2003

Structure/function studies of the alpha-crystallin small heat-shock chaperone proteins

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Structure/function studies of the α-crystallin small heat-shock chaperone proteins

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Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

Department of Chemistry and Department of Biological Sciences

University of Wollongong
Wollongong, AUSTRALIA

December, 2003
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.

Teresa Mary Treweek.
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Chapter 9: Conclusions and future directions
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<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<td>Ap</td>
<td>ampicillin</td>
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<td>A</td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-galactopyranoside</td>
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List of Publications


Abstract

α-Crystallin is a member of the small heat shock protein (sHsp) family which exists as a multimer of αA- and αB-crystallin subunits in the ratio of 3:1 in the lens, where it was first identified. It is an intracellular molecular chaperone, capable of interacting with a multitude of target proteins to prevent their aggregation and precipitation. Initially considered to be solely a lens protein, individual αA- and αB-crystallin proteins have since been found in other organs with αB-crystallin in particular appearing to play a role in many neurodegenerative disorders (e.g. Alzheimer’s, Parkinson’s and Creutzfeldt-Jakob diseases). Due to the dynamic nature of α-crystallin oligomers and the propensity for subunit exchange, crystallisation of the protein has been impossible. As a result, the mechanisms by which α-crystallin functions remain elusive, as does a complete picture of the chaperone’s quaternary structure.

In this study, recombinant human αA- and αB-crystallin were expressed and purified using conventional methods (Horwitz, J., Huang, Q-L., Ding, L. and Bova, M. P. (1998) Methods in Enzymology 290:363-383). A series of mutants of αA- and αB-crystallin were also constructed, with mutation sites concentrated in the C-terminal region of the protein and in particular the solvent-exposed and flexible C-terminal extension. This extension, which comprises 10 and 12 amino acids in human αA- and αB-crystallin, respectively, behaves in a similar manner to an unstructured peptide in solution. Previous NMR spectroscopic studies have indicated that it serves a crucial role in binding target proteins.

C-Terminal extension mutants K175L, K174A/K175A, E164A/E165A, I159A/I161A and R163STOP (αB-crystallin) and S172L, T168L and R163STOP mutants
(αA-crystallin) were produced and purified in the same manner as for the wildtype proteins. The expected masses of mutants were confirmed by electrospray mass spectrometry (ESI-MS). Complete purification, however, of S172L and R163STOP αA-crystallin was not achieved due to decreased aggregate size and excessive hydrophobicity, respectively. Purified wildtype and mutant proteins were structurally and functionally characterised using a variety of spectroscopic techniques. These included chaperone assays under both reduction and heat stress with insulin and βL-crystallin as target proteins, respectively, intrinsic tryptophan fluorescence spectroscopy, far- and near-UV circular dichroism (CD) spectroscopy, thermostability studies, size-exclusion high-performance liquid chromatography (HPLC), mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Many of the αB-crystallin mutants purified successfully and provided insight into specific amino acid residues that are important for the chaperone action of the protein. These include the “I-X-I motif” at the C-terminal end of the protein which is highly conserved throughout sHsps and is thought to be critical for oligomeric assembly. Mutation of both isoleucine residues in the αB-crystallin I-X-I motif to alanine resulted in a protein which formed larger oligomeric complexes than the wildtype protein. Truncation of the C-terminal extension of αB-crystallin resulted in a protein with severely impaired chaperone ability, increased tendency to aggregate and disrupted secondary, tertiary and quaternary structures. These results suggest that the polar and flexible C-terminal extension is also necessary for uniform oligomeric assembly as well as for the solubility of α-crystallin as a whole. Chaperone and thermostability studies on the double glutamic acid mutant (E164A/E165A) showed that these highly charged residues are critical to the solubility of αB-crystallin at higher temperature. Consistent with this, ion-pairs and the formation of salt bridges between charged amino acids on the surfaces of thermophilic proteins are
thought to be responsible for their increased thermostability. Recombinant human αA- and αB-crystallin were uniformly $^{15}$N-labelled for the purposes of 2D Nuclear Magnetic Resonance spectroscopy (NMR) studies. Measurement of $^{15}$N relaxation time constants ($T_1$ and $T_2$) and $^{15}$N Nuclear Overhauser Effects (NOEs) for both wildtype proteins and the K175L and I159/I161A mutants of αB-crystallin have provided detailed information on the relative flexibilities of residues in the protein's C-terminal extension. Substitution of a leucine residue for the C-terminal lysine (K175) increased extreme C-terminal mobility and substitution of the isoleucine pair of the I-X-I motif with alanine residues led to a disruption of flexibility throughout the C-terminal extension. $^{15}$N $T_1$ and $T_2$ and $^{15}$N NOE values were also determined for $^{15}$N-labelled αA-crystallin in the presence of reduced α-lactalbumin in order to gain information on changes in the flexibility of the C-terminal extension upon chaperone interaction with a stressed target protein. Upon formation of a chaperone-target protein complex, the flexibilities of C-terminal residues of αA-crystallin were equalised across the extension indicating that the entire extension was involved in interaction with the target protein to some extent.

The R120G αB-crystallin mutant, which is associated with desmin-related myopathy and cataract in humans was also expressed and purified for the purposes of further structural characterisation. Previous studies on this mutant have provided some ambiguous results with regard to its chaperone ability and general structural stability. It was found that in addition to being intrinsically unstable and susceptible to unfolding, R120G αB-crystallin underwent C-terminal proteolysis with time. Furthermore, R120G αB-crystallin exhibited marked substrate specificity and in fact, acted as an “anti-chaperone” in the presence of reduced α-lactalbumin. Under these conditions, R120G αB-crystallin promoted the aggregation of the molten globule state of α-lactalbumin and co-precipitated with it out of solution. This study, therefore provided several
insights into structural and functional aspects of α-crystallin small heat shock chaperone proteins.