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Structural and functional characterisation of the extracellular chaperone clusterin

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Structural and Functional Characterisation of the Extracellular Chaperone Clusterin

By

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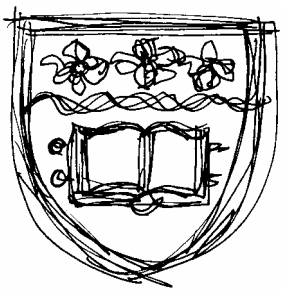

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Declaration of Authenticity

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 previously 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.

Elise Marie Stewart

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Yipee!!

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Abbreviations

AD	Alzheimer's disease
AFU	arbitrary fluorescence units
Apo E	apolipoprotein E
Ap^r	ampicillin resistance
ATP	adenosine triphosphate
Aβ protein	amyloid beta protein
BCA	bicinchoninic acid
bisANS	5, 5'-bis(8-anilino-1-naphthalenesulfonate)
BSA	bovine serum albumin
CD	circular dichroism
CHO	Chinese hamster ovary
CL	clusterin
CO_2	carbon dioxide
CS	citrate synthase
CSF	cerebrospinal fluid
DAB	diaminobenzidine
DG	deglycosylated
dH_2O	distilled water
DMEM: F-12	Dulbecco's modified Eagles medium: Hams F-12
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERAD	ER associated degradation
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	relative centrifugal force 9.8 $m.s^{-2}$
GST	glutathione S transferase
HBS	Hank's buffered saline
HDC	heat denatured casein
HMW	high molecular weight
HRP	horseradish peroxidase
IAA	iodoacetic acid

K_d	dissociation constant
Km^r	kanamycin resistance
LB	Luria-Bertani
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LMW	low molecular weight
MRE	mean residue ellipticity
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MTOC	microtubule organising centre
NFT	neurofibrillary tangle
PBS	phosphate buffered saline
PCD	protein conformational disorder
PCR	polymerase chain reaction
PD	Parkinson's disease
PF	protein free
PI	propidium iodide
ROS	reactive oxygen species
rpm	revolutions per minute
SAP	serum amyloid P component
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
SEC	size exclusion chromatography
sHSP	small heat shock protein
SPR	surface plasmon resonance
TAE	tris acetate EDTA
TE	tris EDTA
TFE	trifluoroethanol
TTR	transthyretin
U	units
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
WT	wild type
βMe	beta mercaptoethanol

Publications and Conference Presentations

Publications

Stewart, E.M., Aquilina, A., Easterbrook-Smith, S.B., Murphy-Durland, D., Jacobsen, C., Moestrup, S., Wilson, M.R., (2007). Effects of Glycosylation on the Structure and Function of the Extracellular Chaperone Clusterin. *Biochemistry*. 46(5):1412-22.

Kumita, J.R., Poon, S., Caddy, G.L., Hagan, C.L., Dumoulin, M., Yerbury, J.J., Stewart, E.M., Robinson, C.V., Wilson, M.R., Dobson, C.M., (2007). The Extracellular Chaperone Clusterin Potently Inhibits Human Lysozyme Amyloid Formation by Interacting with Prefibrillar Species. *Journal of Molecular Biology*. 369(1):157-67.

Yerbury, J.J., Stewart, E.M., Wyatt, A.R., Wilson, M.R., (2005). Quality Control of Protein Folding in Extracellular Space. *EMBO Reports.* 6(12):1131-6.

Conference Presentations

Stewart, E.M., Wyatt, A.R., Wilson, M.R. Probing the Structure of Clusterin and its Interaction with Megalin. *4th Workshop on Clusterin/ApoJ 2005*, Villars-sur-Ollon, Switzerland (Oral Presentation).

Stewart, E.M., Poon, S., Vine, K.L., Yerbury, J.J., Jones, R.A., Wilson, M.R. The Human Extracellular Chaperone Clusterin: How Does it Work? *29th Annual Lorne Conference on Protein Structure and Function 2004*, Lorne, Australia (Poster Presentation).

Stewart, E.M., Poon, S., Vine, K.L., Yerbury, J.J., Jones, R.A., Wilson, M.R. The Human Extracellular Chaperone Clusterin: How Does it Work?. *ComBio 2003*, Melbourne, Australia (Poster Presentation).

Yerbury, J.J., Stewart, E.M., Wyatt, A.R. Understanding Alzheimer's Disease: Protein Quality Control in the Extracellular Space. *Higher Degree Research Student Conference 2005*, Wollongong, Australia (Poster Presentation).

Abstract

The incidence of protein conformational disorders is a problem affecting the aging population and is an increasing cost on the health system. These diseases involve the breakdown of control mechanisms within and outside of cells leading to accumulation of unfolded and misfolded proteins. The control of protein folding, both inside and outside the cell, is important to maintain the integrity of all proteins. Dysfunctions in this control may lead to the development of protein conformational disorders.

Intracellularly the mechanisms monitoring the correct translation, modification, assembly and transport of synthesised proteins are very well studied. However, in the harsh extracellular environment, mechanisms to control protein folding need further investigation. Extracellular chaperones have recently been proposed as an important part of extracellular quality control for protein folding.

The ubiquitous glycoprotein clusterin has been implicated in this extracellular quality control mechanism. A primary aim of the work described in this thesis was to examine the relationship between clusterin structure and function. This was done using three different approaches; characterisation of mutant clusterin expressed in transfected CHO cells; characterisation of enzymatically deglycosylated clusterin; and characterisation of bacterially expressed recombinant clusterin domains.

Initially a panel of mutants was designed to disrupt predicted features of the protein, consisting of five point mutants and five truncation mutants. The clusterin molecules were expressed in CHO cells and were proteolytically processed with two subunits and appeared to be normally glycosylated. The mutant clusterin molecules appeared to be chaperone-active to some degree also, evidenced by their copurification with non-

specific media proteins. In a rolling culture proteins in the media would be exposed to sheer stress and one of the actions of clusterin is to bind to exposed hydrophobicity on stressed proteins. A decrease in the amount of contaminating proteins copurified with clusterin when static cultures were used instead of rolling cultures supports this argument.

In an effort to understand the importance of the glyscosylation of clusterin, the conjugated sugars were enzymatically removed to determine their effects on clusterin function. The success of the deglycosylation process was first verified using SDS-PAGE and mass spectrometry. Structural features of the deglycosylated molecule were tested, with no change seen in the secondary structure of the deglycosylated protein compared to the native protein. A shift to slightly acidic pH produced no change in secondary structure as assessed by circular dichroism spectroscopy. Exposed hydrophobicity was seen to increase with the removal of the sugars but the K_d of the interaction with bisANS was not significantly different. At slightly acidic pH both the wild type and deglycosylated molecules exposed a greater amount of hydrophobicity to solution.

Binding to the known clusterin ligand megalin was also tested with a similar binding affinity seen for both molecules, however the removal of sugars increased the rate at which the molecule bound to and dissociated from megalin. Binding of the deglycosylated molecule to known native ligands (IgG and GST) and stressed protein ligands (lysozyme and BSA) was not affected by the absence of sugar moieties. The chaperone activity of the deglycosylated molecule was also not different to the wild type molecule.

Following the determination that the sugars appeared to have little effect on the activity of clusterin, a series of domains was cloned into bacterial expression vectors. Two

domains (αN: D23-E112 and βC: R306-E449) were purified and their structure and function was examined. In the presence of a polar solvent both domains exhibited alpha helical secondary structure as expected based on amino acid analysis, though in aqueous buffer the domains had very little or no significant structure. The domains alone did not have any effect on the heat stressed precipitation of citrate synthase. Neither domain alone bound to heat-stressed lysozyme, however the αN domain bound to the native protein ligand IgG and this was enhanced at slightly acidic pH, as seen with native clusterin. The αN domain also bound with high affinity to LRP indicating that this domain of clusterin is likely to be involved in binding interactions of the entire molecule. Interaction between the αN and βC domains was also observed and further investigation is required to determine binding and chaperone activity of the interacting domains.

Understanding the functional relationship between the structure and function of clusterin is important in understanding the role of clusterin in protein folding control. This knowledge may also be important in the development of novel treatments for the prevention or slowing of protein conformational diseases. The work in this manuscript showed that the deglycosylated form of clusterin is as potent a chaperone as the entire clusterin molecule. This indicated that a bacterially expressed form of clusterin may be used to further investigate the function of the molecule. Recombinant domains of clusterin were also studied to localise the regions of the molecule important in clusterin binding and chaperone function. Further investigation is needed, however the use of bacterially expressed recombinant proteins allows the potential development of recombinant chaperone-active clusterin which may ultimately be used as a therapeutic for protein conformational diseases. This research has gone some way to showing the best methods of characterising the structural relevance to the binding and chaperone activity of clusterin, and to providing a knowledge base for future investigations.