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***Structure and Function of the
Mammalian Small Heat Shock
Protein Hsp25***

Amie Michelle Morris, B. Sc. (Hons)

A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy

School of Biological Sciences
and Department of Chemistry
University of Wollongong
Wollongong, Australia



2007

Declaration

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.

Amie Morris

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List of Abbreviations

ADH	alcohol dehydrogenase
ANS	1-anilino-8-naphthalene sulphonate
Ap	ampicillin
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CD	circular dichroism
crys	crystallin
cvHsp	cardiovascular Hsp
D ₂ O	deuterium oxide
DEAE	diethylaminoethyl
DMPK	dystrophia myotonica-protein kinase
DMSO	dimethylsulphoxide
DNase I	deoxyribonuclease I
DRM	desmin-related myopathy
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
FPLC	fast protein liquid chromatography
Hsp	heat shock protein
HSQC	heteronuclear single-quantum coherence
IPTG	isopropyl- β -D-thiogalactosidase
Km	kanamycin
LB medium	Luria-Bertani medium
MG	molten globule
MS	mass spectrometry
NanoESI	nanoscale electrospray ionisation

NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
NOESY	nuclear overhauser effect spectroscopy
ODPF	outer dense fibre protein
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEI	polyethylenimine
PMSF	phenylmethylsulphonyl fluoride
SDS	sodium dodecyl sulphate
SEC	size-exclusion chromatography
sHsp	small heat shock protein
TEMED	N,N,N',N'-tetramethylethylenediamine
TOCSY	total correlation spectroscopy
Tris	tris(hydroxymethyl)aminomethane
WET	water suppression enhanced through T1 effects
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

List of Publications and Presentations

A.M. Morris, T.M. Treweek, J.A. Carver and M.J. Walker (2007) "Glutamic acid residues in the C-terminal extension of Hsp25 are critical for structural and functional integrity". *Under revision*.

A.M. Morris, M.J. Walker and J.A. Carver (2006) "Structure/function studies on the mammalian small heat shock protein Hsp25: the role of the C-terminal extension". Poster presentation at *Molecular Chaperones & the Heat Shock Response meeting, Cold Spring Harbor Laboratory, New York*.

A.M. Morris, J.A. Carver and M.J. Walker (2005) "The role of the C-terminal extension of the mammalian small heat shock protein Hsp25". Poster presentation at *Higher Degree Research Student Conference, University of Wollongong*.

A.M. Morris, J.A. Carver and M.J. Walker (2005) "The role of the C-terminal extension of the mammalian small heat shock protein Hsp25". Poster presentation at *The Lorne Conference on Protein Structure and Function*.

A.M. Morris, J.A. Carver and M.J. Walker (2004) "Structure and function studies on the mammalian small heat shock protein Hsp25". Poster presentation at *The Lorne Conference on Protein Structure and Function*.

T.M. Treweek, A.M. Morris and J.A. Carver (2003) "Intracellular protein unfolding and aggregation: The role of small heat-shock chaperone proteins". *Aust. J. Chem.* 56(5): 357-367.

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Abstract

Hsp25 is the murine equivalent of human Hsp27, both of which are members of the small heat shock protein (sHsp) family. sHsps are a group of intracellular molecular chaperones that protect unstable intermediates of cellular proteins from aggregation and precipitation. Hsp27 and other sHsps play a role in various neurodegenerative diseases such as Alzheimer's, Alexander's, Creutzfeld-Jakob and Parkinson's diseases. Crystallisation of mammalian sHsps has thus far not been achieved due to the polydisperse and dynamic nature of these proteins. As a result, the oligomeric structure of sHsps is unclear, hindering the elucidation of the functional mechanisms of these proteins.

In this study, a series of site-directed Hsp25 mutants was constructed, in which polar residues of the flexible region of the C-terminal extension were replaced with less polar residues. The C-terminal extension of sHsps is typically short and unstructured and is thought to play an important role in solubilising these proteins and the complexes they form with target proteins by counteracting the hydrophobicity of the remainder of the sHsp. The C-terminal extension is also implicated in interaction with target proteins.

Wildtype Hsp25 and various C-terminal extension mutants (E190A, R192A, Q194A, E199A, E204A, Q205A, K209L) were expressed and successfully purified. A truncation mutant, E190stop, was also constructed but became insoluble during the purification process, demonstrating the importance of the C-terminal extension in maintaining the stability of Hsp25. Wildtype and

mutant Hsp25 proteins were characterised structurally and functionally using a range of spectroscopic techniques, including far-UV circular dichroism spectroscopy, tryptophan and ANS binding fluorescence spectroscopy, size-exclusion fast protein liquid chromatography, nuclear magnetic resonance spectroscopy and chaperone assays under both reduction and heat stress conditions. These experiments enabled the identification of residues key to the chaperone ability of Hsp25.

The R192A and Q194A mutants were functionally indistinct from the wildtype protein and exhibited only minor alterations to their structure. It was therefore concluded that the R192 and Q194 residues are not vital for Hsp25 to function as a molecular chaperone.

Each of the glutamic acid residue mutants exhibited significant alterations in tertiary structure, with increases in exposure of hydrophobic regions compared with wildtype Hsp25, and a minor decrease in the oligomeric size of the E190A mutant was observed. Functionally, these mutants showed poor thermostability and disrupted chaperone function. Glutamic acid residues are abundantly present in proteins from thermophilic organisms and are implicated in the stability of these proteins at high temperatures. Replacement of each of the glutamic acid residues in the C-terminal extension of Hsp25 resulted in loss of solubility at elevated temperatures, indicating that these residues perform similar roles in both Hsp25 and thermophilic proteins. The increase in surface hydrophobicity may have contributed to the poor thermostability of these

mutants and also the inefficient capture of target proteins observed in the chaperone assays.

The tertiary and quaternary structures of the Q205A mutant were significantly perturbed and the function of this mutant was completely abolished under heat stress conditions. Alterations to the tertiary structure of the N-terminal domain were observed and oligomerisation was severely disrupted, with three distinct oligomeric forms being present: an oligomer larger than that of the wildtype protein and two smaller oligomers. The chaperone activity of this mutant was comparable to that of wildtype Hsp25 under reduction stress conditions, indicating that each of the oligomeric forms were functional. Under heat stress conditions, however, the Q205A mutant co-precipitated along with the target proteins. Flexibility of the mutated residue was considerably decreased, as assessed by NMR experiments, but the remainder of the C-terminal extension was not significantly altered. Together, these results lead to the conclusion that the Q205 residue is vital for the performance of Hsp25 as a molecular chaperone at elevated temperatures.

Mutation of the K209 residue also showed disruption to the oligomerisation of Hsp25, with the K209L mutant eluting as three peaks after size-exclusion chromatography. This mutant was functionally defective under reduction stress conditions but showed comparable chaperone activity to the wildtype at high temperature, suggesting that the smaller oligomeric species require temperature-induced structural alterations in order to acquire chaperone ability. Because full chaperone activity was observed under reduction stress conditions,

direct interactions between the C-terminal lysine residue of Hsp25 and target proteins is unlikely to be a requirement of chaperone activity.

The stabilisation of α B-crystallin by α A-crystallin is important for the maintenance of the structure and function of α B-crystallin in the eye lens, where these sHsps are present in a \sim 3:1 ratio. Outside the eye lens, however, α A-crystallin is found only in trace amounts in some tissues. Co-complexes between various sHsps have been observed *in vivo*, for example between α B-crystallin and Hsp27, and it has been proposed that one or more ubiquitous sHsps stabilise α B-crystallin in non-lenticular tissues. Whilst α B-crystallin was found to be unstable above \sim 69°C, Hsp25 remained almost completely in solution at 100°C. A 3:1 Hsp25: α B-crystallin mixture showed practically identical thermostability to the Hsp25 homo-oligomer and provides support for the role of this sHsp as a stabiliser of α B-crystallin. Chaperone activity assays of the 3:1 mixture show results closely resembling those of the Hsp25 homo-oligomer and demonstrate that interactions between the two sHsps result in an altered chaperone activity of α B-crystallin.

Significant insights into the structure and function of Hsp25 were gained in this study. Several residues in the C-terminal extension were identified as critical to the structural and functional integrity of this sHsp and analysis of the amino acid composition of the C-terminal extensions of sHsps from various organisms indicate that some of these residues may play similar roles in other sHsps.