteins act as carriers for other extracellular molecules and target them for receptor-mediated endocytosis. The work described in earlier chapters of this thesis provides strong evidence that CLU forms soluble HMW complexes with client proteins under conditions of physiologically relevant stress. Intuitively, it is obvious that these complexes can not accumulate in extracellular spaces indefinitely. Therefore, investigations were carried out to determine where in the body (if at all) HMW CLU-stressed protein complexes are selectively cleared. The experiments described in this chapter were carried out using three different HMW CLU-stressed protein complexes. Specifically, these were (i) HMW CLU-GST formed at high temperature (60°C); (ii) HMW CLU-CS formed at a physiologically relevant temperature (41°C) and (iii) HMW CLU-FGN formed using a potential endogenous plasma client protein at a near physiological temperature (45°C).
7.2 Materials and methods

7.2.1 Purification of HMW CLU-stressed protein complexes

Precipitation assays using heat stress to induce the precipitation of a number of client proteins were performed to generate HMW CLU-stressed protein complexes in vitro (see Section 2.6). Specifically, GST and CLU were co-incubated at 60°C for 50 min, CS and CLU were co-incubated at 41°C for 12 h and FGN and CLU were co-incubated at 45°C for 12 h. Co-incubated solutions of CLU and client proteins were fractionated by SEC using a Superose™ 6 10/300 column (see Section 2.8) and protein eluting at the exclusion limit (≥ 4 x 10^7 Da) was retained and designated HMW CLU-stressed protein complex. HMW CLU-stressed protein complex were stored in PBS at 4°C at between 0.25 and 1 mg/mL and were routinely re-injected prior to experiments back on to the Superose™ 6 10/300 column to ensure that breakdown of the complex had not occurred. Typically, for animal studies HMW CLU-stressed protein complexes were formed and purified no more than one week before their use.

7.2.2 Iodination

A method to label native or residual heated CLU, GST, FGN, CS, and HMW CLU-stressed protein complexes formed between these proteins, was developed with the assistance of Ivan Greguric (Radiopharmaceuticals Research, ANSTO). Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril) (Sigma-Aldrich) was dissolved in dichloromethane (DCM) at 1 mg/mL. Between 30-50 μL was blown dry using N₂ gas in the bottom of a high performance liquid chromatography (HPLC) sample tube. In the sample tube an aliquot containing approximately 40 μg of protein was adjusted to pH 8.5 using 0.5 M Na₂CO₃. NaI¹²³ in 0.02 M NaOH (ARI ANSTO, Lucas Heights, Australia) was added to the solution (typically 5-10 mCi was used in each reaction). The final volume of the reaction mixture was increased by 100 μL using 0.1 M phosphate buffer pH 8.5. The solution was left to incubate at room temperature with shaking between 30-60 min. Zeba™ desalt mini spin columns (Pierce Biotechnology) were washed free of azide and other buffer constituents using distilled water. An appropriate volume (100-150 μL) of the reaction solution was laid on top of the resin and centrifuged according to the manufacturer’s directions. The recovered eluate was passed through a second column in the same manner. The efficiency of labelling was estimated
Clearance of blood-borne HMW CLU-stressed protein complexes in vivo

using the percentage of radioactivity in the eluate (labelled protein) compared to the percentage of radioactivity remaining in the column (free NaI\textsuperscript{123} and labelled iodogen). A sample of the eluate was passed over a Biosep 3,000 PEEK SEC column 75 x 7.5 mm (Phenomenex, Torrance, USA) at 1 mL/min using an high performance liquid chromatography (HPLC) system consisting of a 2998 photodiode array detector, 600s pump and 717s autosampler (Waters, Milford, USA) and an ACE mate\textsuperscript{TM} radioactivity detector (Ortec, Oak Ridge, USA). Profiles of the eluted radioactivity and changes in A\textsubscript{280} nm were obtained using the software program Empower Pro 2002, Version 5 (Waters). When purity greater than 95% was observed the labelled proteins were formulated at the appropriate concentration in PBS for either single photon emission computed tomography (SPECT) imaging or biodistribution studies. In figures, the prefix \textsuperscript{123}I is omitted from the abbreviations of the radiolabelled proteins for simplicity. Residual heated proteins were heated according to Section 2.7. In the case of *CLU, heating was at 60ºC for 50 min. HMW CLU-GST, HMW CLU-FGN and HMW CLU-CS were generated at 60ºC, 45ºC and 41ºC, respectively.

7.2.3 SPECT imaging

Native or residual heated CLU, FGN, GST and HMW CLU-stressed protein complexes formed between these proteins were radiolabelled as previously described (see Section 7.2.2). The radiotracers were formulated to a concentration of approximately 500 \(\mu\text{Ci}/100 \ \mu\text{L}\) in PBS. Tween\textsuperscript{®} 20 (Sigma-Aldrich) was added to achieve a final concentration of 1 \(\mu\text{g}/\text{mL}\). Female Sprague Dawley rats were restrained by hand and injected via the tail vein with 500 \(\mu\text{Ci}\) of the formulation. After 35 min the animals were anaesthetized using inhalant isoflurane with 200 mm/min oxygen via a nose cone fitted to the animal bed of the X-SPECT\textsuperscript{®} SPECT/CT imaging system. Once the animal was no longer responsive to stimuli (pinching between toes) the animal was taped to the bed lying on its stomach and the bed driven into the imaging tunnel. The HRES (5” x 5”) collimators were fitted and rotated to be horizontal above and below the animal, and moved in as close as possible to the animal. Acquisitions were taken around 40-60 min, 3, 6, 24 and 48 h post-injection (p.i.) of tracer (i.e. labelled protein or complex). The animals were removed from the anaesthetic, woken and observed for recovery between each imaging time point and had access to food and water. After the final image was acquired, animals were sacrificed by CO\textsubscript{2} asphyxiation followed by cervical dislocation.
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Three rats were imaged in this manner for each radiolabelled protein or HMW CLU-stressed protein complex.

7.2.4 Preliminary biodistribution

Residual heated CLU, FGN, and GST, and HMW CLU-stressed protein complexes were radiolabelled as previously described (Section 7.2.2). The labelled proteins were formulated to a concentration of approximately 12 μCi/100 μL in PBS. Tween® 20 was added to achieve a final concentration of 1 μg/mL. For each labelled protein or complex, 12 animals were injected via the tail vein with 12 μCi of the formulation. The animals were re-housed and provided access to food and water. At 1, 6 and 24 h p.i. 4 animals were sacrificed by CO₂ asphyxiation followed by cervical dislocation. The animals were exsanguinated and a sample of the blood retained. The liver, spleen, lungs, heart, bladder, stomach, small gastrointestinal tract, large gastrointestinal tract, tail, brain, thyroid, pancreas, caecum and the ovaries were carefully removed in addition to a single kidney and samples of muscle, skin and bone (femur). Each organ/tissue sample was weighed and the radioactivity measured using a Wallac gamma counter 1,480 (LKB Wallac, Turku, Finland) which was calibrated using the radiolabelled protein solution to be injected into the animals. The data was corrected for radioactivity remaining in the tail. Statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey HSD. Total blood volume was calculated according to the formula Blood Volume (mL) = 0.06 x BW(g) + 0.77, which has a reported r = 0.99 and p < 0.0001 for n = 70 (Lee and Blaufox, 1985).

7.2.5 Biodistribution studies investigating the effect of pre-injection with fucoidin

Native CLU, FGN, CS and HMW CLU-stressed protein complexes were radiolabelled as previously described (Section 7.2.2). The labelled proteins were formulated to a concentration of approximately 12 μCi/100 μL in PBS. Tween® 20 was added to achieve a final concentration of 1 μg/mL. For each labelled protein or complex, 32 animals were injected via the tail vein with 12 μCi of the formulation. Half of these animals were pre-injected with 15 mg/kg body weight fucoidin 5 min before injection of the tracer. The animals were re-housed and provided access to food and water. At 5, 15,
30 and 60 min p.i. animals were sacrificed and biodistribution data was collected as described for the preliminary study (Section 7.2.4). In addition, the Z score (also known as the standard score) was used to calculate whether there was a difference in tissue associated activity between animals pre-injected with fucoidin to those not treated with the inhibitor (control). This was performed by calculating the ratio of the percentage of the injected dose/g tissue between animals pre-injected with fucoidin to control animals and comparing this value to 1 (the theoretical value for no difference between the treatments).
7.3 Results

7.3.1 123I labelling of CLU, client proteins and HMW CLU-stressed protein complexes

All HMW CLU-stressed protein complexes and control proteins (residual heated or native CLU, GST, FGN or CS) were efficiently labelled using direct iodination, although the reaction time needed to achieve the best results varied between 30 min and 90 min depending on the protein. The composition of the solution at the conclusion of the reaction was examined by SEC. In all cases a minor peak corresponding to the position of labelled iodogen or free iodine followed the elution of the labelled protein. An example size exclusion profile is shown for 123I-labelled CLU in Figure 7.1. Given the presence of reaction by-products (iodinated iodogen and free iodine), it was necessary to find a method to purify the sample before formulation of the tracer for injection into the rats. The Biosep size exclusion columns used for quality control analysis were not suitable for this purpose. Although enough labelled protein passed through the column to detect, using the reaction volume described the fraction that eluted was dilute and the yield of labelled protein was low. Altering the mobile phase did not overcome this problem. It was found that passing the sample through a Zeba™ desalt mini spin column was sufficient to separate the labelled protein from the contaminating reaction by-products (Figure 7.2). The specific activity of the labelled proteins was typically around 89.8 ± 8.6 μCi/μg of protein with a range of 107 – 59 μCi/μg (n = 6). At these levels the yield and concentration of the labelled protein was suitable for formulation to the concentration required for the standard injected dose in SPECT imaging and biodistribution studies. The reason for variation in specific activity between labelling reactions appeared to correspond with the starting concentration and source of the 123I-Na, however, there was no obvious difference in the labelling efficiency of the different proteins or the HMW CLU-stressed protein complexes (data not shown).
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7.3.2 SPECT imaging

Preliminary SPECT imaging of animals injected with $^{123}$I-labelled HMW CLU-stressed protein complexes, uncomplexed CLU, or uncomplexed GST or FGN was carried out to provide preliminary insight into the clearance rates and localization of the labelled proteins. In all cases, within 45 min p.i., the tracers were targeted to the upper abdomen in the general region of the liver, spleen and nearby organs (Figures 7.3-7.7). The results
were consistent between animals injected with the same labelled protein and no
difference was seen between animals injected with uncomplexed native or residual
heated proteins. Therefore, only representative images are shown for the uncomplexed
controls (obtained using native proteins). Radioactivity in the bladder was also
noticeable within the first 6 h of the experiment. During this time radioactivity in the
thyroid was seen to increase, however, in all cases, for the entire 48 h of the experiment
a significant portion of radioactivity remained localized in the original upper abdominal
location. For $^{125}$I-labelled GST, within this highly radioactive region the tracer appeared
to be most concentrated in a smaller region to one side of the animal at the earlier time
points (Figure 7.7).
Figure 7.3 SPECT imaging of a Sprague Dawley rat injected with $^{123}$I-HMW CLU-FGN.

A) lower body (orientation indicated by the labels ABDOMEN and TAIL) or (B) head and upper body (orientation indicated by the labels HEAD and ABDOMEN). Times shown are p.i. Static summations are the resultant image after combining the acquisitions at the specified time points. The images shown are representative of three separate experiments.
Figure 7.4 SPECT imaging of a Sprague Dawley rat injected with $^{123}$I-FGN. 
(A) lower body (orientation indicated by the labels ABDOMEN and TAIL) or (B) head and upper body (orientation indicated by the labels HEAD and ABDOMEN). Times shown are p.i. Static summations are the resultant image after combining the acquisitions at the specified time points. The images shown are representative of three separate experiments.
Figure 7.5 SPECT imaging of a Sprague Dawley rat injected with ¹²³I-CLU. 
(A) lower body (orientation indicated by the labels ABDOMEN and TAIL) or (B) head and upper body (orientation indicated by the labels HEAD and ABDOMEN). Times shown are p.i. Static summations are the resultant image after combining the acquisitions at the specified time points. The images shown are representative of three separate experiments.
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Figure 7.6 SPECT imaging of a Sprague Dawley rat injected with $^{123}$I-HMW CLU-GST.

(A) lower body (orientation indicated by the labels ABDOMEN and TAIL) or (B) head and upper body (orientation indicated by the labels HEAD and ABDOMEN). Times shown are p.i. Static summations are the resultant image after combining the acquisitions at the specified time points. The images shown are representative of three separate experiments.
Figure 7.7 SPECT imaging of a Sprague Dawley rat injected with $^{123}$I- GST. (A) lower body (orientation indicated by the labels ABDOMEN and TAIL) or (B) head and upper body (orientation indicated by the labels HEAD and ABDOMEN). Times shown are p.i. Static summations are the resultant image after combining the acquisitions at the specified time points. The images shown are representative of three separate experiments.
7.3.3 Preliminary biodistribution study

A preliminary biodistribution study was performed using HMW CLU-GST (formed after incubation at 60°C for 50 min), HMW CLU-FGN (formed after incubation at 45°C for 12 h) and their respective uncomplexed heated controls *CLU, (heated at 60°C for 50 min), *GST (heated at 60°C for 50 min) and *FGN (heated at 45°C for 12 h).

7.3.3.1 Clearance of blood-borne radioactivity after injection with ¹²³I-labelled HMW CLU-stressed protein complex or uncomplexed control proteins

For all ¹²³I-labelled proteins and HMW CLU-stressed protein complexes there was an overall decline in circulating radioactivity over the 24 h of the experiment (Figure 7.8). The amount of radioactivity measured in the blood of animals 1, 6 and 24 h p.i. was statistically different between the ¹²³I-labelled proteins and HMW CLU-stressed protein complexes as determined by ANOVA (F(4,15) = 61.97, F(4,14) = 40.48, F(4, 14) = 6.4, respectively; all p < 0.01). Specifically, at 1 and 6 h p.i. there was less radioactivity per gram of blood in animals injected with either of the two ¹²³I-labelled HMW CLU-stressed protein complexes compared to heated ¹²³I-*CLU or the respective uncomplexed control proteins (¹²³I-*GST or ¹²³I-*FGN) (Tukey HSD, p < 0.01). After 24 h, at the same level of confidence, there was no significant difference between the amounts of radioactivity per gram of blood in animals injected with HMW CLU-stressed protein complexes compared to the relevant uncomplexed control proteins.
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Figure 7.8 Percentage of the injected dose/g blood in Sprague Dawley rats after injection with ¹²³I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Panels show the clearance of circulating radioactivity up to 24 h after animals were injected with ¹²³I-labelled (A) HMW CLU-GST, *CLU or *GST or (B) HMW CLU-FGN, *CLU or *FGN. Data points represent means (n = 4 ± standard deviation) and are corrected for any radioactivity remaining in the tail.

Using the weight of the animals to approximate the total blood volume it was evident that the majority of all ¹²³I-labelled proteins and HMW CLU-stressed protein complexes had been cleared 1 h p.i. (Figure 7.9). At this time, the differences between the clearance of ¹²³I-labelled HMW CLU-stressed protein complexes and the relevant control proteins were most evident and this was confirmed by ANOVA (F(4,15) = 61.97; p ≤ 0.01). At 1 h p.i., for ¹²³I-HMW CLU-GST and ¹²³I-HMW CLU-FGN, less than 6% of the injected dose remained in the blood - this was significantly less than the corresponding fractions for ¹²³I-*CLU, ¹²³I-*GST and ¹²³I-*FGN (Figure 7.9; Tukey HSD, p ≤ 0.01).
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Figure 7.9 Total percentage of radioactivity remaining in the blood of Sprague Dawley rats 1 h after injection of $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins. Animals were injected with $^{125}$I-labelled HMW CLU-stressed protein complex or uncomplexed residual heated control protein. The radioactivity per gram of blood was measured 1 h p.i. and the total remaining activity was calculated using the formula of Lee and Blaufox (1985) to estimate the approximate blood volume of each rat. Data points represent means ($n = 3 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail. ** Denotes significantly reduced circulating radioactivity compared to free *CLU and the relevant uncomplexed control proteins (Tukey HSD, $p \leq 0.01$).

7.3.3.2 Organs of highest uptake

$^{125}$I-labelled HMW CLU-stressed protein complexes displayed a different biodistribution profile to their respective uncomplexed controls (Figure 7.10). With the exception of the stomach and thyroid (the body’s major iodine sinks) there was little radioactivity in any organs other than the liver, spleen and kidney regardless of the $^{125}$I-labelled protein or HMW CLU-stressed protein complex injected. Generally, there was higher radioactivity in the liver and spleen of animals injected with $^{125}$I-labelled HMW CLU-stressed protein complexes compared to $^{125}$I-*CLU, $^{125}$I-*GST or $^{125}$I-*FGN. However, there was higher radioactivity in the kidneys of animals injected with these uncomplexed control proteins compared to animals injected with $^{125}$I-labelled HMW CLU-stressed protein complexes. As evident from the results of the clearance of the $^{125}$I-labelled proteins and HMW CLU-stressed protein complexes from the bloodstream, the majority of activity had been cleared to tissues by 1 h p.i. Due to time-dependent proteolytic degradation following cellular internalization (and the consequent release of radioactive iodine), it is
expected that measurements of tissue-associated radioactivity would most accurately reflect uptake of radiolabelled protein at the earliest time point measured.

Figure 7.10 Percentage of the injected dose/g of tissue in Sprague Dawley rats 1 h after injection of $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins. Panels show the results for animals injected with $^{125}$I-labelled (A) HMW CLU-GST, *CLU or *GST or (B) HMW CLU-FGN, *CLU or *FGN. Data points represent means ($n = 3 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.
Consistent with their clearance from the bloodstream being completed by 1 h p.i.,
regardless of the $^{125}$I-labelled protein or HMW CLU-stressed protein complex injected,
tissue-associated radioactivity was seen to decrease between 1 and 24 h p.i. in the liver,
spleen and kidney (Figure 7.11 and Figure 7.12). Comparison of the different treatment
groups showed there were differences in the accumulation of radioactivity in the liver at
1, 6 and 24 h p.i. between HMW CLU-stressed protein complexes and the uncomplexed
controls according to ANOVA ($F(4,15) = 151.69$, $F(4,15) = 98.73$, $F(4,13) = 146.39$, respectively; all $p \leq 0.01$). Animals injected with $^{125}$I-HMW CLU-GST or $^{125}$I-HMW CLU-FGN recorded significantly higher radioactivity in the liver at all time points
compared to animals injected with $^{125}$I-*CLU, $^{125}$I-*GST or $^{125}$I-*FGN (Tukey HSD, $p \leq 0.01$; Figure 7.11; panel A and Figure 7.12; panel A). Similarly, $^{125}$I-labelled HMW
CLU-stressed protein complexes also showed preferential accumulation in the spleen at
1, 6 and 24 h p.i. ($F(4,15) = 22.21$, $F(4,15) = 108.02$, $F(4,13) = 44.71$, respectively; $p \leq 0.01$). For animals injected with $^{125}$I-HMW CLU-GST, greater radioactivity was
measured in the spleen at all time points compared to $^{125}$I-*CLU or $^{125}$I-*GST (Tukey
HSD, $p \leq 0.01$; Figure 7.11; panel B). At the same level of significance the difference
between the radioactivity in the spleen of animals injected with $^{125}$I-HMW CLU-FGN or
$^{125}$I-*CLU was only significant at 24 h p.i. (Figure 7.12; panel B). However, compared to
animals injected with uncomplexed $^{125}$I-*FGN, much greater radioactivity was measured
in the spleen of animals injected with $^{125}$I-HMW CLU-FGN at all time points (Tukey
HSD, $p \leq 0.01$; Figure 7.12; panel B). Preferential accumulation of radioactivity in the
kidney of animals injected with the uncomplexed control proteins was measured at 1, 6
and 24 h p.i. ($F(4,15) = 90.76$, $F(4,15) = 25.65$, $F(4,13) = 12.53$, respectively; $p \leq 0.01$).
For animals injected with $^{125}$I-labelled HMW CLU-stressed protein complexes
(incorporating either GST or FGN), at 1 h p.i. there was significantly less radioactivity
in the kidneys compared to animals injected with $^{125}$I-*CLU or the respective residual
heated control protein (Figure 7.11; panel C and Figure 7.12; panel C; Tukey HSD, $p \leq 0.01$). Beyond this, there was higher radioactivity in the kidney in animals injected
with $^{125}$I-*GST compared to animals injected with $^{125}$I-HMW CLU-GST at 6 h p.i., but
no other apparent differences between $^{125}$I-HMW CLU-stressed protein complexes and
their respective uncomplexed control proteins (Tukey HSD, $p \leq 0.01$).
Figure 7.11 Percentage of the injected dose/g tissue in Sprague Dawley rats 1, 6 and 24 h after injection with $^{125}$I-labelled HMW CLU-GST, *CLU or *GST.

Animals were injected with $^{125}$I-labelled HMW CLU-GST, *CLU or *GST. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail. Individual tissues are identified in the corresponding panels. + Denotes significantly higher tissue-associated activity and – denotes significantly lower tissue-associated activity in animals injected with $^{125}$I-labelled HMW CLU-GST compared to those injected with $^{125}$I-*CLU (blue) or $^{125}$I-*GST (maroon) (Tukey HSD, $p \leq 0.01$).
Figure 7.12 Percentage of the injected dose/g tissue in Sprague Dawley rats 1, 6 and 24 h after injection of $^{123}$I-labelled HMW CLU-FGN, *CLU or *FGN.

Animals were injected with $^{123}$I-labelled HMW CLU-FGN, *CLU or *FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail. Individual tissues are identified in the corresponding panels. + Denotes significantly higher tissue-associated activity and – denotes significantly lower tissue-associated activity in animals injected with $^{123}$I-labelled HMW CLU-FGN compared to those injected with $^{123}$I-*CLU (blue) or $^{123}$I-*FGN (maroon) (Tukey HSD, $p \leq 0.01$).
7.3.4 Biodistribution studies investigating the effect of pre-injection with fucoidin

Given that the preliminary biodistribution results suggested that the majority of all $^{123}$I-labelled proteins and HMW CLU-stressed protein complexes were cleared from the blood at 1 h p.i., a second study investigating time points at 5-60 min p.i. was carried out. In this study the biodistribution of HMW CLU-FGN (formed after incubation at 45°C for 12 h) and HMW CLU-CS (formed after incubation at 41°C for 12 h) was compared to the biodistribution of native CLU, FGN and CS. Native control proteins were chosen as a suitable control for biodistribution studies after SPECT imaging showed no difference in the time-dependent localization of native and residual heated proteins. Moreover, circular dichroism analysis suggested that the structure of HMW CLU-FGN was closer to that of native FGN and CLU than to residual heated *FGN and *CLU (Figure 5.7; panel E and Figure 5.8; panel E). Similar results were obtained for CS after the formation of HMW CLU-CS (data not shown). The results are divided into the subsections which address (i) blood-borne clearance (Section 7.3.4.1), (ii) the organs of highest uptake (Section 7.3.4.2) and (iii) other organs (Section 7.3.4.3).

7.3.4.1 Clearance of blood-borne radioactivity after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or control proteins

The consistently low blood-borne radioactivity (even at 5 min p.i.) in animals injected with $^{123}$I-FGN, $^{123}$I-HMW CLU-FGN or $^{123}$I-HMW CLU-CS suggests that the majority of these labelled proteins were cleared from the bloodstream prior to any measurements being taken (Figure 7.13). However, measurements of the radioactivity remaining in the blood of animals injected with $^{123}$I-CLU and $^{123}$I-CS indicate that larger proportions of these two proteins remained in the blood at up to 30 min p.i. ANOVA comparison of the different treatments showed that there was significantly less of the injected dose per gram of blood in animals injected with $^{123}$I-HMW CLU-stressed protein complexes compared to the relevant uncomplexed control proteins ($F(4,13) = 310.99$, $p \leq 0.0001$; Tukey HSD, $p \leq 0.01$). Comparing the uncomplexed control proteins, the radioactivity in the blood of the $^{123}$I-CLU-injected animals was higher than in those injected with $^{123}$I-CS or $^{123}$I-FGN (Tukey HSD, $p \leq 0.01$).
Clearance of blood-borne HMW CLU-stressed protein complexes in vivo

Figure 7.13 Percentage of the injected dose/g blood in Sprague Dawley rats after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Panels show the clearance of circulating radioactivity up to 60 min after animals were injected with $^{123}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.

There were clear differences in the levels of radioactivity in the blood of the treatment groups 5 min p.i. and this was verified by ANOVA ($F(4,13) = 347.71; p \leq 0.0001$). The results indicated that approximately 95% of both $^{123}$I-labelled HMW CLU-stressed protein complexes were cleared within 5 min p.i. At this time point the remaining blood-borne radioactivity in animals injected with $^{123}$I-HMW CLU-stressed protein complexes was much less than the amount of radioactivity in animals injected with $^{123}$I-labelled CLU, CS or FGN (Tukey HSD, $p \leq 0.01$; Figure 7.14). Comparing animals injected with the uncomplexed control proteins, there was significantly more radioactivity in the blood of animals injected with $^{123}$I-CLU compared to animals injected with $^{123}$I-labelled CS or FGN (Tukey HSD, $p \leq 0.01$).
Figure 7.14 Percentage of the injected dose remaining in the blood of Sprague Dawley rats 1 h after injection of $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Animals were injected with $^{123}$I-labelled HMW CLU-stressed protein complex or uncomplexed control protein. The radioactivity per gram of blood was measured 1 h p.i. and the total remaining activity was calculated using the formula of Lee and Blaufox (1985) to estimate the approximate blood volume of each rat. Data points represent means ($n = 3 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail. Significantly reduced blood-borne radioactivity compared to animals injected with (i) $^{123}$I-CLU or (ii) $^{123}$I-CLU and the relevant uncomplexed control protein are denoted by * and **, respectively (Tukey HSD, $p \leq 0.01$).

In all cases, when compared with animals injected with labelled protein/complex in the absence of fucoidin, fucoidin pre-treatment resulted in significantly greater radioactivity measured in the blood at 5 min p.i. ($Z_{CLU} = 7.76$, $Z_{CS} = 7.35$, $Z_{HMW\ CLU-CS} = 2.83$, $Z_{FGN} = 3.77$, $Z_{HMW\ CLU-FGN} = 5.66$; $p \leq 0.05$; Figure 7.15; panel A). This effect was most significant for animals injected with $^{123}$I-HMW CLU-FGN, where fucoidin produced close to a 4-fold increase in the amount of radioactivity present in the blood at this time point. The corresponding increase was approximately 3-fold for $^{123}$I-HMW CLU-CS and $^{123}$I-FGN and closer to 2-fold for $^{123}$I-CLU and $^{123}$I-CS. At 15 min p.i. of the labelled protein/complex, fucoidin pre-treatment had no significant effect on the levels of radioactivity in the blood of animals injected with $^{123}$I-HMW CLU-stressed protein complexes, however it still significantly increased blood-borne radioactivity in animals injected with $^{123}$I-CLU, $^{123}$I-CS and $^{123}$I-FGN ($Z_{CLU} = 2.05$, $Z_{CS} = 2.52$, $Z_{HMW\ CLU-CS} = 0.39$, $Z_{FGN} = 2.54$, $Z_{HMW\ CLU-FGN} = 0.91$; $p \leq 0.05$; Figure 7.15; panel B).
Figure 7.15 Ratios of the proportion of injected dose in the blood of fucoidin pre-treated versus control Sprague Dawley rats injected with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 5 min, and (B) 15 min p.i.

The data represent means (n = 4 ± standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); Z score, $p \leq 0.05$.

### 7.3.4.2 Organs of highest uptake

As seen in the preliminary biodistribution data, there were some differences in the biodistribution profiles of animals injected with $^{123}$I-labelled HMW CLU-stressed protein complexes compared to the relevant uncomplexed control proteins. Again, relatively little radioactivity was seen in organs other than the liver, spleen and kidney. Consistent with the preliminary study, at 5 min p.i. there was more radioactivity in the kidneys of animals injected with the uncomplexed control proteins compared to those injected with $^{123}$I-labelled HMW CLU-stressed protein complexes, while there was
generally more radioactivity in the liver and spleen of animals injected with $^{123}$I-labelled HMW CLU-stressed protein complexes compared to those injected with the uncomplexed control proteins (Figure 7.16; panel A and Figure 7.17; panel A). Pre-injection with fucoidin changed the biodistribution profile for all proteins (Figure 7.16 panel B and Figure 7.17; panel B). At 5 min p.i., pre-treatment with fucoidin reduced the amount of radioactivity in the liver and spleen and increased the amount of radioactivity in the lungs. There were unusually high levels of radioactivity in the bladder of some animal injected with $^{123}$I-FGN and this contributed to the very large variation in this group, which is probably the result of renal clearance of free iodine (Figure 7.17; panel A).
Figure 7.16 Percentage of the injected dose/g of tissue in Sprague Dawley rats 5 min after injection with $^{125}$I-labelled HMW CLU-CS, CLU or CS without (A) or with (B) fucoidin pre-treatment. Data points represent mean percentages ($n = 3 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.
Figure 7.17 Percentage of the injected dose/g of tissue in Sprague Dawley rats 5 min after injection with $^{125}$I-labelled HMW CLU-FGN, CLU or FGN without (A) or with (B) fucoidin pre-treatment. Data points represent means ($n = 3 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.
7.3.4.2.1 Liver

Time-dependent decreases in liver-associated radioactivity suggested that uptake of $^{123}$I-labelled complexes/proteins into the liver was essentially complete at 5 min p.i. and that free $^{123}$I was subsequently being released following intracellular degradation of the labelled proteins (Figure 7.18). At 5 min p.i. there was a significant difference between the amounts of liver-associated radioactivity in animals injected with $^{123}$I-HMW CLU-stressed complexes versus the uncomplexed control proteins ($F(4,14) = 53.87, p \leq 0.0001$). More radioactivity was found in the livers of animals injected with $^{123}$I-HMW CLU-FGN complexes compared to those injected with $^{123}$I-labelled CLU or FGN (Tukey HSD, $p \leq 0.01$). Furthermore, in animals injected with $^{123}$I-HMW CLU-CS, there was significantly more radioactivity in the liver at 5 min p.i. compared with animals injected with $^{123}$I-CLU (Tukey HSD, $p \leq 0.01$). Using Tukey HSD, the level of radioactivity in the liver of animals injected with $^{123}$I-CS was not significantly different to animals injected with $^{123}$I-HMW CLU-CS, however, this difference was significant according to the Student’s $t$-test ($t(6) = 3.027; p \leq 0.03$). The time-dependent decrease in liver-associated radioactivity in animals injected with $^{123}$I-HMW CLU-CS was less than that measured for other $^{123}$I-labelled proteins and $^{123}$I-HMW CLU-FGN, suggesting that degradation of $^{123}$I-HMW CLU-CS by the liver was relatively slow. The time-dependent decrease in liver-associated radioactivity of animals injected with $^{123}$I-labelled CLU, CS and HMW CLU-FGN occurred largely within the first 30 min, while that in animals injected with $^{123}$I-FGN continued to decrease steadily for the entire 60 min of the study suggesting that the latter was being degraded slowly for the entire duration of the study.
Clearance of blood-borne HMW CLU-stressed protein complexes in vivo

Figure 7.18 Percentage of the injected dose/g liver in Sprague Dawley rats after injection with $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Panels show the clearance of circulating radioactivity up to 60 min after animals were injected with $^{125}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.

In all cases, at 5 min p.i., fucoidin pre-treatment reduced the level of liver-associated radioactivity (Figure 7.19; panel A; $Z_{CLU} = -3.97$, $Z_{CS} = -11.42$, $Z_{HMW\ CLU-CS} = -2.36$, $Z_{FGN} = -2.58$, $Z_{HMW\ CLU-FGN} = -3.90$; $p \leq 0.05$). Furthermore, even at 15 min p.i., animals that had been pre-treated with fucoidin still showed significantly reduced liver-associated radioactivity if they had been injected with $^{125}$I-labelled CLU or CS ($Z_{CLU} = -4.95$, $Z_{CS} = -5.11$) but not $^{125}$I-labelled HMW CLU-CS or FGN ($Z_{HMW\ CLU-CS} = -0.07$, $Z_{FGN} = -0.10$) or $^{125}$I-labelled HMW CLU-FGN ($Z_{HMW\ CLU-FGN} = 1.69$) (Figure 7.19; panel B).
Figure 7.19 Ratios of the proportion of liver-associated injected dose for fucoidin pre-treated versus control Sprague Dawley rats injected with $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 5 min, and (B) 15 min p.i.

The data shown represent means ($n = 4 \pm$ standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); Z score, $p \leq 0.05$.

7.3.4.2.2 Spleen

The fact that spleen-associated radioactivity did not increase during the 60 min of the study suggests that uptake of the $^{125}$I-labelled complexes/proteins into the spleen was largely complete by 5 min p.i. (Figure 7.20). Degradation of the proteins and the subsequent release of free $^{125}$I (indicated by decreasing spleen-associated radioactivity) appeared to be slower than in the liver. At 5 min p.i., the amount of radioactivity in the spleen was significantly different between animals injected with $^{125}$I-labelled HMW
CLU-stressed protein complexes versus the uncomplexed control proteins (F(4,13) = 8.98; p ≤ 0.001). At 5 min p.i., there was a higher proportion of the injected dose in the spleen of animals injected with $^{123}$I-labelled HMW CLU-stressed protein complex versus $^{123}$I-CLU; the same was true when comparing animals injected with $^{123}$I-HMW CLU-FGN versus $^{123}$I-FGN (Tukey HSD, p ≤ 0.05). Using Tukey HSD, with the same level of confidence, there was no significant difference between the proportion of the injected dose in the spleens of animals injected with $^{123}$I-labelled HMW CLU-CS or CS. However, comparison of the two treatments by Student’s t-test found that there was a higher proportion of spleen-associated injected dose in animals injected with $^{123}$I-HMW CLU-CS versus those injected with $^{123}$I-CS (t(5) = 4.047; p ≤ 0.01193).

Figure 7.20 Percentage of the injected dose/g spleen in Sprague Dawley rats after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins. Panels show the clearance of circulating radioactivity up to 60 min after animals were injected with $^{123}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means (n = 4 ± standard deviation) and are corrected for any radioactivity remaining in the tail.
In all cases, pre-treatment with fucoidin significantly reduced the proportion of the injected dose present in the spleen at 5 min p.i. (Figure 7.21; panel A; $Z_{CLU} = -6.68$, $Z_{CS} = -4.39$, $Z_{HMWCLU-CS} = -3.99$, $Z_{FGN} = -2.39$, $Z_{HMWCLU-FGN} = -2.72$; $p \leq 0.05$). However, at 15 min p.i., fucoidin pre-treatment had no significant effect in animals injected with $^{123}$I-labelled HMW CLU-CS or CLU-FGN (Figure 7.21; panel B; $Z_{HMWCLU-CS} = 0.79$, $Z_{HMWCLU-FGN} = -1.51$; $p \leq 0.05$). Nevertheless, at this time point, fucoidin pre-treatment still significantly reduced the proportion of the injected dose associated with the spleen of animals injected with $^{123}$I-labelled CLU, CS or FGN ($Z_{CLU} = -2.98$, $Z_{CS} = -6.48$, $Z_{FGN} = -1.68$; $p \leq 0.05$).

![Figure 7.21](image)

**Figure 7.21** Ratios of the proportion of spleen-associated injected dose for fucoidin pre-treated versus control Sprague Dawley rats injected with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 5 min, and (B) 15 min p.i.

The data represent means ($n = 4 \pm$ standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); Z score, $p \leq 0.05$. 
7.3.4.2.3 Kidney

As observed for the liver and spleen, between 5 and 60 min p.i. there was no time-dependent increase in tissue-associated radioactivity, suggesting that uptake of the $^{123}$I-labelled complexes/proteins into the kidney was complete at or before 5 min p.i. (Figure 7.22). At 5 min p.i., there were statistically significant differences between the proportions of kidney-associated injected dose for the various $^{123}$I-labelled proteins and HMW CLU-stressed protein complexes injected ($F(4,14) = 704.6; p \leq 0.0001$). The most dramatic difference was the high proportion of the injected dose present in the kidneys of animals injected with $^{123}$I-FGN compared to all other $^{123}$I-labelled proteins and complexes (Figure 7.22; panel B; Tukey HSD, $p \leq 0.01$). Notably, at 5 min p.i., animals injected with $^{123}$I-HMW CLU-stressed protein complexes had significantly less of the injected dose associated with the kidney compared to animals injected with $^{123}$I-labelled CLU or CS (Figure 7.22; Tukey HSD, $p \leq 0.01$).

![Figure 7.22 Percentage of the injected dose/g kidney in Sprague Dawley rats after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.](image)

Panels shows the kidney-associated activity up to 60 min after animals were injected with $^{123}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.
In contrast to the results for liver and spleen, at 5 min p.i. there was no significant effect of fucoidin pre-treatment for any of the $^{125}$I-labelled proteins/complexes (Figure 7.23; panel A; $Z_{CLU} = 1.21$, $Z_{CS} = 1.64$, $Z_{HMW\,CLU-CS} = 0.16$, $Z_{FGN} = 0.03$, $Z_{HMW\,CLU-FGN} = -0.73$; $p \leq 0.05$). This was also the case at 15 min p.i., with the exception of animals injected with $^{125}$I-CLU, for which fucoidin pre-treatment slightly increased the proportion of the injected dose associated with the kidney (Figure 7.23; panel B; $Z_{CLU} = 2.75$, $Z_{CS} = 0.68$, $Z_{HMW\,CLU-CS} = -1.42$, $Z_{FGN} = 0.35$, $Z_{HMW\,CLU-FGN} = -0.73$; $p \leq 0.05$).

**Figure 7.23** Ratios of the proportion of kidney-associated injected dose for fucoidin pre-treated versus control Sprague Dawley rats injected with $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 5 min, and (B) 15 min p.i. The data represent means ($n = 4 \pm$ standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); $Z$ score, $p \leq 0.05$. 
7.3.4.3 Other organs

7.3.4.3.1 Lungs

There was little time-dependent change in the proportion of the injected dose in the lungs between 5 and 60 min p.i. (Figure 7.24). In the absence of fucoidin pre-treatment, compared to the liver, spleen and kidney, the level of lung-associated radioactivity was relatively low. The proportion of the injected dose present in the lungs at 5 min p.i. varied significantly with the individual $^{125}$I-labelled complex/protein ($F(4,15) = 44.53$; $p \leq 0.0001$). There was not a consistent pattern of preferential uptake of HMW CLU-stressed protein complexes. For example, the proportion of the injected dose in the lungs of animals injected with $^{125}$I-HMW CLU-CS was lower than that of animals injected with $^{125}$I-CLU (Tukey HSD, $p \leq 0.01$) but no different to that of animals injected with $^{125}$I-CS (Figure 7.24; panel A). In contrast, a higher proportion of the injected dose was associated with the lungs of animals injected with $^{125}$I-HMW CLU-FGN compared to those injected with $^{125}$I-labelled CLU or FGN (Figure 7.24; panel B; Tukey HSD, $p \leq 0.01$).
Clearance of blood-borne HMW CLU-stressed protein complexes in vivo

Figure 7.24 Percentage of the injected dose/g lung in Sprague Dawley rats after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Panels shows the proportion of the injected dose associated with the lungs up to 60 min after animals were injected with $^{123}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.

For all $^{123}$I-labelled proteins and both HMW CLU-stressed protein complexes examined, pre-treatment with fucoildin dramatically increased the proportion of the injected dose present in the lungs at 5 min p.i. (Figure 7.25; panel A; $Z_{CLU} = 2.46$, $Z_{CS} = 3.65$, $Z_{HMW \ CLU-CS} = 1.62$, $Z_{FGN} = 2.73$, $Z_{HMW \ CLU-FGN} = 2.55$; $p \leq 0.05$). At 15 min p.i., pre-treatment with fucoildin had no significant effect for $^{123}$I-labelled CLU, CS or HMW CLU-FGN but it still significantly increased the proportion of the injected dose associated with the lungs in animals injected with $^{123}$I-labelled HMW CLU-CS and FGN (Figure 7.25; panel B; $Z_{CLU} = 0.95$, $Z_{CS} = 0.84$, $Z_{HMW \ CLU-CS} = 2.80$, $Z_{FGN} = 4.44$, $Z_{HMW \ CLU-FGN} = 0.54$; $p \leq 0.05$).
Figure 7.25 Ratios of the proportion of lung-associated injected dose for fucoidin pre-treated versus control Sprague Dawley rats injected with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 5 min, and (B) 15 min p.i.
The data represent means ($n = 4 \pm$ standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); Z score, $p \leq 0.05$.

7.3.4.3.2 Thyroid
In the time period 5-60 min after injection of $^{123}$I-labelled complexes/proteins, the thyroid and stomach were the only organs to show a time-dependent increase in associated radioactivity. These organs are known to be in vivo iodine "sinks" (Venturi and Venturi, 1999). This suggests that during this timeframe intracellular degradation of $^{123}$I-labelled complexes/proteins was occurring, leading to the release of $^{123}$I and
Clearance of blood-borne HMW CLU-stressed protein complexes in vivo

$^{123}$I-tyrosine derivatives back into the bloodstream and the subsequent accumulation of $^{123}$I in the iodine sink organs.

The uptake of radioactivity into the thyroid was similar for animals injected with $^{123}$I-labelled HMW CLU-CS, CLU or CS (Figure 7.26; panel A). Animals injected with $^{123}$I-FGN also showed a time-dependent increase in the proportion of the injected dose associated with the thyroid (Figure 7.26; panel B); the 30 min treatment group showed very large variation, however, none of the four animals could be validly excluded as an "outlier". At 60 min p.i., there was no significant difference in the proportion of the injected dose associated with the thyroid for animals injected with $^{123}$I-labelled HMW CLU-FGN, CLU or FGN.

![Figure 7.26](image.png)

Figure 7.26 Percentage of the injected dose/g thyroid in Sprague Dawley rats after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Panels shows the proportion of the injected dose associated with the thyroid up to 60 min after animals were injected with $^{123}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.
Fucoidin pre-treatment significantly inhibited the accumulation of radioactivity in the thyroid in animals injected with $^{125}$I-labelled HMW CLU-CS and CLU-FGN (at 60 min and 30 min p.i., respectively) (Figure 7.27; $Z_{\text{HMW CLU-CS}} = -3.01$, $Z_{\text{HMW CLU-FGN}} = -2.94$; $p \leq 0.05$). In contrast, under the same conditions, fucoidin pre-treatment had no significant effect in animals injected with the relevant uncomplexed control proteins (60 min p.i. $Z_{\text{CLU}} = -0.79$, $Z_{\text{CS}} = -1.27$; panel A; 30 min p.i. $Z_{\text{CLU}} = 0.82$, $Z_{\text{FGN}} = -1.35$; panel B; $p \leq 0.05$).

Figure 7.27 Ratios of the proportion of thyroid-associated injected dose for fucoidin pre-treated versus control Sprague Dawley rats injected with $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 60 min, and (B) 30 min p.i.

The data represent means ($n = 4 \pm$ standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); $Z$ score, $p \leq 0.05$. 
7.3.4.3.3 Stomach

Like the thyroid, from 0-60 min after the injection of $^{123}$I-labelled complexes/proteins, the stomach showed essentially a continuous time-dependent accumulation of radioactivity (Figure 7.28). This probably resulted largely from the uptake of free $^{123}$I released into the blood following the intracellular degradation of the labelled complexes/proteins in other tissues. At 60 min p.i., the proportion of the injected dose associated with the stomach was significantly different between some individual complexes/proteins ($F(4,15) =10.53; p \leq 0.0003$). Specifically, the proportion of the injected dose in the stomach of animals injected with $^{123}$I-labelled FGN or HMW CLU-FGN was significantly less than that in animals injected with $^{123}$I-labelled CLU, CS or HMW CLU-CS (Tukey HSD, $p \leq 0.05$).

![Figure 7.28](image)

**Figure 7.28** Percentage of the injected dose/g stomach in Sprague Dawley rats after injection with $^{123}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins.

Panels shows the stomach-associated activity up to 60 min after animals were injected with $^{123}$I-labelled (A) HMW CLU-CS, CLU or CS, or (B) HMW CLU-FGN, CLU or FGN. Data points represent means ($n = 4 \pm$ standard deviation) and are corrected for any radioactivity remaining in the tail.
In animals injected with $^{125}$I-labelled HMW CLU-CS or CLU (but not CS), at 60 min p.i., fucoidin pre-treatment significantly reduced the proportion of the injected dose associated with the stomach (Figure 7.29; $Z_{CLU} = -1.96$, $Z_{CS} = -1.64$, $Z_{HMW CLU-CS} = -2.24$; $p \leq 0.05$). Furthermore, at 30 min p.i., fucoidin pre-treatment significantly reduced the proportion of the injected dose associated with the stomach of animals injected with $^{125}$I-HMW CLU-FGN but not $^{125}$I-CLU or $^{125}$I-FGN ($Z_{CLU} = -0.002$, $Z_{FGN} = -0.97$, $Z_{HMW CLU-FGN} = -3.03$; $p \leq 0.05$).

![Figure 7.29](image)

**Figure 7.29** Ratios of the proportion of stomach-associated injected dose for fucoidin pre-treated versus control Sprague Dawley rats injected with $^{125}$I-labelled HMW CLU-stressed protein complexes or uncomplexed control proteins at (A) 60 min, and (B) 30 min p.i. The data represent means ($n = 4 \pm$ standard deviation). Blue diamonds indicate instances where fucoidin had a significant effect and red diamonds indicate instances where it did not (i.e. ratio close to 1); $Z$ score, $p \leq 0.05$. 

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7.4 **Discussion**

7.4.1 **SPECT imaging**

SPECT imaging and a preliminary biodistribution study were necessary to design the final biodistribution study, from which a great deal of information was gathered. The initial time points for SPECT imaging and biodistribution (between 45 min - 48 h p.i.) were chosen because in rats, intravenously injected plasma proteins may have plasma half-lives that extend to several days (Campbell *et al.*, 1956; Morgan, 1968; Sell, 1974); however, it is common for normally intracellular enzymes to display much shorter half-lives (Bijsterbosch *et al.*, 1981; Bijsterbosch *et al.*, 1985). Using only two-dimensional SPECT imaging it was very difficult to localize radioactivity to individual organs that are physically adjacent. For example, the upper abdominal region where all radiolabelled proteins appeared to accumulate was in the approximate location of the liver, but the liver overlays the spleen, pancreas, stomach and to some degree the kidneys. Therefore, on the basis of the SPECT imaging analyses alone, it could only be reported (with any degree of confidence) that the radiolabelled proteins were localized to organs within this region within 45 min p.i. The steadily increasing radioactivity in the thyroid during the first 6 h of the study (Figures 7.3-7.7; panel B) suggested that the $^{123}$I-labelled complexes/proteins were being intracellularly degraded in other tissues to release free $^{123}$I or $^{123}$I-tyrosine derivatives into the blood which were subsequently sequestered by the thyroid.

7.4.2 **Preliminary biodistribution study**

As for *in vitro* analysis of cell surface binding (see Chapter 6), kinetic analysis of the molar rate of clearance for blood-borne HMW CLU-stressed protein complexes was not possible due to the fact that the precise molecular weight of the complexes was unknown. Furthermore, variation in the specific activity of the labelled product after individual protein labelling reactions complicated comparisons of the uptake of HMW CLU-stressed protein complexes and the uncomplexed control proteins on a per unit mass basis. Consequently, the percentage of the injected dose, which was standardized between the different treatments, was used to analyse the biodistribution of the labelled complexes/proteins.
In preliminary studies, at 1 h p.i., around 95% of the injected dose of $^{123}$I-HMW CLU-stressed protein complexes had been cleared from the blood (Figure 7.9) - this provided the rationale for investigating the biodistribution of HMW CLU-stressed protein complexes after much shorter intervals p.i. Moreover, tissue associated radioactivity in the liver, spleen and kidney was seen to decrease with time for the duration of the 1 h experiment. Collectively these results suggested that the uptake of the $^{123}$I-labelled complexes/proteins was largely complete by 1 h p.i. and that at points in time beyond this, changes in tissue-associated radioactivity were due to intracellular degradation of $^{123}$I-labelled complexes/proteins and the subsequent release of free $^{123}$I or iodinated metabolites into the blood. On the basis of this limited data, it appeared that compared to CLU or control proteins, a greater proportion of HMW CLU-stressed protein complexes were quickly localized to the liver and spleen. However, it was expected that investigations at earlier time points would yield more information about the relative efficiency with which different proteins and the HMW CLU-stressed protein complexes were cleared from the blood.

### 7.4.3 Blood-borne clearance of HMW CLU-stressed protein complexes

The nature of the effect of the very large physical size of CLU-stressed protein complexes on their clearance from the blood is unknown. Previous studies showed that covalent attachment of PEG to proteins (i) significantly increases their half-lives if the protein is routinely cleared by renal filtration (Knauf et al., 1988), and (ii) can increase their accumulation in the liver (Caliceti et al., 1999). The respective explanations put forward for these observations were that (i) the increase in size caused by the attachment of PEG meant that the derivatized proteins were excluded by the glomerular filter, and (ii) the increased size caused the derivatized proteins to travel more slowly though the liver increasing the efficiency of internalization at this site. It is important to note that these studies on the effect of size on the rate of clearance of molecules from the blood did not include PEG-protein conjugates with molecular weights within the range expected for HMW CLU-stressed protein complexes. The maximum effective sizes of PEG conjugates tested were $3.26 \times 10^5$ Da (Knauf et al., 1988) and $5.3 \times 10^5$ Da (Caliceti et al., 1999) compared to $\geq 4 \times 10^7$ Da for HMW CLU-stressed protein complexes. In any case, it appears that the very large physical size does not impede the clearance of HMW CLU-stressed protein complexes from the blood. For decades it had
been accepted that plasma protein catabolism was a random process occurring by first-order kinetics, but an increasing body of evidence supports the preferential clearance of aged or damaged proteins (Margineanu and Ghetie, 1981). This includes the reported enhanced clearance of proteins modified by gross denaturation, oxidation or conformation altering mutations (Benacerraf et al., 1957; Bocci et al., 1968; Iwao et al., 2006). Of previous studies, experiments involving colloidal albumin (which represents a different type of heat-denatured protein complex) may be useful in drawing some comparisons with the results of this study. Heat-denatured colloidal albumin is comprised of aggregates of uniform thickness (14-15 nm) and variable length (of the order of several hundred nm) with an estimated molecular weight in the millions of Daltons. The reported half-life of these in the blood of rats is 2 min, suggesting that their very large size does not impede their rapid clearance (Bijsterbosch et al., 1985). Clearance of colloidal albumin is predominantly via the liver, with a much smaller contribution by the spleen and kidney, and release of $^{131}$I follows degradation of the radiolabelled colloids (Bijsterbosch et al., 1985).

When the blood-borne clearance of $^{125}$I-labelled HMW CLU-stressed protein complexes was examined 5-60 min p.i., it was apparent that their clearance was indeed very rapid. Following injection of $^{125}$I-labelled HMW CLU-CS or HMW CLU-FGN, around 95% of the injected dose had been cleared from the blood within 5 min. Moreover, the radioactivity remaining in the blood did not subsequently significantly decrease, suggesting that internalization of radiolabelled proteins was effectively completed by 5 min p.i. Comparing animals injected with $^{125}$I-labelled HMW CLU-stressed protein complexes versus $^{125}$I-labelled control proteins, at 5 min p.i., a significantly lower proportion of the injected dose remained in the blood of animals injected with the complexes. This indicates that HMW CLU-stressed protein complexes were cleared from the blood more quickly than the uncomplexed control proteins. While, on the basis of the percentage of the injected dose, the clearance of $^{125}$I-labelled CLU, CS and FGN from the blood appeared to be less efficient than that of $^{125}$I-HMW CLU-stressed protein complexes, in all cases the clearance was rapid - this was especially the case for animals injected with $^{125}$I-FGN. Although at 5 min p.i. the proportion of the injected dose of $^{125}$I-FGN in the blood was greater than that for $^{125}$I-HMW CLU-FGN, the former did not decrease significantly with time, suggesting that clearance of $^{125}$I-FGN was largely complete within 5 min p.i. (Figure 7.13; panel B). In contrast, in animals
injected with $^{123}$I-labelled CLU or CS, there a time-dependent decrease in blood-borne radioactivity between 5-30 min p.i. suggesting that these radiolabelled proteins were being cleared more slowly (Figure 7.13; panel A). However, an accurate estimate of the plasma half-life was not possible for the $^{123}$I-labelled HMW CLU-stressed protein complexes or the uncomplexed control proteins given that at the earliest practical time point for which animals could be sacrificed (5 min p.i.) much greater than 50% of the injected dose had been cleared from circulation in all cases.

Reports in the literature suggest that plasma half-lives of intravenously injected proteins vary markedly. The reasons for this are not understood. Little work has focused on identifying whether proteins from foreign species are cleared differently to species-matched proteins, however, given the rapidity of their clearance from the bloodstream it is unlikely that immune responses played a significant role in the uptake of the radiolabelled proteins/complexes in this study. Even between studies of the same protein injected intravenously, comparison of reported half-lives may not always be appropriate since experimental design may influence the results (Bouma, 1982). Therefore, the choice of time points for measuring the remaining radioactivity in the bloodstream is of critical importance. Given that in this study the radioactivity remaining in the bloodstream was measured from the earliest possible time point p.i., and the methodology was consistent between the HMW CLU-stressed protein complexes and the uncomplexed controls, the differences measured are likely to accurately represent their relative rates of clearance. Moreover, the measurements of radioactivity remaining in the bloodstream were highly reproducible. While there are reports of intravenously injected proteins persisting in plasma for several hours or even days, it is not uncommon for other proteins to be cleared within a few minutes as in the case of albumin colloids (Bijsterbosch et al., 1985).

The rapidity of clearance of the $^{123}$I-labelled complexes/proteins complicates analysis of their biodistribution. Iodinated tyrosine derivatives and free $^{123}$I are not recycled by cells and as a result the label is usually sequestered in the thyroid or stomach or excreted in the urine following release after the radiolabelled molecules are internalized and degraded (Bouma, 1982). The results of this study suggest that the uptake of labelled complexes/proteins was quickly followed by their intracellular degradation and the release of the $^{123}$I label from the original organ of uptake. As over 95% of the injected
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dose appeared to be cleared from the bloodstream within 5 min p.i. it is possible that
greater tissue-associated radioactivity may have been seen at earlier time points and that
the radioactivity measured in the blood at 5 min p.i. is in fact partly comprised of free
$^{123}$I or $^{123}$I-labelled metabolites. This interpretation is supported by the high levels of
radioactivity measured at 5 min p.i. in the bladder of animals injected with $^{123}$I-FGN.
While measurements of tissue-associated radioactivity at 5 min p.i. (the earliest practical
time point for which results could be obtained) represent the best estimate of the overall
contribution of each organ to protein uptake in this experiment, in future studies,
amendment to the methodology to incorporate the use of lysosome inhibitors (to inhibit
breakdown of the radiolabelled proteins and subsequent release of the tracer) may allow
for more accurate estimates of the uptake of HMW CLU-stressed protein complexes.

7.4.4 Major organs of uptake

The liver was identified as the major organ responsible for the in vivo uptake of
$^{123}$I-labelled HMW CLU-stressed protein complexes. Liver kupffer cells, endothelial cells
and parenchymal cells are all known to have important roles in plasma protein clearance.
Non-specific pinocytosis by hepatocytes, also known as fluid-phase endocytosis, is
thought to contribute to the uptake and subsequent catabolism of all plasma proteins
(Hoffenberg et al., 1970; Bouma, 1982). Intuitively, the relative contribution of
pinocytosis will depend on the concentration of the protein in plasma and whether
more efficient specific uptake mechanisms exist to target the protein for clearance. Since
the clearance of all radiolabelled proteins in this study was rapid, it is unlikely that
pinocytosis into hepatic cells was a major contributor to their clearance from the
circulation. Liver cells are known to express many receptors with important roles in
targeting plasma proteins for clearance. This includes certain members of the LDL
receptor superfamily and several SRs, which are all capable of binding and facilitating
the subsequent degradation of plasma proteins. Pre-treatment with fucoidin inhibited
the uptake of all radiolabelled proteins in this study (Figure 7.15). This suggests that SRs
were involved in the clearance of these both radiolabelled proteins and HMW
CLU-stressed protein complexes, however, this does not exclude the potential
involvement of other receptors.
Fucoidin is an inhibitor of class A SRs (Platt et al., 1996), LOX-1 (Oka et al., 1998), SREC-1 (Berwin et al., 2004) but not class B SRs (Acton et al., 1994). Given that the effect of fucoidin (to inhibit clearance of 125I-labelled complexes/proteins from the blood) was most evident at 5 min p.i., this suggests that the uptake and degradation of fucoidin was rapid (Figure 7.15, Figure 7.19 and Figure 7.21). The results indicate that the uptake of uncomplexed control proteins also involve fucoidin-inhibitable mechanisms. Whether or not these mechanisms are the same as those already described for damaged or modified ligands such as oxLDL, modified albumin and AGE remains to be confirmed. A possible explanation for reduced uptake of the radiolabelled proteins may be that fucoidin interacts with cell surface receptors other than SRs. Fucoidin is known to bind L-selectin and as such interferes with leukocyte rolling (Yednock et al., 1987; Kansas et al., 1991; Ley et al., 1993; Granert et al., 1994). Additionally, fucoidin inhibits phagocytosis of apoptotic leukocytes by endothelial cells where the expression of (fucoidin-inhibitable) SRs could not be detected (Johnson et al., 2003). However, SRs bind a diverse array of polyanionic ligands and as such, specific binding of CLU, CS or FGN (all negatively charged at physiological pH) to SRs is possible. As the constituents of the complexes are all anionic at physiological pH it is likely that the complexes are also anionic under these conditions. Given that HMW CLU-stressed protein complexes are very large but remain in solution it is fair to assume that a significant portion of the exposed surface is covered by charged residues that may also play a role in preferentially targeting the complexes to SRs.

The percentage of the injected dose per gram of tissue was higher in the liver than the spleen at 5 min p.i. (Figure 7.18 and Figure 7.20). Given that the mass of the liver is about 19 times greater than that of the spleen the total contribution of the liver towards uptake of the radiolabelled proteins was much greater than that of the spleen. However, on a per gram basis, the percentage of injected dose accumulated by the spleen was much higher than for other organs (excluding the liver), suggesting that the spleen may have specific mechanisms for the uptake of HMW CLU-stressed protein complexes. Supporting this idea, pre-treatment with fucoidin significant inhibited the uptake of the radiolabelled ligands into the spleen (Figure 7.21). The spleen has a large population of resident macrophages that are known to express class A SRs (Hughes et al., 1995; van der Laan et al., 1999). It is therefore feasible that the fucoidin inhibitable accumulation
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of radioactivity into the spleen was due to clearance of the $^{123}$I-labelled proteins and HMW CLU-stressed protein complexes by these resident macrophages.

Given that the glomerular filter exclusion limit is around 50 kDa, the results obtained confirmed the expectation that the kidneys would not be a primary site of accumulation for $^{123}$I-labelled HMW CLU-stressed proteins complexes. While the kidney did appear to play a role in the clearance of CLU and CS the uptake of these proteins into the liver was considerably more. Compared to the other $^{123}$I-labelled proteins studied, a greater proportion of the injected dose accumulated in the kidneys of animals injected with $^{123}$I-FGN (Figure 7.22; panel B). The accumulation of radiolabelled FGN in the kidneys after intravenous injection in rats was previously reported (Dawiskiba, 1996). While the large molecular weight of FGN (340 kDa) and net negative charge (pI 5.5) suggests that FGN would not freely permeate the glomerular filter, it becomes concentrated in the kidney subendothelium where it has a role in limiting the access of other plasma proteins to the basement membrane (Masri et al., 1985). The progressive time-dependent decrease in the kidney-associated radioactivity of animals injected with $^{123}$I-FGN (Figure 7.22; panel B) suggests that uptake into the kidney is followed by intracellular degradation of the radiolabelled protein.

7.4.5 Other organs

Like the liver and spleen the lungs are a reticuloendothelial rich organ and contain abundant tissue-associated macrophages. Clearly the mechanism(s) responsible for the uptake of radioactivity into the lungs are different to those of the liver and spleen since pre-injection of fucoidin increased the accumulation of radioactivity in lung tissue 5 min p.i. (whereas fucoidin had an inhibitory effect on the uptake of radioactivity into the liver and spleen). Measurements of the clearance of radioactivity from the blood suggest that in the absence of fucoidin the liver rapidly and efficiently removes HMW CLU-stressed protein complexes from circulation. It is possible that the greater accumulation of radioactivity in the lungs after pre-injection with fucoidin may be accounted for by increased trapping of the HMW CLU-stressed protein complexes in lung tissue as a direct result of their prolonged circulation in the bloodstream. Macroaggregates of protein (10-100 nm), including labelled albumin colloids, are used in lung perfusion scintigraphy because of their propensity to become trapped in the lung
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pulmonary arteriolar bed. Given that the physical sizes of the radiolabelled proteins used in this study fall within this range (Figure 5.3) it is possible that a portion of the radioactivity measured in the lung was due to entrapment of the radiolabelled protein in the capillaries of this highly vascularised organ. However, specific mechanisms for the uptake of the radiolabelled proteins in the lung can not be discounted based on the data obtained, since pre-injection with fucoidin had comparable effects on the uptake of the uncomplexed native proteins and the HMW CLU-stressed proteins complexes. If size-related non-specific trapping was the sole cause of increased tissue accumulation then the effect of fucoidin would be expected to be biased towards the trapping of HMW CLU-stressed protein complexes due to their much larger size compared to the uncomplexed control proteins.

7.4.5.1 Uptake of free $^{123}$I and iodinated metabolites

The sustained time-dependent accumulation of radioactivity measured in the stomach and thyroid of rats injected with $^{123}$I-labelled complexes/proteins (Figure 7.26 and Figure 7.28) is consistent with their rapid uptake and intracellular degradation in other organs followed by (i) the release into the blood of free $^{123}$I and small $^{123}$I-labelled molecules, and then (ii) the accumulation of the latter by the stomach and thyroid. The thyroid and stomach both concentrate iodine via iodide pumps, however the stomach iodide pump is phylogenetically more primitive and has a lower affinity for iodide (Venturi and Venturi, 1999). It is therefore unsurprising that both organs showed time-dependent increases in tissue-associated radioactivity but that the thyroid accumulated markedly more radioactivity than the stomach.

7.4.6 Summary

In summary, results of SPECT imaging and biodistribution show that $^{123}$I-labelled HMW CLU-stressed protein complexes are rapidly cleared from the bloodstream with a half-life of < 5 min. Furthermore, in terms of the percentage of the injected dose, the clearance of protein in HMW CLU-stressed protein complexes is more efficient than that of $^{123}$I-labelled CLU or the uncomplexed control proteins. The liver was found to be the primary organ responsible for the uptake of $^{123}$I-labelled HMW CLU-stressed protein complexes, while the spleen appeared to play a lesser role. For both organs,
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fucoidin-inhibitable mechanisms of uptake are implicated and the uptake of $^{125}\text{I}$-labelled HMW CLU-stressed protein complexes appears likely to be followed by rapid intracellular degradation as indicated by the time-dependent accumulation of radioactivity in the thyroid and stomach. While the identity of the precise receptor(s) involved remains to be elucidated, these results suggest that HMW CLU-stressed protein complexes are recognized by fucoidin-inhibitable SRs that facilitate their uptake and disposal. The persistence of CLU in the bloodstream for longer than HMW CLU-stressed protein complexes suggests that the role of CLU may be limited to stabilizing unfolding proteins in large soluble complexes and that some inherent characteristic of the complexes, whether it be size or disruption of the native secondary structure, of either the stressed client protein, CLU or both targets the complex for clearance. However, it is also possible that binding to stressed client proteins exposes a conformationally-sensitive site on CLU which binds to an endocytic receptor. Further characterization of HMW CLU-stressed protein complex receptor interactions will be important in increasing our understanding of mechanisms of extracellular protein folding quality control. Regardless of the precise mechanism targeting HMW CLU-stressed protein complexes for clearance, the results of this study strongly support the model presented in Chapter 1 in which complexation with CLU targets misfolded extracellular proteins to endocytic receptors (predominately in the liver) where they are internalized and degraded (Figure 1.6).
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8 CONCLUSIONS

Like their intracellular counterparts, extracellular chaperones including CLU are likely to be important components of systems for protein folding quality control. In the absence of any known specific mechanisms for the degradation of non-native extracellular proteins, rescue by chaperones and subsequent intracellular degradation is a likely route for their clearance and to prevent their potentially pathological accumulation. The work presented here strongly supports a model for the disposal of stressed proteins outlined in the introductory chapter of this thesis (Figure 1.6). In this model the stabilization of stressed proteins via the formation of complexes with CLU is an important first step. Following complex formation it appears that CLU-stressed protein complexes are targeted to scavenger-like fucoxanthin inhibitable receptor(s). In vitro studies suggest that receptors of this nature are present on numerous cell types including peripheral monocytes, cells of yolk sac lineage and hepatocytes. However, at least in a rat model, the clearance of blood-borne HMW CLU-stressed protein complexes in vivo is largely the role of the liver. Cellular internalization of HMW CLU-stressed protein complexes is followed by lysosomal degradation according to the classical route of endocytosis.

The inherent difficulties involved in the formation and purification of CLU-stressed protein complexes in vitro have previously been discussed (see Section 3.4). It is expected that in vivo protein unfolding results from the combined effects of numerous different stresses, including shear, heat and oxidation all of which may be intermittently more severe. Moreover, compared to low concentrations of purified protein in buffered solutions, protein unfolding in complicated biological fluids may be influenced by factors such as macromolecular crowding. Given the complexity of the extracellular environment in vivo, precise replication of these conditions in vitro is virtually impossible. It is therefore common practice to use a single stress at supraphysiological levels (or in some cases chemical denaturing agents) to induce protein unfolding. Systematic testing found that several structurally unrelated proteins unfold at near physiological temperatures, although their precipitation was far more rapid at higher temperatures. The events of unfolding are the same regardless of the temperature (Day et al., 2002), thus the use of higher temperatures to induce protein unfolding is largely a matter of experimental convenience. However, changes in the secondary structure of CLU, including an increase in disordered content at physiologically relevant temperatures...
(between 37-41°C) as determined by CD analysis, could be important in CLU’s activity \textit{in vivo} (Figure 3.13). Further experiments should be aimed at identifying the importance of these observed structural changes. The formation of complexes with a HMW in the range of \(\geq 4 \times 10^7\) Da was more common at supraphysiological temperatures, however, it was important to identify whether HMW CLU-stressed protein complex formation was achievable at physiologically possible temperatures given that it was only possible to purify complexes of this size for use in further experiments. Dot blot analysis of shear stressed human plasma suggested that HMW complexes in the range of \(\geq 4 \times 10^7\) Da were formed at 37°C (Figure 4.16) and HMW CLU-stressed CS complexes were also formed in buffered solution at 41°C (Figure 3.7). Importantly, when HMW CLU-CS complexes formed at the physiologically relevant temperature of 41°C were compared with HMW CLU-stressed protein complexes formed at higher non-physiological temperatures it appeared that the physical properties of these complexes were largely similar. HMW complexes of CLU-CS (formed at 41°C), CLU-FGN (formed at 45°C) and CLU-GST (formed at 60°C) displayed remarkable consistency in terms of having an amorphous morphology, mass ratio of CLU to stressed client protein of 1:2, and exposed hydrophobicity less than or approximately equal to that of the uncomplexed control proteins. While HMW CLU-CS and HMW CLU-GST had a similar estimated physical size (~ 50 nm in diameter), HMW CLU-FGN was approximately twice the diameter of the other two complexes. This may be due to FGN (340 kDa) having an individual size much greater than the two other client proteins examined. The shared characteristics of HMW CLU-stressed protein complexes suggests that regardless of the temperature used to induce protein precipitation the complexes formed will be comparable, however, the overall physical size of the complex may vary according to the client protein. Investigating the complexes formed by a larger number of CLU client proteins will be needed to definitively confirm this conclusion.

Using a sandwich ELISA technique, several abundant plasma proteins displayed increased association with CLU after plasma was incubated at 37°C under mild shear stress or under oxidative stress compared to static storage at 4°C (Figure 4.11). Given that CLU recognizes stressed proteins via exposed regions of hydrophobicity and that this is a characteristic shared by unfolding proteins, it quite possible that complexes formed between CLU and stressed proteins in plasma may contain more than one client protein. Moreover, the increased association of CLU with the two other known
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extracellular chaperones, αM and Hp (after treatment of the plasma using shear stress), suggests that these proteins may work together to stabilize unfolding proteins as a result of environmental stress, perhaps forming a diverse array of heterogeneous complexes under these conditions. FGN was selected as a representative putative endogenous client protein and used to generate HMW CLU-FGN complexes in vitro. In plasma incubated at 37°C under mild shear stress for 10 days (but not in control plasma stored static at 4°C), both CLU and FGN were detected as HMW species (in the range of \( \geq 4 \times 10^7 \) Da) using dot blot analysis (Figure 4.16). The inability to form HMW CLU-FGN complexes when the purified proteins were incubated in buffered solution at temperatures less than 45°C is probably due, at least in part, to the lack of macromolecular crowding in vitro versus in plasma. In the future, it will be important to further confirm the identity of endogenous plasma client proteins for CLU. This will be particularly significant if the confirmed client proteins included examples with known disease relevance, such as FGN. While the ELISA method used in this study indicated increased association of specific plasma proteins with CLU after exposure to stress, confirmation of these associations by an independent approach would be useful. One such approach is to use mass spectrometry to identify proteins co-purifying with CLU from stressed biological fluids. FGN was identified as a client protein for CLU by C. McKay and J. A. Aquilina (University of Wollongong, Australia) using this technique - however, in that study the treated plasma was incubated at 43°C, slightly higher than those temperatures expected in vivo. Ideally, this should be repeated, stressing plasma at (physiologically relevant) 41°C. This was commenced in the current study, however only 1D-SDS-PAGE was performed, which was probably insufficient for mass spectrometry identification of individual proteins co-purifying with CLU. Although Western blot analysis indicated that FGN co-purified with CLU from plasma after shear-stress, mass spectrometry analyses of protein spots eluted from 2D-SDS PAGE gels should be used in future to provide definitive identification of endogenous CLU client proteins co-purifying with CLU from stressed plasma.

It would be desirable to purify CLU-stressed protein complexes directly from biological fluids for use in experiments, however, it is likely that after they are formed in vivo they are quickly taken up by tissues and degraded, limiting their availability in freshly obtained body fluid samples. Furthermore, using immunoaffinity chromatography to purify CLU and co-purifying proteins from stressed plasma of necessity involves a harsh
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denaturing step to elute bound proteins from the column - this could potentially disrupt CLU-stressed protein complexes. Thus, obtaining CLU-stressed protein complexes generated in vivo or in situ in collected plasma is impractical. Therefore, complexes were generated in vitro using purified proteins. Where possible, physiologically relevant temperatures were used to form complexes in vitro, and physiological conditions of pH and ionic strength. However, other aspects of conditions encountered in vivo were not thoroughly addressed e.g. macromolecular crowding. While it was obvious that macromolecular crowding caused FGN to precipitate even at subphysiological temperatures, it was not possible to purify CLU-stressed FGN complexes using this approach (Figure 4.17). Further experiments manipulating both temperature and the concentration of macromolecular crowding agents used may identify physiologically relevant conditions under which HMW CLU-FGN complexes can be formed. This could also be a useful approach to develop physiologically relevant systems to form and purify HMW CLU-stressed protein complexes using other endogenous client proteins.

Surface plasmon resonance suggested that while CLU is a ligand of megalin, HMW CLU-stressed protein complexes are only poorly recognized or not recognized by this receptor. Taken together with the results of in vitro cell binding experiments (see Section 6.3), these results suggest that LDL receptors are unlikely to play a significant role in the disposal of similar complexes in vivo. However, the preferential binding of HMW CLU-stressed protein complexes to peripheral monocytes, BN rat yolk sac cells and rat hepatocytes, suggest that they are recognized by specific fucoidin-inhibitable cell surface receptor(s) (see Section 6.3.2). Perplexingly, fucoidin-inhibitable SRs are not known to be expressed by BN cells or mammalian hepatocytes. The identification of the precise receptors involved in the uptake of HMW CLU-stressed protein complexes will be important to better define the details of the model proposed in this thesis for extracellular protein folding quality control. Potential approaches include screening candidate hepatic receptors for their possible interaction with HMW CLU-stressed protein complexes using a protein binding microarray, surface plasmon resonance or binding assays using transfected cell lines. Considering that fucoidin inhabitable SRs display the unique ability to recognize damaged or modified ligands and not their native counter parts, it is feasible that a shared physical characteristic of the stressed proteins in HMW CLU-stressed protein complexes targets them to these receptors for subsequent internalization and lysosomal degradation. This is supported by the consistency of the
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results obtained using different HMW CLU-stressed protein complexes in the various cell binding experiments (e.g. preferential binding compared to the uncomplexed control proteins and a high degree of inhibition by fucoidin regardless of the client protein used to form the complex). However, the possibility that a receptor binding site is exposed on CLU as a result of conformational changes during the formation of complexes cannot be excluded.

HMW CLU-stressed protein complexes intravenously injected into Sprague Dawley rats were rapidly cleared from the blood-stream predominantly via the liver and this mechanism was inhibited by pre-injection with fucoidin (see Section 7.3.4). The time-dependent decrease in liver-associated radioactivity and the corresponding increase in radioactivity in the body’s iodine sinks (thyroid and stomach) suggest that uptake in tissues was quickly followed by degradation of the HMW CLU-stressed protein complexes and release into the blood of the iodine label. In vitro, this was supported by the results of confocal microscopy which showed that HMW CLU-stressed protein complexes are internalized by isolated rat hepatocytes and transported to acidified compartments likely to include lysosomes (see Section 6.3.2.2). Degradation of HMW CLU-stressed protein complexes by lysosomes is also implied since incubation of hepatocytes with the lysosomal inhibitor chloroquine inhibited measurable proteolysis of the complexes (Figure 6.26). Taken together, the in vitro and in vivo studies described here strongly support that CLU may play an important role in the disposal of stressed proteins via receptor-mediated disposal. While the focus of this thesis is on the role of CLU in extracellular protein folding quality control, it is likely that other extracellular chaperones (e.g. α₂M and Hp) may play similar roles. Other yet to be identified extracellular chaperones may also be involved.

A large number of serious human diseases are characterized by the deposition of extracellular protein aggregates. The discovery of extracellular chaperones presents an exciting avenue for the development of strategies to combat these diseases. However, these strategies are unlikely to be as simple as the upregulation of extracellular chaperone expression in affected individuals. Upregulation of CLU is known to be associated with cancer progression and protects cells from chemotherapy drugs such as paclitaxel (Park et al., 2008). Additionally, while α₂M protects LRP expressing cells from
Aβ toxicity, in the absence of LRP the extracellular chaperone is not protective (Fabrizi et al., 2001). Therefore, it appears that the pathways that clear aggregating species from extracellular spaces and not chaperones alone are needed to elicit a protective effect. Further characterization of the route(s) by which CLU-stressed protein complexes are disposed of will be very important to define the role of CLU in extracellular protein folding quality control and to develop new CLU-based therapies for treating extracellular PDDs (Wyatt et al., 2009a). It is hoped that the pioneering work described in this thesis will help draw attention to the importance of unravelling the mechanisms underpinning extracellular protein folding quality control.
9 REFERENCES


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References


APPENDIX 1.1

Publication:

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Publication:

APPENDIX 1.3

Publication:

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