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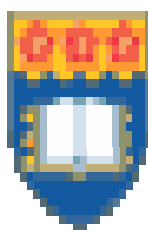
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The Chaperone Action of α -Crystallin

Arezou Ghahghaei, Master of Science in Chemistry

Submitted in fulfillment of the requirement
for Degree of Doctor of Philosophy



Department of Chemistry
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Wollongong, AUSTRALIA

March, 2006

Declaration of authenticity

This thesis is submitted in accordance with the regulation of the University of Wollongong in fulfillment 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 University.

Arezou Ghahghaei

March 2006

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List of abbreviation used

1D	one-dimensional
^1H	proton
α T-crystallin	purified α -crystallin containing α A and α B subunits
AIAS	4-acetamido-4'-((iodoacetyl) amino) stilbene-2, 2'-disulfonic acid
ANS	1-anilino-8-naphthalene sulfonic acid
CD	Circular Dichroism
DTT	dithiothreitol
D ₂ O	deuterium oxide
EDTA	ethylenediaminetetraacetic acid
FRET	Fluorescence resonance energy transfer
g	gram
KDa	kilodalton
L	litre
LYI	Lucifer yellow iodoacetamide
mg	milligram
μL	microlitre
mL	milliliter
mM	mili molar
μM	micro molar
min	minute
MW	molecular weight
NMR	nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect Spectroscopy

ppm	parts per million
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sHsp	small heat-shock proteins
TEM	transmission electron microscopy
ThT	Thioflavin T
TOCSY	Total correlation spectroscopy

Abstract

α -Crystallin is the principal lens protein. It is a member of the small heat shock protein family (sHsp) and acts as a molecular chaperone by stabilizing proteins under stress conditions through the formation of a soluble sHsp target protein complex to prevent their aggregation.

Macromolecular crowding is ubiquitous in all types of cells and describes the normal conditions inside a cell. The concentration of macromolecules inside a cell is very high (up to 300 mg/mL) arising from species such as polysaccharides, proteins and nucleic acids and therefore it greatly promotes the self-assembly of proteins. Thus, there is a major difference between *in vivo* and *in vitro* conditions such as those used in most studies of protein behaviour and properties.

Appropriate destabilizing conditions (i.e. heating or reduction) cause damage and misfolding of proteins, which as a result expose previously buried hydrophobic regions. Hydrophobic interactions of nearby molecules cause self-association and aggregate formation. Aggregation of intermediately folded peptide or protein molecules also leads to the formation of amyloid fibrils, highly-ordered β -sheet structures associated with a number of neuro-degenerative disorders such as Alzheimer's, Parkinson's, and Creutzfeldt-Jakob diseases.

In vitro, much work has been published on the interactions of α -crystallin with target proteins in dilute solutions. In order to better understand the chaperone activity of α -crystallin under conditions more closely resembling the intracellular environment, its interaction with a range of destabilized proteins (ovotransferrin, β_L -crystallin, insulin, α -lactalbumin, α_s - and κ -casein) in the presence of dextran (68 kDa) have been examined using visible absorption spectroscopy, tryptophan fluorescence spectroscopy, ANS binding, TEM, HPLC and NMR spectroscopy studies.

In the presence of dextran, the rate and extent of aggregation of reduced ovotransferrin, insulin, α -lactalbumin and β_L -crystallin was accelerated. Under these conditions, α -crystallin was less effective in preventing aggregation and precipitation of target proteins. It is proposed that a kinetic competition exists between aggregation of target proteins and the chaperone action of α -crystallin which supports the hypothesis that α -crystallin interacts more effectively with slowly aggregating rather than rapidly aggregating target proteins.

Amyloid fibril formation by α -lactalbumin, α_s - and κ -casein was verified by a sigmoidal increase in Thioflavin T fluorescence over time. α -Crystallin prevented amyloid formation in α_s - and κ -casein. In the presence of dextran, the rate of amyloid formation by α -lactalbumin, α_s - and κ -casein was enhanced. Under these conditions, α -crystallin was less effective in preventing amyloid formation of κ -casein and this was supported by TEM, CD, NMR spectroscopy and HPLC

studies.

Subunit exchange is an important feature of sHsp chaperone action. This study found that subunit exchange of α A-crystallin increased with increasing temperature and decreased as a result of interaction with reduced ovotransferrin. It was further demonstrated that the presence of the dextran markedly reduced the rate of subunit exchange of α A-crystallin and with increasing temperature, this effect was exacerbated. Moreover, in the presence of reduced ovotransferrin, dextran further slowed the subunit exchange of α A-crystallin.

Aggregation of β -lactoglobulin occurs mainly via intermolecular disulphide bond exchange. Upon heating, β -lactoglobulin aggregated which increased with increasing pH. The presence of dextran or DTT led to more rapid aggregation and precipitation of β -lactoglobulin. α -Crystallin prevented the aggregation of heat-stressed β -lactoglobulin and was a more efficient chaperone at higher pH values. In the presence of DTT, however, α -crystallin was a less efficient chaperone due to faster aggregation of heated and reduced β -lactoglobulin.

In order to obtain further structural and functional information on the C-terminal extension, α -crystallin from dogfish (*Squalus acanthias*) was studied to allow comparisons with bovine α -crystallin to be made. Chaperone assays under heat and reduction stresses as well as NMR spectroscopy showed that the C-terminal extension of dogfish α -crystallin was very flexible and had a similar

structure and function to that of bovine α -crystallin. Its chaperone action under heat stress was found to be comparable to bovine α -crystallin but it was a better chaperone under reduction stress.

Chapter 1

Introduction

1.1 Molecular Chaperones

Proteins are linear polymers synthesized by ribosomes from activated amino acids. The product of this biosynthetic process is a polypeptide chain, which has to adopt a unique three-dimensional structure required for its function in the cell (Nicholls et al. 1991, Gething et al. 1992, Haslbeck et al. 1999). Anfinsen et al. (1973) demonstrated that the information needed for a protein to reach its native conformation is encoded within its amino acid sequence. Protein folding is a process that leads from the linear polypeptide chain to a native structure. Proteins are known to reach their correct conformation from many different unfolded states. A large variety of folding and unfolding processes can be guided by specific proteins, molecular chaperones, throughout the life cycle of proteins, from their synthesis to their degradation (Mogk et al. 2002).

Molecular chaperones are a group of proteins that interact with unfolded or partially folded states of proteins (Ranson et al. 1998, Carver et al. 2001). In so doing, they prevent irreversible aggregation of non-native states and keep proteins on the productive folding pathway (Buchner et al. 1996). In the absence of chaperones, these unfolded proteins could mutually associate via exposed hydrophobic regions and precipitate out of the solution (Carver et al. 1995). Chaperones recognize these unfolded proteins and prevent their aggregation by formation of a complex with proteins and in the case of some chaperones e.g. GroEL by providing encapsulated hydrophobic environments that allow the proteins to fold correctly (Ellis et al. 1991).

Molecular chaperones are also called heat-shock proteins (Hsps) because under conditions of cellular stress, such as heat, denaturant, oxidation, infection, osmotic variation, etc. their expression is upregulated in order to minimize inappropriate interactions between destabilized proteins (Treweek et al. 2000). These types of stresses promote the unfolding of proteins, so that increased levels of molecular chaperones are required to interact with the large number of partially folded molecules (Treweek et al. 2003).

There are several structurally unrelated families of molecular chaperones (Kim et al. 1998, Treweek et al. 2003). The names of the chaperone families are derived from the molecular weight of their monomeric subunits e.g. Hsp70, Hsp60, Hsp90, Hsp100, Hsp16.5 (Mogk et al. 2002, Treweek et al. 2003). Amongst these molecular chaperones, Hsp60 and Hsp70 (*DnaJ* in *GroEL* and *DnaK* in *E. coli*), are intimately involved in regulating the correct folding of some proteins in the crowded environment of the cell, whilst another class of intracellular molecular chaperones, the small heat-shock proteins (sHsps), are not directly involved in protein folding. Instead, sHsps are the major group of protein chaperones associated with stabilizing other proteins under stress conditions to prevent their large-scale precipitation (Michel et al. 1997, Poon et al. 2002).

The Hsp60 and Hsp70 families constitute two ubiquitous chaperone systems which cooperate in the folding of newly synthesized polypeptides, as well as preventing protein aggregation under conditions of cellular stress (Heyrovska. 1997). Hsp70 chaperones assist a broad spectrum of protein-folding processes

in the cell, ranging from the folding and translocation of newly synthesized proteins to the disaggregation of aggregated protein deposits (Ellis et al 1999a and b, Laufen et al. 1999). These proteins share the ability to associate with extended linear segments of polypeptides in an ATP-dependent manner. ATP regulates the affinity of Hsp70 for substrates (Ellis et al 1999a and b, Laufen et al. 1999).

During protein folding, Hsp70 and its cofactors bind to unfolded protein states to form a complex. After complexation, the release of a polypeptide from Hsp70 is coupled to ATP hydrolysis. From there, it may be transferred to members of the Hsp60 family to fold to its native form (Buchberger et al. 1996, Rudiger et al. 2000). Hsp90 is one of the most abundant proteins in the cytosol of eukaryotic cells and functions as a dimer of 90 kDa subunits (Grallert et al. 2001). The Hsp90 dimer is a molecule with an unusual N-terminal ATP binding site (Richter et al. 2000) whereas the main dimerisation site occurs in the C-terminal domain (Walter et al. 2002). The intra cellular level of Hsp90 increases several fold under stress conditions. The function of this chaperone is not well understood but it has been shown that the chaperone action of Hsp90 is ATP-independent (Grallert et al. 2001, Walter et al. 2002). Finally, members of the Hsp100 family are ATP-dependent chaperones capable of dissolving pre-formed protein aggregates (Arrigo et al. 2001).

1.1.1 Small Heat-Shock Proteins (sHsps)

The sHsp family has been recognized in all organisms with the exception of a few bacteria (van Montfort et al. 2002). sHsps are involved in the stabilization of other proteins to prevent their precipitation under normal cellular conditions (Carver et al. 2001, van Montfort et al. 2002). In contrast to other chaperone families, sHsps bind several non-native state molecules per oligomeric complex (Arrigo et al. 2001, van Montfort et al. 2002). Small heat-shock proteins recognize and complex with aggregation-prone, partially folded proteins and keep them in a refoldable conformation. The complex of the small heat shock protein with the unfolded target protein then interacts with another chaperone system, such as Hsp70, in an ATP-dependent process to refold the target protein (Haslbeck et al. 1999, Horwitz et al. 2003).

The folding and unfolding of proteins occurs along the same pathway and proceeds via a series of partially folded intermediates (or molten globule states) to the final folded or unfolded state (Carver et al. 2001, Figure 1). sHsps perform their functions by complexing with stressed proteins in a molten globule state, on the relatively slow off-folding pathway to prevent their large-scale aggregation. Hsp70 and Hsp60 recognize and complex these types of intermediates and refold them to their native conformation (Carver et al. 2001).

Insight into the mechanism of sHsp chaperone action (Carver et al. 2001, Figure 1) was gained from real-time ^1H NMR spectroscopy of the complexation of α -crystallin with chemically destabilized proteins such as α -lactalbumin. The

folding/unfolding pathway is relatively fast and reversible, i.e. the various molten globule intermediates (I_1 and I_2) are transient species. The off-folding pathway, however, is much slower and irreversible, particularly the step from aggregation to precipitation (Carver et al. 2001, Treweek et al. 2003). α -Crystallin (and probably all sHsps) interacts with target proteins in their relatively disordered I_2 state prior to large-scale aggregation. The interaction occurs in the dissociated, probably dimeric, form of α -crystallin that has its chaperone binding site exposed and accessible (Carver et al. 2002, Poon et al. 2002, van Montfort et al. 2002, Treweek et al. 2003). Once I_2 interacts with the α -crystallin dimer, the complex is sequestered into a high-molecular-mass complex between the two proteins. Recovery of I_2 back to its native state (N) can occur via the interaction of the complex with Hsp70, involving ATP hydrolysis (Carver et al. 2002, Poon et al. 2002, van Montfort et al. 2002, Treweek et al. 2003).

1.1.2 sHsp Structure

sHsps have molecular masses in the range of 12 to 43 kDa and often assemble into large oligomers with a total molecular mass of 140 to over 800 kDa. sHsps have an N-terminal domain and a C-terminal extension, that is diverse and varied in length (Lentze et al. 2003). The C-terminal region of 80 to 100 residues, referred to as the α -crystallin domain, is homologous within the family and is probably essential for the aggregate formation as well as for solubilising high-molecular weight complexes with target proteins (Muchowski et al. 1999). The N-terminal domain is relatively hydrophobic and variable in length and sequence (Gusev et al. 2002, Usui et al. 2003). Because of its hydrophobic

Figure 1: The putative mechanism of chaperone action of α -crystallin to prevent aggregation and subsequent precipitation of stressed proteins (Carver et al. 2002). Along this folding pathway, the protein under stress adopts a partially folded state (MG). The ordered molten globule states (MG) of proteins are transiently present whereas the disordered states (I_2), which are highly dynamic and expose the hydrophobic area to solution, are present for a significant period of time. This exposed area has the propensity to aggregate along the off-folding pathway, which leads to irreversible aggregation (Carver et al. 2002, van Montfort et al. 2002). In this mechanism the dimeric form of the chaperone binds to I_2 , which is sequestered into a high molecular weight complex and refolds I_2 to its native state (N) via the interaction of the complex with Hsp70, which is ATP-dependent (Carver et al. 2002).

nature it has a crucial role in self-assembly and possibly in the capture of target proteins during chaperone action (Gusev et al. 2002, Park et al. 2002, Usui et al. 2004).

In mammalian cells, sHsps form large heterogeneous complexes, often exceeding 500 kDa in mass. On the other hand, some non-mammalian sHsps have an ordered quaternary structural arrangement (Harndahl et al. 2001). For

example, two non-mammalian sHsp structures were determined recently by X-ray crystallography: Hsp16.5 from *Methanococcus jannaschii* and *Triticum aestivum* (wheat) Hsp16.9 (Figure 2) (Kim et al. 1998, van Montfort et al. 2001). The structure of Hsp16.5 revealed 24 subunits arranged with octahedral symmetry forming a hollow spherical complex with a small window opening. Each subunit in the complex contains a disordered N-terminal domain, a core α -crystallin domain composed of nine β -strands arranged into two antiparallel β -sheets and a C-terminal extension. Hsp16.9 is organized as a hollow spherical complex of 12 subunits. Despite different quaternary structures, the monomeric subunits of Hsp16.5 from *Methanococcus jannaschii* and *Triticum*

Figure 2: (a) Oligomeric arrangement of subunits in *T. aestivum* Hsp16.9 (left) and *M. jannaschii* Hsp16.5 (right) reported by Kim et al. 1998 and van Montfort et al. 2001. Hsp16.9 is a dodecamer containing three tetramers structured to form two rings of six subunits and Hsp16.5 is a 24-mer. The central cavity in both aggregate is apparent (b) Secondary structure of Hsp16.9 (red) and Hsp16.5 (blue) superimposed upon on each other to show their similar folds. The N- and C-terminals of each protein are indicated.

aestivum exhibit very similar secondary and tertiary structure. In both sHsps, the α -crystallin domain is located in the β -strands (from $\beta 2$ to $\beta 9$) which fold into a β -sandwich with two hydrophobic edges of N and C-terminals. (Figure 2b) (Kim et al. 1998, van Montfort et al. 2002, StamLer et al. 2005).

1.2 α -Crystallin

The eye lens contains of a group of proteins called crystallins whose high concentration and specific interactions enable the lens to maintain highly refractive capabilities. They make up more than 90% of the total dry mass of the lens (Carver et al. 1994). Lens transparency is dependent on the maintenance of the crystallins at this high concentration in their native conformation to ensure the proper refraction of light. Modification of the crystallins, for example by truncation, glycation or oxidation, can result in altered conformation, precipitation, and lens opacity leading to cataract (Garnera et al. 2000).

In mammals, there are three types of crystallin proteins: α -, β - and γ - classified on the basis of their elution from a size-exclusion column (Carver et al. 1996). α -Crystallin is the major protein component of the mammalian eye lens and comprises 50% of its dry weight (Raman et al. 1995, Datta et al. 1999). Most of the α and β crystallins exist as aggregates. α -Crystallin is the most aggregated crystallin in having a broad molecular mass distribution (300 to 1000 kDa) with an average mass of around 800 kDa, comprising approximately 40 subunits of 20 kDa each (Lindner et al. 1998). The β -crystallin fraction is a mixture of proteins which form smaller aggregates than α -crystallin, comprising 20-30 kDa

subunits, with a mass range of approximately 50-200 kDa. The γ -crystallins are monomers with molecular masses of around 20 kDa (Groenen et al. 1994, Carver et al. 1996, Lindner et al. 1998). The β - and γ -crystallins are related to each other but share no sequence similarity to α -crystallin.

There are two α -crystallin subunits, A and B, with molecular mass around 20 kDa, which in the lens are generally found in a 3:1 molar ratio (Horwitz et al. 2000, Horwitz et al. 2003, Lentze et al. 2003, Augusteyn et al. 2004). These two polypeptides of 173 and 175 residues in length, respectively, share 57% amino acid sequence similarity (Horwitz et al. 1999, Abgar et al. 2000). Until 1985, it was thought that the crystallins were lens-specific proteins. However, Bhat and Nagineni (1989) found that α B-crystallin was also abundant in non-lenticular tissues such as heart, skeletal muscle, skin, brain, spinal cord and lung. By contrast, outside of the lens, α A-crystallin is only expressed in trace amounts in the spleen and thymus (Carver et al. 1996, Haley et al. 1998).

1.3 Structure of α -crystallin

The secondary, tertiary and quaternary structure of α -crystallin is not known. However, recent studies have predicted a mostly β -pleated sheet conformation with little or no helical structure (Thomson et al. 1989, Kim et al. 1998). Secondary structural analysis suggested that α -crystallin possesses approximately 50% of β -sheet and β turns and 10-15% of helical structure (Thomson et al. 1989, Augusteyn et al. 2004). Koteiche et al. (1999) showed that the β -sheet arrangement of α A-crystallin is very similar to that of Hsp16.9

and Hsp16.5. The quaternary structure of α -crystallin has been a subject of investigation for many years. Various hypothetical models have been proposed for the structure of α -crystallin (Carver et al. 1996, Haley et al. 1998). For example, a model for the structure of α -crystallin was proposed (Figure 3, Carver et al. 1994a) that consisted of two layers of approximately 20 subunits.

Figure 3: A model for quaternary structure of α -crystallin (Carver et al. 1994a). It consists of two annuli of 20 subunits laid on top of each other. The smaller spheres are hydrophobic N-terminal domains whilst hydrophilic C-terminal domains are indicated by the larger spheres.

These subunits are mobile and exchangeable between aggregates under normal conditions (van den Oetelaars et al. 1990). The smaller spheres are the hydrophobic N-terminal domains while the larger spheres are the hydrophilic C-terminal domains (Carver et al. 1994a, Augusteyn et al. 2004). The model is

characterized by a large central cavity and highly mobile and solvent-exposed C-terminal extensions. The presence of the large cavity was subsequently confirmed by cryo-electron microscopy studies (Haley et al. 1998). NMR spectroscopic studies have shown the inherent flexibility of the C-terminal regions (Carver et al. 1994 a).

1.3.1 Chaperone action of α -crystallin

α -Crystallin belongs to the small heat-shock protein family and acts as a molecular chaperone (Ignolia et al. 1982, Horwitz et al. 1992) by preventing abnormal aggregation of lens crystallins resulting from protein aging and accumulation of environmental stresses. It achieves this through the formation of a stable, soluble, high molecular-mass sHsp-substrate complex (Lindner et al. 2001, Carver et al. 2002). It binds with unfolding target proteins and keeps them in a refoldable conformation (Wang et al. 2001). In the lens, β - and γ -crystallin are its normal substrates (Horwitz et al. 1992 a, Horwitz et al. 2003). *In vitro* α -crystallin has been shown to suppress aggregation of many stressed target proteins, e.g. alcohol dehydrogenase, insulin, β_L -crystallin as well as γ -crystallin (Horwitz et al. 1992a, Horwitz et al. 1992b, Wang et al. 1994, Boyle et al. 1994, Datta et al. 1999, Bloemendal et al. 2004).

Previous studies have demonstrated that the chaperone-like activity of α -crystallin is enhanced at elevated temperature (Das et al. 1997, Lindner et al. 1998, Reddy et al. 2000, Regini et al. 2004). Upon heating, α -crystallin undergoes a conformational transition which is associated with a partial

unfolding of the protein and a marked increase in surface hydrophobicity. The increase in surface hydrophobicity correlates with the increased chaperone activity of the protein, which indicates that hydrophobic interactions play a major role in the chaperone action of α -crystallin (Das et al. 1997, Lindner et al. 1998, Reddy et al. 2000, Regini et al. 2004). It is believed that regions in the C-terminal region of α -crystallin are responsible for recognizing the exposed hydrophobic regions of denatured proteins (Das et al. 1997, Sharma et al. 1997, Derham et al. 1999).

α -Crystallin, therefore, has a dual function in the lens: to act as a molecular chaperone to stabilize the β - and γ -crystallins and prevent their aggregation, and in a structural manner to ensure proper refraction of light. Disruption of crystallin order and precipitation can lead to opacification and, potentially, cataract formation (Lindner et al. 1998, Treweek et al. 2000, Lindner et al. 2001).

1.4 Amyloid fibril formation

Amyloid fibril formation arises from the slow aggregation of intermediately folded peptide or protein molecules. During this process the protein goes from its native soluble form to insoluble fibril which is highly β -sheet in characters (Come et al. 1993, Chiti et al. 1999, Arrigo et al. 2001, van Montfort et al. 2002). Amyloid formation is associated with a number of diseases e.g. Alzheimer's, Parkinson's, Huntington's, Creutzfeldt-Jakob diseases (CJD) and late-onset (type II) diabetes (Haley et al. 1998, Arrigo et al. 2001, Hatters et al. 2002). All

these diseases are primarily associated with old age. Amyloid fibrils are insoluble, high-molecular-weight aggregates of peptides or proteins (Tyco et al. 2000). Structural studies of Alzheimer's amyloid fibrils (from the amyloid β -peptide) have revealed information about their structure at different levels (Tyco et al 2000, Petkova et al 2002, Wentzel et al. 2002, Inouye et al. 2006).

Negative-stain electron microscopy (EM) showed that the amyloid fibrils associated with the various diseases most appear straight, and unbranched, and are 30-120 Å in diameter. They consist of two or more filamentous subunits, which twist around each other with 11.5 nm, 24 β -strand periodicity resulting in a protofilament diameter of 5-6 nm. The amyloid fibril, shown in Figure 4, is about 25-35 Å wide and consists of two or three subunits strand helically arranged with a 35-50 Å diameter repeat (Shirahama et al. 1967, Cohen et al. 1982, Serpell et al. 1995, Jimenez et al. 1999, Chamberlain, et al. 2000, Conway et al. 2000).

X-ray fibre diffraction showed that all types of fibrils have a common core structure (Kirschner et al. 1986, Blake et al. 1996, Sunde et al. 1997, Guijarro et al. 1998). They consist of helical array of β -sheets along the length of fibre. This indicates that the polypeptide chain in fibres are hydrogen-bonded together along their entire length which increases their stability (Kirschner et al. 1986, Blake et al. 1996, Sunde et al. 1997, Guijarro et al. 1998). Cross β -sheets consist of two dominant reflections: a sharp and intense meridional reflection at 4.8 Å, which corresponds to the average distance between the hydrogen-bonded β -strands that comprise β -sheets and strong and more diffuse reflection

on the equator between 9-11 Å arising from the distance between stacked β -sheets, (Kirschner et al. 1986, Blake et al. 1996, Sunde et al. 1997, Guijarro et al. 1998). The X-ray diffraction pattern of PI3-SH3 fibril is shown in Figure 5 (Guijarro et al. 1998). The fibril contains two reflections: the main reflection, which is intense, occurs at 4.71 Å while the weaker reflections appear at 9.42 Å.

Figure 4: Modeling of an amyloid fibril structure. (a) Overview of the fibril structure showing protofilaments extended in regular helical twists along the length of the fibre (b) Side view of inter-sheet spacing between subprotofilaments of 9-11 Å perpendicular to the axis (c) Cross-section of amyloid fibril (d) Slightly tilted side view of the fibril showing inter-strand spacing of 4.7 Å along the fibre axis (Jimenez et al. 1999).

Figure 5: X-ray diffraction pattern of PI3-SH3 fibrils. The meridional (interstrand spacing) and equatorial (intersheet spacing) reflections are indicated at 4.7 Å and 9.4 Å, respectively (Guijarro et al. 1998).

The aggregation of the amyloid β peptide has been examined in various solvents and conditions and this led to a model by which a conformational switching occurs from α -helix or random coil to a β -sheet structure early on the amyloid-forming pathway (Serpell et al. 2000), prior to a nucleation-dependent process leading to the elongation of the fibril. Along this pathway, small oligomeric intermediates and short fibrillar structures (protofibrils) have been observed. In a cross-section, the fibril appears to be composed of several

subfibrils or protofilaments. Each of these protofilaments is rich in stacked β -sheet structure in which hydrogen bonded β -strands run perpendicular to the fibril's axis (Tycko et al. 2000, Serpell et al. 2000, van Montfort et al. 2002).

Like amorphous aggregation, amyloid fibrils arise from the aggregation of intermediately folded protein states (Figure 6) (Dobson et al. 2001, Treweek et al. 2003). Figure 6 suggests that the various fate awaiting a polypeptide chain,

Figure 6: A proposed mechanism for lysozyme amyloid fibril formation (Dobson et al. 2001). An intermediate structural state form of the protein (II) aggregates through the β domain (IV) to commence fibril formation. This intermediate (IV) shows a pattern for the development of the fibril.

once it has been synthesized in the cell, will depend on the kinetics and thermodynamics of the various equilibria between various possible states (Serpell et al. 2000, Dobson et al. 2001).

In its monomeric state, the protein is believed to fold from its highly disordered unfolded state (III) through a partially structured intermediate (II) to a globular native state (I) (Dobson et al. 1999, Dobson et al. 2001). The unfolded and partially folded states can form aggregated species that are frequently disordered. Highly ordered amyloid fibrils can form through a mechanism of nucleation and elongation (Canet et al. 1999, Dobson et al. 1999, Dobson et al. 2001). Canet et al. (1999) showed that the β -domain is the destabilized region of the human lysozyme. This implies that the aggregation process may be initiated by the intermolecular association rather than intramolecular association (Canet et al. 1999, MacPhee et al. 2000, Dobson et al. 2001).

In vitro studies have shown that small heat shock proteins (sHsps) can suppress amyloid formation (Kudva et al. 1997, Stege et al. 1999, Hatters et al. 2001, Rekas et al. 2004). Kudva et al. (1997) demonstrated that human Hsp27 inhibited amyloid formation by the Alzheimer's A β (1-42) polypeptide. A similar result was reported with α B-crystallin preventing amyloid formation by A β , but the toxic effects of the peptide are enhanced (Stege et al. 1999). The effect of α -crystallin to suppress amyloid formation by apolipoprotein C-II has been demonstrated (Hatters et al. 2001a). In addition, the effect of the extracellular chaperone, clusterin, to inhibit amyloid formation by apolipoprotein C-II, in a manner similar to α -crystallin, was demonstrated (Hatters et al. 2002b).

Chaperone action of the milk proteins, α_s - and β -casein to prevent both amyloid fibril formation and amorphous aggregation by bovine milk, κ -casein, α -lactalbumin and milk whey protein, β -lactoglobulin, has also been established (Thorn. 2003, Morgan et al. 2005). Likewise the chaperone action of α B-crystallin to prevent amyloid formation of α -synuclein was reported by Rekas et al. (2004) by increased amorphous aggregation of both proteins.

1.5 Macromolecular crowding

Macromolecular crowding is ubiquitous in all types of cells and describes the normal conditions inside a living cell. It greatly accelerates numerous physiological processes, e.g. the self-assembly of proteins (Berg et al. 1999, Ellis et al. 2001a). The concentration of macromolecules inside a cell is very high (up to 300 mg/mL) arising from the presence of species such as polysaccharides, proteins and nucleic acids interacting with each other. As a result, the intracellular environment is highly crowded. By contrast, reactions that are carried out *in vitro* (e.g. in a test tube) are mainly undertaken in dilute solution (Figure 7) (Ellis et al. 1997, van den Berg et al. 1999, Ganea et al. 2001, Ellis et al. 2001a). Cells are surrounded by a high concentration of salts and other small molecules, which balance the extracellular osmolarity (Garner et al. 1994). Moreover, the activity of water molecules is lower inside a cell compared to in a dilute solution (Garner et al. 1994). In order to mimic cellular conditions in a test tube, inert macromolecular crowding agents such as PEG (polyethylene glycol) or dextran can be added (Zimmerman et al. 1993, Hatters

et al. 2002). This promotes the rate and extent of association between macromolecules (Zimmerman et al. 1993, Hatters et al. 2002).

Crowding influences a variety of biological phenomena and systems by altering the reactivity of individual macromolecules, both qualitatively and quantitatively. It has both thermodynamic and kinetic effects on the properties of macromolecules resulting in large quantitative effects on both the rates and the equilibria of interactions involving macromolecules (van den Berg et al. 1999,

Figure 7: Representation of macromolecules inside a cell of *Escherichia coli*. The approximate number, shape and density of packing of macromolecules are shown (Ellis et al. 2001b).

Ellis et al. 2001a and b, Rivas et al. 2004). Crowding promotes macromolecular association, catalytic activity of enzymes and cell volume regulation (Zimmerman et al. 1993, Ellis et al. 2001a).

Macromolecular crowding is termed 'the excluded volume effect' because of the non-specific steric repulsion that occurs between the solute molecules. The amount of intracellular volume which is unavailable to other macromolecules depends on the number, size, shapes and concentration of all the molecules. In general, cellular environments are ~30% volume occupied by the background macromolecules. The remaining 70% of the space is the area which can be occupied by the molecules of interest or the test species (Ellis et al. 2001a and b, Minton et al. 2001). If the test molecule is small relative to background molecule (Figure 8a), it can access virtually all the space between macromolecules, i.e. 70%. However, if the test molecule is larger or similar in size to the background species, the volume available is much less, as the centre of any background molecule can approach the centre of the test molecule to no less than the distance at which the surfaces of the two molecules meet; this distance is indicated by the open circle around each macromolecule. Thus, depends on the size and concentration of interacting molecules, the effect of crowding on equilibria and reaction rates are highly non-linear (Figure 8b) (Ellis et al. 2001a and b, Minton et al. 2001).

The presence of macromolecules reduces the entropy of the solute. Although in a cell there is a great deal of free space to distribute macromolecules, by increasing the numbers of macromolecules and eventually increasing the

excluded volume, the free spaces are diminished due to the increase in the total concentration of solute molecules (Figure 8). This causes the entropy of the solute to decrease. Hence, with a decrease in entropy, both the free energy of a solution and the chemical potential of each species of solute in solution increase. (Figure 8) (Ellis et al. 2001a and b, Minton et al. 2001). As volume exclusion in a crowded solution results in the destabilization of any test species of solute, crowding ordinarily affects equilibria by preferentially destabilizing either reactants or products, because it causes the association constant to increase and the reaction rate to decrease (Figure 8). Macromolecular crowding provides a non-specific force for molecular compaction and self-association in a

Figure 8. Excluded volume available in macromolecular solutions (showing in yellow). (a) Volume available for introduced small molecules (b) volume available for an introduced molecule of size comparable with the background molecule (Ellis et al. 2001b).

crowded solution. On the other hand, crowding reduces the diffusion mobility of macromolecules, and the rates of diffusion-controlled reactions are thus lowered. However, although crowding reduces diffusion, it increases thermodynamic activities. Therefore, it increases the association and aggregation of proteins (Ellis et al. 1997, van den Berg et al. 1999, Minton et al. 2000, Ganea et al. 2002, Ellis et al 2001a and b, Minton et al. 2001).

The effect of macromolecular crowding can be expressed in a mathematical format as follows:

1.5.1 Effects of crowding on equilibria

The chemical potential (μ) of solute species i , can be partitioned into ideal and non-ideal contributions (Ellis et al. 2001a and b).

$$\mu_i = \mu_i^{\text{ideal}} + \mu_i^{\text{non-ideal}} \quad (1)$$

This is necessary because in biological systems, the total concentration of molecules is often very high and molecules are not ideal. The ideal contribution is the free energy change expected in the absence of solute-solute interactions (Ellis et al. 2001 a and b).

$$\mu_i^{\text{ideal}} = \mu_i^0 + kT \ln c_i \quad (2)$$

where μ_i^0 is chemical potential of species i in its standard state, k is Boltzmann's constant, T the absolute temperature, and c_i is the concentration in molar or w/v relative to the density of molecules. The non-ideal component of the chemical potential of a particular molecule occurs in systems where their total concentration is very high.

The non-ideal contribution is the free energy change associated with the equilibrium constant between molecules of solute species i and all other solute molecules in the solution (Ellis et al. 2001 a and b)

$$\mu_i^{\text{non-ideal}} = kT \ln \gamma_i \quad (3)$$

Where γ_i , called the activity coefficient, is defined as $\gamma_i = \exp(\mu_i^{\text{non-ideal}}/kT)$.

Equations 1-3 can be combined to yield Equation 4 (Ellis et al. 2001 a and b):

$$\mu_i = \mu_i^0 + RT \ln a_i \quad (4)$$

where $a_i = \gamma_i c_i$ is an effective concentration called the thermodynamic activity. Chemical equilibrium is based on the use of activities rather than concentration. In dilute solution, solute-solute interactions, and thus the non-ideal contribution to the chemical potential, diminishes to zero and $a_i = c_i$ for all solute species. Under these conditions, the solution can be said to behave in an ideal fashion (Minton et al. 2000, Ellis et al. 2001a and b). However, in concentrated solutions, solute-solute interactions cannot be neglected and a_i is not equivalent

to c_i . In particular, under crowded conditions, the interaction between macromolecules changes the free energy and equilibrium constant, which affects the excluded volume.

1.5.2 Effects of crowding on reactions of macromolecules

Studies on protein stability *in vitro* are routinely performed using dilute solutions of proteins in order to avoid aggregation during the reaction and/or to maintain the physiologically relevant concentration of the studied protein. A major difference between these idealized conditions and those encountered within cells is that the intracellular environment is highly crowded due to the presence of high concentrations of various soluble and insoluble macromolecules in the cytosol (Ganea et al. 2002, Dobson et al. 2001, Sasahara et al. 2003).

Research with cell extracts has shown that the high concentration of macromolecules within *E. coli* cells produces a large increase in the activity coefficient values of macromolecules. In addition, the activity coefficient increases dramatically with molecular mass. The activity coefficient triples as the molecular mass exceeds 3 kDa, and increases by two orders of magnitude as the molecular mass exceeds 50 kDa (Ellis et al. 2001a and b). Biochemical equilibrium is affected by crowding via association of macromolecules such that the reaction equilibrium constants can be increased by as much as two to three orders of magnitude, depending on the sizes and shapes of the reactants, products and on those of the background macromolecules (Tyco et al. 2000, Minton et al 2000, Ellis et al. 2001 a and b). Thus, the equilibrium constant in

dilute solution between a spherical homodimer and a monomer of mass 40 kDa will shift the reaction towards dimerisation by a factor in the range 8-40 fold, if the protein is expressed in *E. coli*. If a protein exists as a tetramer, the shift in equilibrium towards the aggregate is in the range of 10^3 - 10^5 . Thus, the effect of crowding on thermodynamic activity is exerted by large molecules on other large molecules (Minton et al. 2000, Ganea et al. 2002).

As discussed above, according to the excluded volume theory, macromolecular thermodynamic activities increase by several orders of magnitude under crowding conditions. Therefore, biochemical rates and equilibria in a living cell may be quite different from those under ideal conditions. The biophysical theory of macromolecular crowding, however, is well developed to mimic the crowded conditions within the cell by the addition of macromolecular crowding agents (Minton et al. 2000, Ganea et al. 2002, Sasahara et al. 2003).

1.6 Research purposes

As discussed above, the ideal test tube is very different from biological media due to high concentration of soluble macromolecules inside the cell. So, to understand better what happens inside a cell, the intracellular environment can be mimicked by the addition of macromolecular crowding agents, such as dextran.

The aim of this project is to study the structural and functional aspects of the chaperone action of α -crystallin, in preventing the aggregation and precipitation

of stressed proteins and their amyloid fibril formation in the presence of macromolecular crowding agent. This aim is achieved by the use of various spectroscopic methods such as visible absorption, fluorescence spectroscopy, NMR spectroscopy, size exclusion HPLC, transmission electron microscopy (TEM) and Circular Dichroism (CD) spectroscopy. In summary, this approach is divided into five stages, which are to investigate:

1. The effect of a macromolecular crowding agents on protein amorphous aggregation and the chaperone action of sHsps, particularly α -crystallin which will provide greater insight into understanding the action of α -crystallin *in vivo*.
2. The effects of a macromolecular crowding agents on amyloid fibril formation and on the ability of sHsps, e.g. α -crystallin, to prevent amyloid formation under crowding conditions.
3. The effect of a macromolecular crowding agent on the rate of subunit exchange of α A-crystallin in order to understand better the role of subunit exchange in α A-crystallin and its chaperone activity under conditions of molecular crowding.
4. The chaperone ability of α -crystallin to stabilize heat-stressed β -lactoglobulin (which aggregates via both intra- and inter-molecular disulphide bond exchange) at different pH values via visible absorption spectroscopy to determine whether α -crystallin would be able to prevent aggregation of heated β -lactoglobulin.

5. Using NMR spectroscopy and chaperone assays of α -crystallin from dogfish (*Squalus acanthias*), which is evolutionarily very old, to obtain structural information on the flexible C-terminal extensions of its α A- and α B-crystallin subunits and their respective chaperone abilities by comparing these properties with those of bovine α -crystallin the current study will allow us observe the evolutionary relationship between structure and function in α -crystallin

Chapter 2

Materials and Methods

2.1 Materials

A local abattoir (Wollondilly Abattoirs, Picton, NSW) provided all bovine lenses, Sephacryl S-300HR size exclusion resin was obtained from Amersham Biosciences (Uppsala, Sweden). α -Lactalbumin, β -lactoglobulin, α_s - and κ -casein, dextran (68,800 Da), insulin, 1,4-dithiothreitol (DTT), sodium azide (NaN_3), ethylenediaminetetraacetic acid (EDTA), Trizma base (Tris [hydroxymethyl] aminomethane), Trizma acid (Tris [hydroxymethyl] amino ethane hydrochloride), sodium hydrogen phosphate (Na_2HPO_4), ovotransferrin, thioflavin T (ThT), 8-anilino-1-naphthalene sulphonate (ANS) and glycine were obtained from Sigma-Aldrich (St. Louis, U.S.A.). Deuterated dithiothreitol (DTT) was obtained from Cambridge Isotopes Laboratories.

Lucifer yellow iodoacetamido (LYI) and 4-acetamido-4' ([iodoacetyl] amino) stilbene-2, 2'-disulfonic acid (AIAS) were purchased from Molecular probes, Inc (Eugene, U.S.A.). Diaflo ultrafiltration membranes (YM 10) were obtained from Amicon Inc. (UK). Snakeskin dialysis tubing with a 3.5 kDa cut-off was obtained from Pierce Chemical Company (Rockford, U.S.A). SealPlateTM, a transparent adhesive film used for sealing cuvettes, was purchased from Excel Scientific (USA).

Dogfish α -crystallin was a gift from Prof. R. Augusteyn who purified it from dogfish eyes (University of Melbourne) using the method of Thomson and Augusteyn, (1983).

2.2 Methods

2.2.1 Isolation and purification of α -, β - and γ -crystallins from bovine eye lenses

The purification of α -crystallin was performed according to the method of Slingsby and Bateman (1990). This method was also used for the purification of β - and γ -crystallin. Bovine lenses were homogenized using a glass homogeniser in 50 mM Tris buffer, pH 7.2, 5 mM EDTA, 1 mM DTT, 0.04% (w/v) NaN_3 , 0.1 mM PMSF. For each calf lens, 3 mL volume of buffer was used. This solution was then centrifuged at 12,000 rpm for 15 minutes at 5°C using an Allegra 21R centrifuge (Beckman Coulter, U.S.A). The pellet was discarded and supernatant fractionated by size-exclusion chromatography. The soluble lens proteins were separated on a Sephacryl S 300HR column with a diameter of 2.6 cm and length of 100 cm. Protein was eluted as the column buffer (50 mM Tris, 0.04% (w/v) NaN_3 , pH 7.5) was passed through at a flow rate of 20 mL/hr and fractions were collected at 20 minute intervals. Fractions containing α -crystallin with an elution time of approximately 8-10 hrs were combined and concentrated to a volume of approximately 3 mL using an Amicon ultrafiltration cell fitted with a Diaflo membrane with a 10 kDa molecular mass cut-off. In order to ensure optimal purity, the concentrate obtained was rechromatographed using the method described above.

The solution was again concentrated and dialyzed extensively against MilliQ water and then freeze-dried and stored. Fractions from the initial calf-

lens homogenate containing β_L -crystallin with elution times of approximately 12-14 hrs and γ -crystallin of approximately 14-18 hrs elution times were also collected using the method described above.

2.2.2 Visible Absorption Spectroscopy

2.2.2.1 Reduction stress assay

The chaperone action of α -crystallin in preventing aggregation and precipitation of stressed proteins was assessed in the presence and absence of a macromolecular crowding agent, dextran, using variety of spectroscopic techniques. Insulin (at the final concentration of 0.43 mg/mL) and ovotransferrin (at the final concentration of 1.4 mg/mL) were studied for aggregation in 50 mM sodium phosphate, pH 7.4, 0.05% (w/v) NaN_3 , 37°C following reduction of their disulfide bonds with dithiothreitol (DTT) in the presence of various concentrations of α -crystallin from 0 to 6 mg/mL. α -Lactalbumin (at final concentration of 2 mg/mL) was studied in the same conditions except that 100 mM NaCl was added to buffer. To investigate the effect of macromolecular crowding on the aggregation of insulin, ovotransferrin and α -lactalbumin and on the chaperone action of α -crystallin, dextran was added up to a final concentration of 10% w/v. Aggregation of all proteins was monitored as an increase in light scattering at 360 nm using a Spectramax 250 multiwell plate reader spectrophotometer (Molecular Devices) with temperature control.

The chaperone activities of α -, αA -, and αB -crystallin were measured at different temperatures to investigate the ability of these proteins to prevent

aggregation of reduced ovotransferrin or β_L -crystallin in the presence and absence of dextran. Ovotransferrin, (at a concentration of 0.66 mg/mL) in the presence and absence of equimolar amount of α - or α_A -crystallin and 0% or 10% w/v dextran was incubated at 37°C, 42°C, and 45°C in 50 mM phosphate buffer, pH 7.4, 0.05% (w/v) NaN_3 . DTT was added at a concentration of 20 mM to commence the unfolding and aggregation of ovotransferrin. Light scattering was then monitored at 360 nm, using a Spectramax microplate reader spectrophotometer.

The rate constant of aggregation of target protein was determined by fitting an exponential function $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the light scattering data using Sigmaplot software (version 8.0). A_1 and A_2 are constants such that $F(0) = A_1 + A_2$, k is the rate constant and t is the time (in minutes).

2.2.2.2 Heat-stress assay

β_L -Crystallin (at final concentrations of 0.3 mg/mL) in 50 mM sodium phosphate pH 7.4, 0.05% (w/v) NaN_3 was incubated at 60°C in the presence of α -, α_A - or α_B -crystallin at concentrations from 0 to 6 mg/mL in the absence and presence of 10% w/v dextran. Samples were prepared in 1 cm pathlength plastic cuvettes to a final volume of 1 mL.

β -Lactoglobulin (at a final concentration of 5 mg/mL) in 50 mM sodium phosphate, 100 mM NaCl and 2.5 mM EDTA, 0.05% (w/v) NaN_3 was incubated at 70°C at different pH values (6.5, 7.0, 7.5, and 8.0) in the presence and absence of 1:1 w:w ratio of β -lactoglobulin: α -crystallin and/or in

the absence and presence of 5% or 10% w/v dextran as a macromolecular crowding agent and/or DTT. Samples were prepared in 1 cm quartz cuvettes to a final volume of 1 mL. To avoid evaporation from cuvettes, a 'Seal Plate' strip was used made of plastic stable up to 120°C.

Aggregation of β_L -crystallin and β -lactoglobulin was monitored as light scattering at 360 nm over 3 hrs with data collected every 2 minutes using a Cary 500 Scan UV-VIS- NIR spectrophotometer.

2.2.3 Fibril formation and kinetic evaluation

Amyloid fibril formation of α -lactalbumin (1.75 mg/mL) in the presence and absence of 5%, and 10% w/v dextran was investigated by incubation of α -lactalbumin in 100 mM NaCl, pH 2.0 at 37°C in a Bioline 472 Incubator (Edwards Instrument company, Australia). To accelerate fibril formation, solutions were shaken at 220 rpm. Amyloid formation of bovine milk, α_s -casein (2.5 mg/mL), was induced by incubation of protein in 50 mM phosphate buffer, 20 mM DTT and pH 7.4. The amyloid formation of α_s -casein was studied with and without 10% w/v dextran and an equivalent amount of α -crystallin (1:1 molar ratio). Fibrillation of α -lactalbumin and α_s -casein in different conditions were monitored using a Hitachi F-4500 spectrofluorimeter. Aliquots of 10 μ L were sampled and amyloid formation was detected by the increase in ThT fluorescence (0.4 μ M in 50 mM Na_2HPO_4 , 0.05% (w/v) NaN_3 , pH 7.4). The excitation and emission wavelengths were set to 378 nm and 479 nm with a 2.5 nm and 5 nm slit widths, respectively.

Fibrillation of bovine milk, κ -casein, (2.5 mg/mL), was monitored in a BioluminTM960 Kinetic Fluorescence Absorbance plate reader (Molecular Dynamics, Sweden) using a method adapted from Nielsen et al. (2001). The protein solution was prepared in 50 mM sodium phosphate buffer, pH 7.4 and 20 mM DTT and incubated at 37°C in Costar 96 microwell black plates (Corning, Australia) with a 200 μ L of sample value in each well. To prevent evaporation, the plate was sealed with a transparent, inert sealing tape. Fibril formation was detected by the increase in ThT fluorescence (0.4 μ M in 50 mM Na₂HPO₄, 0.05% (w/v) NaN₃, pH 7.4) with emission and excitation at 485 nm and 450 nm respectively at 15 min intervals for a specified period of time. Between readings, the plate was shaken for 10 min. Fibril formation of α _S- and κ -casein was induced with 20 mM DTT at 37°C (Thorn et al. 2005).

2.2.4 Intrinsic fluorescence spectroscopy

Intrinsic fluorescence intensity was measured on samples containing combinations of insulin, κ -casein, α -lactalbumin, α -crystallin and 10% w/v dextran to investigate the effect of dextran on the environment of the tryptophan residues of α -crystallin and on the amorphous and amyloid formation of α -lactalbumin and κ -casein respectively. Samples containing κ -casein (1 mg/mL), α -lactalbumin (10 μ M), α -crystallin (in 1:1 molar ratio) and 10% w/v dextran together and separately with 20 mM DTT were incubated in 50 mM phosphate buffer, 0.05% (w/v) NaN₃ and pH 7.4 for 3 hrs at 37°C. Insulin (10 μ M) and α -crystallin (10 μ M), separately or in the presence of each other and/or 10% w/v dextran, were incubated in 50 mM phosphate buffer, 0.05% (w/v) NaN₃ and pH 8.5 for 1 h at 37°C after the

addition of DTT. Control experiments with the same sample components were carried out without the addition of DTT.

Intrinsic fluorescence intensity was measured on α -crystallin and L-tryptophan in the presence and absence of 10% w/v dextran in 10 mM phosphate buffer, pH 7.0 at different temperatures (25°C, 37°C, 42°C, 45°C and 48°C) to investigate the effect of dextran on the environment of the tryptophan residues of α -crystallin and L-tryptophan with increasing temperature

Fluorescence spectra were obtained on a Hitachi F4500 fluorescence spectrofluorimeter equipped with a thermostated circulating water bath. Tryptophan residues were excited at 295 nm (Freifelder et al. 1982) using a 2.5 nm slit width and emission spectra were recorded from 300-400 nm with a 5 nm slit width. Protein samples were 15 μ M in a 10 mm pathlength quartz cuvette. The instrument was set to 700 V with a scan speed of 240 nm/min

2.2.5 ANS binding experiments

The 1-anilino-8-naphthalene sulphonic acid (ANS) binding assay was used to assess changes in clustered exposed hydrophobicity upon interaction of proteins with α -crystallin and/or dextran. ANS binds to exposed hydrophobic clusters of residues of proteins with a resultant increase in fluorescence (Matulis et al. 1998 a and b). κ -Casein (10 μ M), α -lactalbumin (10 μ M) and α -crystallin (10 μ M) were incubated in the presence and absence of each other and 10% w/v dextran with 20 mM DTT at 37°C for 3 hrs. Insulin (10 μ M)

and α -crystallin (10 μ M) were incubated in the presence and absence of each other and 10% w/v dextran with 20 mM DTT at 37°C for 1 h. The experiments were performed in 50 mM phosphate buffer, pH 7.4 for κ -casein and α -lactalbumin and pH 8.0 for insulin, and 0.05% (w/v) NaN_3 . The ANS fluorescence was measured on a Hitachi F-4500 spectrofluorimeter with a thermostated circulating water bath at 37°C. The excitation and emission wavelengths were set to 400 nm and 550 nm, with the 5 and 10 nm slit widths, respectively. The fluorescence emission intensity was measured in a 10 mm pathlength quartz cuvette in 1 mL samples titrated with 3 μ L aliquots of a 10 mM ANS stock solution in 50 mM phosphate buffer, pH 7.4 and 0.05% (w/v) NaN_3 , with 1 minute of stirring after each addition, until fluorescence intensity reached a plateau.

2.2.6 Circular Dichroism (CD) spectroscopy of κ -casein and α -crystallin

The overall tertiary structure of κ -casein in the presence and absence of dextran was examined using near-UV CD spectroscopy (250-350 nm). The sample consisted of 4 mg/mL κ -casein, 10% w/v dextran and 20 mM DTT in 10 mM phosphate buffer, pH 7.0. The measurements were taken in 1 cm pathlength cuvette using a JASCO J-810 spectropolarimeter (Jasco, Victoria, Canada) connected to Jasco water bath. Experiments were conducted at 25°C.

The temperature dependence of tertiary structure of α -crystallin in the presence and absence of dextran was studied using near-UV CD

spectroscopy (250-350 nm). The sample consisted of 3 mg/mL α -crystallin in 10 mM sodium phosphate buffer, pH 7.0 with or without 10% w/v dextran. The measurements were taken in a 1 mm pathlength cell with a JASCO J-810 CD spectropolarimeter (Jasco, Tokyo, Japan) connected to a Jasco CDF 426S temperature controller. Experiments were conducted between 25°C and 48°C

Spectra were recorded with a data interval of 1 nm, response time of 4 seconds and a scan rate of 100 nm/min. Each spectrum was an average of 4 scans, with a baseline scan subtracted. The data were normalized using the following formula: $[\Theta] = \frac{\Theta \times 100 \times MW}{c \times \alpha \times NA}$ in which Θ is the molar ellipticity (deg), MW is molecular mass (in kDa), c is the concentration of protein (mg/mL), α is the path length (cm) and NA is the number of amino acid residues of the protein concerned.

2.2.7 Nuclear Magnetic Resonance (NMR) Spectroscopy

2.2.7.1 ^1H NMR spectroscopy of α -lactalbumin and κ -casein in the presence and absence of α -crystallin and dextran

To investigate the effect of dextran on α -lactalbumin, κ -casein and the chaperone action of α -crystallin all NMR samples contained α -lactalbumin (2 mg/mL) and κ -casein (2.5 mg/mL) alone and in the presence or absence of α -crystallin and/or dextran (10% w/w) spectra were acquired in 0.6 mL of 50 mM sodium phosphate buffer, pH 7.4 in 100% D_2O , 1 mM EDTA at 37°C. In the case of α -lactalbumin 0.1 M NaCl was also present.

Reduction of the disulphide bonds of α -lactalbumin and κ -casein was achieved by the addition of deuterated DTT to a final concentration of 20 mM. The residual water resonance was suppressed by presaturation during the recycle delay using a low powered pulse via the transmitter (Smallcombe et al. 1995). A series of spectra were acquired over 4 hrs following reduction. Acquisition time for each spectrum was 46 seconds. ^1H NMR spectra were acquired at 500 MHz on a Varian Inova 500 MHz spectrometer. A spectral width of 6000 Hz and 16 scans per spectrum was used for ^1H 1D spectra. The spectra were Fourier transformed after exponential multiplication of the free induction decay with a line broadening of 3.0 Hz.

2.2.7.2 TOCSY and NOESY experiments

Samples of bovine α -lactalbumin (4.2 mg/mL) were prepared in 0.6 mL of 50 mM phosphate buffer, pH 7.4, 0.1 M NaCl, 1 mM EDTA, made up with 90% MilliQ H_2O and 10% D_2O . Samples containing κ -casein at a concentration of 2.5 mg/mL were prepared in 0.6 mL of 50 mM phosphate buffer, 2 mM EDTA, pH 7.4 made up with 100% D_2O . Dogfish α -crystallin at concentration of 20 mg/mL was dissolved in 0.6 mL of 10 mM phosphate buffer, pH 6.5, 0.03% (w/v) NaN_3 made up with 90% MilliQ H_2O and 10% D_2O .

Total Correlation Spectroscopy (TOCSY) ^1H - ^1H 2D spectra of α -lactalbumin and κ -casein were acquired with 256 t_1 increments with 80 and 64 scans per increments and a 70 ms mixing time. TOCSY and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were employed in the phase sensitive

mode using hypercomplex conjugation (Jeener et al. 1979, States et al. 1982). The intense water peak was suppressed by the WATERGATE pulse sequence in the TOCSY experiments (Piotto et al. 1992) and by wet methods in the NOESY experiments (Smallcombs et al. 1995). A spin-lock period of 65 ms was employed in TOCSY experiments of dogfish α -crystallin. In TOCSY experiments, 32 scans were acquired for each of the 256 t_1 increments. The FID in t_2 consisted of 4096 data points over a spectral width of 8000 Hz. A mixing time of 100 ms was used in the NOESY spectra with 80 scans for each of the 256 t_1 increments. The FID in t_2 consisted of 2048 data points over a spectral width of 6000.6 Hz. Prior to the second Fourier transformation, all spectra were processed using a shifted Gaussian function in both directions.

The processing of spectra obtained from 1D and 2D NMR spectra were conceded on a Sun Microsystems Sunblade 150 workstation with Varian VNMR software. Real time 1D ^1H NMR data were analyzed using SigmaPlot software (version 8.0).

2.2.8 Size exclusion HPLC

2.2.8.1 Size exclusion HPLC of κ -casein in the presence and absence of different concentration of dextran

The size of κ -casein aggregates in the absence and presence of different concentrations of dextran was determined by size-exclusion chromatography. A Phenomenex[®] BioSep[™] SEC S4000 column, 300 × 4.60 mm

(Phenomenex[®], Torrance, U.S.A.) with a mobile phase of 50 mM sodium phosphate buffer, pH 7.2, 0.05% (w/v) NaN₃, 1 mM EDTA was used on a Shimadzu HPLC system with a SLC-10A vp pump system organizer, with a PDA detector (Tokyo, Japan). This column has a mass range of 14.6 MDa to 2 kDa for native proteins. Samples containing κ -casein (10 mg/mL) in different conditions and standards at 10 mg/mL, including blue dextran (2.0 MDa), bovine thyroglobulin (670 kDa), catalase (250 kDa), ovotransferrin (78 kDa), and bovine serum albumin (BSA) (67 kDa) were prepared in 50 mM sodium phosphate buffer, pH 7.2, 0.05% (w/v) NaN₃, 1 mM EDTA. Before injection, samples were centrifuged at 14000 rpm in a Beckman Ultrafuge E benchtop for 5 minutes and 25 μ L of the supernatants were injected onto the column. The buffer flow rate was 0.5 mL/min and protein elution was detected at 280 nm. Blue dextran elution was detected at 600 nm.

2.2.8.2 Time course size exclusion HPLC of κ -casein in the presence and absence of 10% w/v dextran

The size of κ -casein (10 mg/mL) aggregates in the absence and presence of 10% w/v dextran at different time (0, 2, 4, 6, 8, and 24 min) after incubation in 10 mM phosphate buffer, pH 7.0 at 37°C were determined by size-exclusion chromatography after injecting 25 μ L of the samples. The column used was the same as that for κ -casein in different concentrations of dextran (Section 2.2.8) on Shimadzu HPLC system by a SLC-10A vp pump system organizer, with a Dual wavelength detector and manual injection (Tokyo, Japan). The manual injection caused different retention time. The aggregate size of samples calculated using standard curve and compared.

2.2.9 Transmission electron microscopy (TEM) of α_s - and κ -caseins

Formvar and carbon-coated nickel TEM grids (SPI Supplies, Wester Chester, USA) were prepared by the application of 2 μ L protein sample at a concentration of 1 mg/mL. The grids were then washed three times with 10 μ L MilliQ water and negatively stained with 10 μ L uranyl acetate (2% w/v; Agar Scientific, UK). The grids were dried with filter paper between each step. The samples were viewed under 25-64 K magnifications at 80 kV excitation voltages using a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands). The dimensions of the fibrils were estimated by observing the images at 200% zoom in paintbrush (Microsoft Corporation, USA) and by comparing them with the length of the scale bar using ImageJ software.

2.2.10 Isolation and purification of α A- and α B-crystallin from bovine α -crystallin

The purification of bovine α A- and α B-crystallin was performed according to the method of Stevens et al. (1987). These proteins were separated using size-exclusion chromatography on a Sephadex G-75 column with a diameter of 2 cm and a length of 80 cm. 20 mg of α -crystallin was loaded on the column after being dissolved in a column buffer (0.1 M glycine, pH 2.5). The buffer was passed through the column at a flow rate of 17 mL/hr and fractions collected at 9 minute intervals. Fractions containing α A-crystallin (elution time of approximately 6 hrs) were combined and concentrated to a volume of

about 2 mL using an Amicon ultrafiltration cell fitted with a Diaflo membrane of 10 kDa cut-off, and then dialyzed against 2 liter MilliQ water for 24 hrs at 4°C. The protein was stored in a freeze-dried form. Fractions containing bovine α B-crystallin (elution time 8 hrs) were also collected using the method described above. The chaperone activity of α A-crystallin was measured as described previously (Section 2.2.2).

2.2.11 Protein characterization by sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the purity of crystallin fractions and α A- and α B-crystallin subunits following chromatographic separation. It was also used to assess the extent of aggregation of heated β -lactoglobulin in the presence and absence of α -crystallin and dextran at different pH values.

The samples of protein were prepared in 10 μ L of distilled water and 10 μ L of loading dye (4% (w/v) SDS, 0.1 M Tris-HCl, 0.2% (w/v) bromophenol blue, and 20% (v/v) glycerol). For reduced samples 0.2 M DTT was added to the loading dye buffer. For β -lactoglobulin samples, 100 μ L of heated β -lactoglobulin solution was taken and pellets were removed by centrifugation. For reduced sample, the protein was dissolved in 25 μ L of phosphate buffer and 25 μ L of reduced loading dye buffer (1 M DTT was added to cracking buffer) was added. For non-reduced samples, proteins were dissolved in 25 μ L of phosphate buffer and 25 μ L of non-reduced loading dye buffer. To obtain information on heated β -lactoglobulin, which

was not aggregated, 25 μ L of supernatant of heated sample were taken and then 25 μ L of reduced buffer were added to supernatant sample.

Samples were boiled for 10 to 15 min and then loaded on to 15% (v/v) polyacrylamide resolving and 4% (v/v) stacking gel for separation. A Bio-Rad MiniProtein II or III apparatus with a Bio-Rad 1000/ 500 power supply were used to electrophorese the gels. Proteins were separated at 140-160 V for 1-1.5 hrs using SDS-PAGE running buffer (.01 M Tris base, 0.1 M glycine, 0.5% (w/v) SDS). Gels were stained overnight by shaking in Coomassie R Brilliant Blue. The gel background was destained in 10% (v/v) acetic acid. Prior to imaging, gels were replaced into a final destain solution which contained 4% (v/v) glycerol for 30 min to prevent cracking upon drying. Gels were imaged using a Canon 9950f image scanner.

2.2.12 Subunit exchange of α A-crystallin

2.2.12.1 Labeling of recombinant α A-crystallin with fluorescence probes

Labeling of α A-crystallin with fluorescent probes 4-acetamido-4'-((iodoacetyl) amino) stilbene-2, 2'-disulfonic acid (AIAS) or Lucifer yellow iodoacetamide (LYI) was performed as described by Bova et al. (1997). 1 mg/mL α A-crystallin was mixed with solid AIAS or LYI, separately to final concentration of 3.2 mM and 8.4 mM, respectively in 100 mM NaCl, 20 mM MOPS, pH 7.9. The reaction proceeded for 12 hrs at room temperature (22°C) in the dark with an additional 6 hrs for LYI at 37°C in the dark. Labeled α A-crystallin with

AIAS or LYI was separated from unreacted fluorescent probes on a Sephadex G-25 size exclusion column. Fractions were eluted with the column buffer containing 100 mM NaCl, 2 mM DTT, 50 mM sodium phosphate, pH 7.5, at a flow rate of 20 mL/hr and collected at 4 minute intervals. Fractions containing labeled α A-crystallin at an elution time of approximately 2 hrs were pooled and concentrated in an Amicon ultrafiltration cell fitted with a Diaflo membrane with a 10 kDa cut-off to a volume of approximately 2 mL and then dialyzed against MilliQ water over night, and freeze-dried before storage.

2.2.12.2 Measurement of the rate of subunit exchange

The rate of subunit exchange was measured by the detection of fluorescence energy transfer (FRET) as described by Bova et al. (1997) at 37, 42, and 45°C. Equal amounts (0.4 mg/mL) of AIAS-labeled α A-crystallin and LYI-labeled α A-crystallin were combined to initiate exchange reaction in 100 mM NaCl, 2 mM DTT, 50 mM sodium phosphate, pH 7.5 at the temperature concerned (Bova et al. 1997). The fluorescence emission spectra were recorded using Hitachi F-4500 spectrofluorimeter with a 2.5 and 10 nm slit widths for emission and excitation, respectively. For AIAS labeled α A-crystallin the excitation wavelength was 335 nm, while for LYI labeled α A-crystallin, 435 nm was used. The fluorescence intensity of α A-crystallin was measured at 415 nm and 545 nm for AIAS labeled α A-crystallin and LYI labeled α A-crystallin, respectively. Aliquots of 20 μ L were sampled over 4 hrs and the rate constants (k) of subunit exchange were determined by fitting an exponential function to the fluorescence intensity data, i.e. $F(t) = C_1 + C_2 e^{-kt}$

for LYI and $F(t) = C_1 + C_2 (1 - e^{-kt})$ for AIAS respectively, using Sigmaplot software (version 8.0). $F(t)$ is the exponential function of the fluorescence intensity at 415 nm for AIAS and 545 nm for LYI for each given time point, C_1 and C_2 are fluorescence intensities determined using the condition $C_1 + C_2 = 1$ at time 0, C_1 is the fluorescence intensity at any given time and t is the time (in minutes).

2.2.12.2.1 The effect of 10% w/v dextran on the rate of subunit exchange in α A-crystallin in the presence and absence of reduced ovotransferrin

The effect of dextran on the rate of subunit exchange was determined by the addition of 10% w/v dextran to the α A-crystallin labeled with either AIAS or LYI before combining these two solutions. The exchange rates were determined as described above.

To investigate the effect of reduced ovotransferrin in its molten-globule conformation on the rate of subunit exchange of α A-crystallin, 0.66 mg/mL of reduced ovotransferrin was added to the labeled α A-crystallin (0.4 mg/mL). AIAS-labeled and LYI-labeled α A-crystallin was incubated separately with ovotransferrin and 20 mM DTT for 3 hrs at 37°C prior to mixing equal amounts of both solutions. The rate of subunit exchange was determined as described above.

To investigate the effect of reduced ovotransferrin in the presence of dextran on the rate of subunit exchange in α A-crystallin, 0.66 mg/mL of ovotransferrin

and 10% dextran (w/v) were added to the labeled α A-crystallin in 100 mM NaCl, 2 mM DTT, 50 mM sodium phosphate, pH 7.5. AIAS-labeled and LYI labeled α A-crystallin were incubated separately with ovotransferrin and 20 mM DTT and 10% w/v dextran for one hour at 37°C. After incubation the rate of subunit exchange was determined as described above.

Chapter 3

**The effect of dextran on the amorphous
aggregation of target proteins and the
chaperone action of α -crystallin**

Many studies have shown that change in solution conditions like heating or mutation cause damage and misfolding of protein structures (Benjamin et al. 1998, Dobson et al. 2001). As a result of their misfolding, proteins expose their normally buried hydrophobic regions to the environment. Hydrophobic interactions of nearby molecules cause self association and aggregate formation (Carver et al. 1995, Benjamin et al. 1998). However, sHsps (which are molecular chaperones) prevent the aggregation and precipitation of proteins on their irreversible unfolding pathway (Hendrick, et al. 1993).

α -Crystallin acts as a molecular chaperone interacting with the non-native conformations of proteins to prevent their aggregation. The chaperone action of α -crystallin has been until now, investigated in the absence of crowding agents (Lindner et al. 1997, Lindner et al. 2001, Carver et al. 2002), a situation which was not representative of the behaviour of this protein *in vivo* (Section 1.4). Thus to mimic the chaperone action of α -crystallin *in vivo*, this study explored the effects of dextran (a well known crowding agent) on protein aggregation and α -crystallin's ability to suppress it. Dextran has been chosen because it is an uncharged, inert polymer (Sasahara et al. 2003, Banks et al. 2005, Winzor et al. 2006).

Reduction experiments were performed with different target proteins: ovotransferrin, α -lactalbumin and insulin, all of which have disulfide bonds and have been well characterized (Farahbaksh et al. 1995, Lindner et al. 1998, Carver et al. 2002). Different target proteins in size were used to investigate the effect of dextran on the rate their aggregation. At 37°C, the disulfide bonds in

these proteins can be reduced by the addition of the reducing agent DTT without the complication of alteration in the structure of α -crystallin which does not contain any disulphide bonds. Disruption of these bonds leads to the unfolding and aggregation of target proteins. The kinetics of aggregation and the effectiveness of α -crystallin to prevent the aggregation of these target proteins were compared in the presence and absence of dextran by visible absorption spectroscopy and NMR spectroscopy. Intrinsic tryptophan fluorescence spectroscopy and ANS-fluorescence binding were used to explore the effects of dextran on the chaperone action of α -crystallin.

β_L -Crystallin, a protein of the eye lens, is a natural target protein for α -crystallin as a chaperone. The aggregation of heat-stressed β_L -crystallin was monitored at 60°C, in the same manner as for a reduction assay, and the efficiency of the chaperone action of α -crystallin in the presence and absence of dextran was compared.

3.1 Purification of α -crystallin

The purification of α -crystallin was performed using the method of Slingsby et al. (1990) as described in the Materials and Methods (Section 2.2.1). Figure 3.1 shows a typical elution profile for crystallins from a single lens on a size exclusion Sephacryl S-300 column. Elution of protein was detected at 280 nm. The elution time of α -crystallin was approximately 10.5 hours followed by 12.3, 14.5, 16.2 and 17.7 hours for β_H -, β_L -, γ_S - and γ_T -crystallins, respectively. To achieve better resolution and maximum purity of the crystallins, pooled and

reconcentrated fractions from the first chromatographic run of crystallins were rechromatographed. The purity of α -crystallin was checked by SDS-PAGE electrophoresis (not shown).

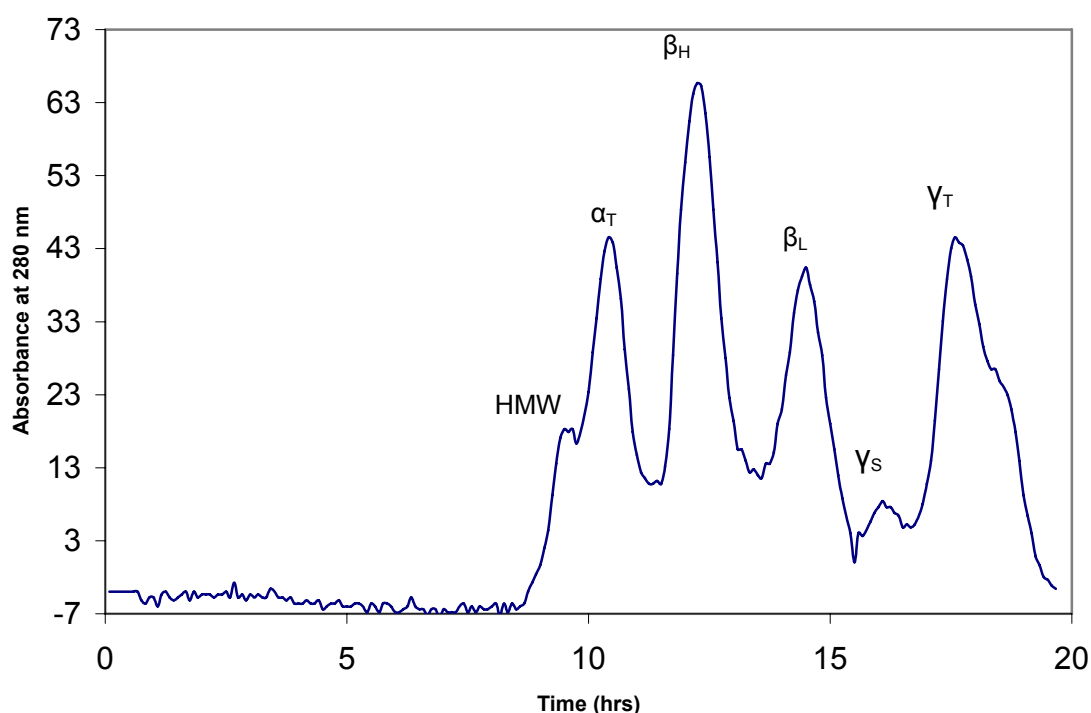


Figure 3.1: Elution profile for lens crystallins on a Sephacryl S-300 column. Column buffer (50 mM Tris, pH 7.4, 0.05% (w/v) NaN₃) was passed through the column (2.6 cm diameter and 100 cm length) at a flow rate of 20 mL/hr and fractions were collected at 20 minute intervals. The first peak to elute is HMW, which is due to α -crystallin forming a high molecular weight aggregate, followed by α -crystallin, β_H - and β_L -crystallins and lastly γ -crystallins: γ_S - and then γ_T -crystallin.

3.2 Visible Absorption Spectroscopy

In order to study the role of α -crystallin as a molecular chaperone preventing aggregation of proteins and the effect of crowding agents on the chaperone action of α -crystallin, visible absorption spectroscopy was applied. The unfolding, aggregation and precipitation of these proteins was induced in the case of ovotransferrin, α -lactalbumin and insulin by the reduction of their

disulfide bonds using DTT, and in the case of β_L -crystallin by heating. One can monitor these processes by observing the increase in light scattering at 360 nm over time. The ability of α -crystallin to prevent protein aggregation can be measured if a reduction in light scattering is observed relative to the control sample. The chaperone action of α -crystallin which prevents the aggregation and precipitation of stressed, destabilized target proteins has been demonstrated in a variety of studies (Horwitz et al. 1992, Horwitz et al. 1998, Lindner et al. 1998, Lindner et al. 2001).

3.2.1 The effect of dextran on the aggregation of reduced ovotransferrin

Chicken ovotransferrin has a mass of 78 kDa and is a primary egg protein with antibacterial properties (Awade et al. 1994). Ovotransferrin controls the levels of iron, copper and aluminum in the body fluids of vertebrates, and its concentration is elevated during infection or inflammation (Kurokawa et al. 1999, Narayan et al. 2002, Narayan et al. 2003). Ovotransferrin consists of two similarly sized homologous N- and C-lobes (Kurokawa et al. 1995, Kurokawa et al. 1999). Each lobe contains a single iron-binding site, which is divided into dissimilar domains (N_1 and N_2 for the N-terminal lobe and C_1 and C_2 for the C-terminal lobe). The two iron-binding sites are located within the inter domain cleft of each lobe (Kurokawa et al. 1995, Mizutani et al. 1999). Chicken ovotransferrin has 6 disulfide bridges in the N-lobe: 2 bridges in the N_1 and 4 bridges in the N_2 lobes (Kurokawa et al. 1995 Kurokawa et al. 1999). The

C-terminal lobe has these 6 disulfide bridges and three additional disulfides bridges (Kurokawa et al. 1995).

Ovotransferrin aggregates readily after addition of DTT to reduce its disulphide bonds, as observed before (Lindner et al. 1998). Thus, the increase in light-scattering at 360 nm with time reflects the aggregation of reduced ovotransferrin. The effect of macromolecular crowding on the rate of aggregation and precipitation of ovotransferrin and other proteins and on the chaperone activity of α -crystallin was investigated by mimicking the crowded conditions within the cell by the addition of a macromolecular crowding agent, dextran (68,800 kDa) at levels of (0%, 2%, 5% and 10% w/v). According to Figure 3.2, the rate of aggregation of reduced ovotransferrin increased and the time to the onset of aggregation (the lag phase) decreased as the concentration of dextran increased. This implies that macromolecular crowding enhanced aggregation of ovotransferrin.

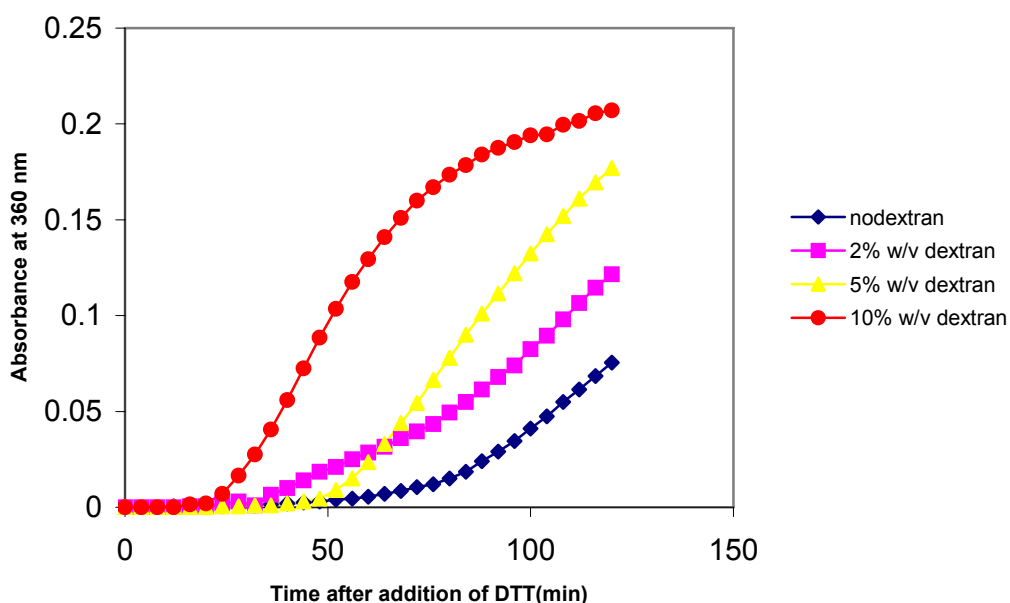


Figure 3.2: Ovotransferrin (1.4 mg/mL) aggregation in the presence of dextran (w/v). The protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , 1 mM EDTA, pH 7.4, and the incubating temperature was 37°C. The aggregation of ovotransferrin was initiated by the addition of 20 mM DTT. Note that the experiment was done in duplicate.

3.2.2 The effect of dextran on the chaperone action of α -crystallin preventing the aggregation of reduced ovotransferrin

A better insight into the mechanism of the molecular chaperone action of sHsps *in vivo* can be achieved through an examination of protein stabilization under crowded conditions. Therefore, in order to study the chaperone action of α -crystallin under such conditions, dextran was added to 10% w/v, a level that led to a significant enhancement of aggregation of ovotransferrin (Figure 3.2). The aggregation of ovotransferrin was monitored in the absence and presence of 10% w/v dextran and varying concentration of α -crystallin. In the presence of 10% w/v dextran, aggregation proceeded much more rapidly than in its absence (Figure 3.3B), with the onset of aggregation at 60 minutes after the addition of

DTT, compared to 96 minutes in the absence of dextran. The extent of aggregation of ovotransferrin was three times higher in the presence of dextran than in its absence (Figures 3.2A and B). As shown in Figure 3.3A, α -crystallin significantly delayed the onset of the aggregation of ovotransferrin and reduced the rate of this process in the absence of the crowding agent. In the absence of dextran, at the time point of 174 minutes, the aggregation of ovotransferrin was prevented by 89% at a 3:1 molar ratio of α -crystallin to ovotransferrin. The efficiency of α -crystallin as a molecular chaperone decreased significantly in the presence of dextran as demonstrated by the limited protection against the aggregation of ovotransferrin. In the presence of dextran and at a 3:1 molar ratio of α -crystallin to ovotransferrin only 23% of the aggregation of ovotransferrin was prevented after 174 min. Moreover, the first-order rate

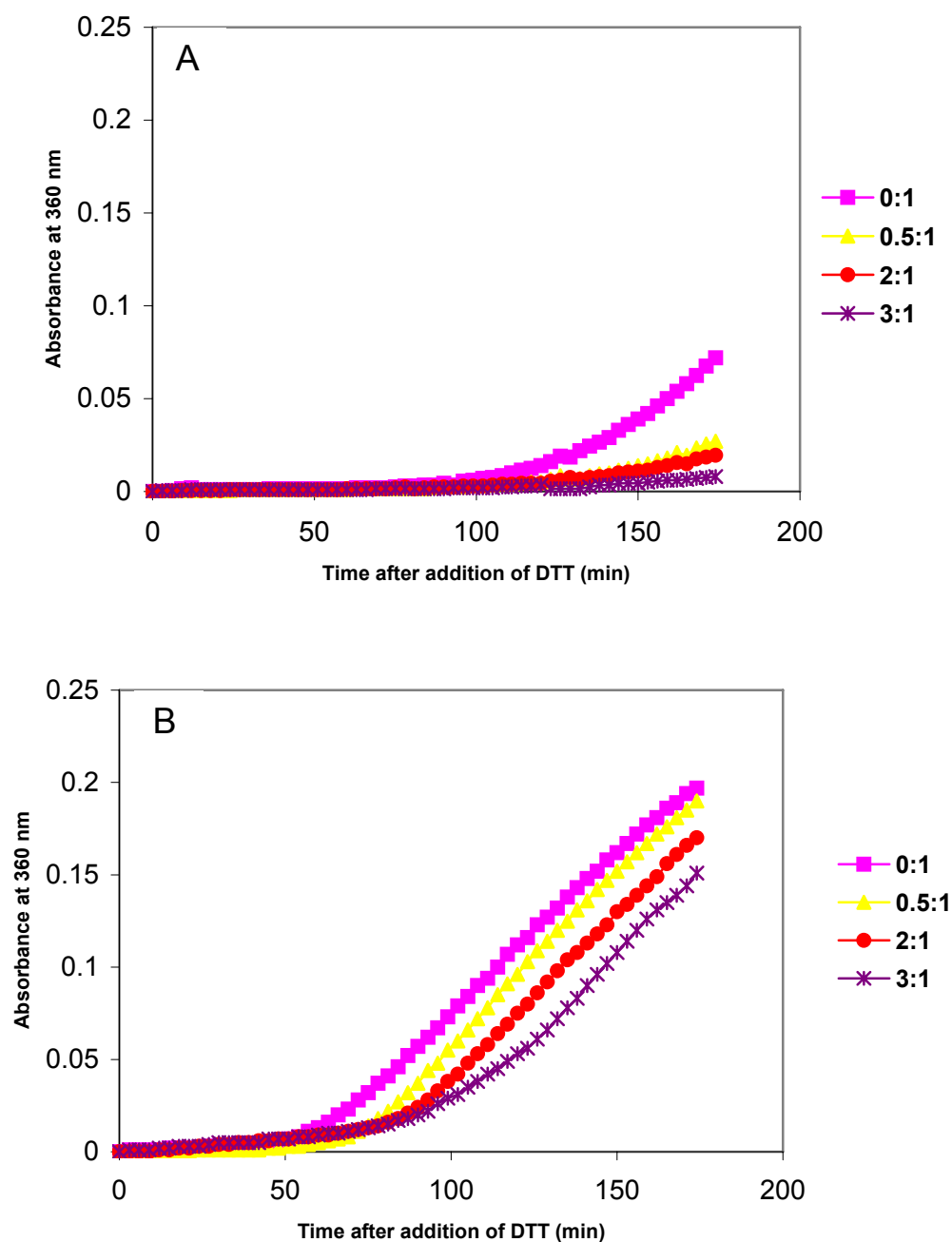


Figure 3.3: Ovotransferrin (1.4 mg/mL) aggregation in the presence of (A) α -crystallin and (B) α -crystallin and 10% w/v dextran. The proteins were dissolved in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , 1 mM EDTA, pH 7.4, and incubated at 37°C. The aggregation of ovotransferrin was initiated by the addition of 20 mM DTT. The ratios indicated are of α -crystallin:ovotransferrin on a molar subunit ratio. Note that the experiment was done in duplicate.

constant for the increase in aggregation of ovotransferrin in the presence of α -crystallin (at a 1:3 molar ratio) increased from $(1.86 \pm 1.07) \times 10^{-2} \text{ min}^{-1}$ to $(3.62 \pm 3.80) \times 10^{-2} \text{ min}^{-1}$ with the addition of 10% w/v dextran. Thus, based on the measurement in the presence of molecular crowding agent, the effectiveness of α -crystallin to protect ovotransferrin from aggregation was reduced ~ 2 fold.

3.2.3 The effect of dextran on the aggregation of reduced insulin

Insulin, with a molecular mass of 5.7 kDa, is a hormone secreted from pancreas in response to an increase in blood sugar levels. Insulin helps the cells take in glucose from the bloodstream and thus plays a role in energy metabolism (Nielsen et al. 2001, Bluher et al. 2002, Bluher et al. 2004). Insulin is comprised of two chains, A and B, which are joined by two disulphide bonds. There is also an intra-chain disulphide bond between residues A6 and A11-Ser in the A chain (Yuan et al. 1999). Insulin molecules have a tendency to form dimers in solution due to hydrogen-bonding between the C-termini of the B chains. Additionally, in the presence of zinc ions, insulin dimers associate into hexamers. Upon reduction of insulin, the B chain aggregates and precipitates whereas the A chain remains in solution (Horwitz et al. 1992).

The effect of macromolecular crowding on the aggregation and precipitation of a much smaller protein, insulin (5.7 kDa), was studied by visible absorption spectroscopy. Different concentrations of dextran (0%, 2%, 5% and 10% w/v)

were used to simulate the crowded cellular interior. An increase in light scattering of reduced insulin with the concentration of dextran was observed (Figure 3.4) which indicates that dextran increased the rate of aggregation and precipitation of reduced insulin, even though the time to the onset of aggregation was not affected. The effect of dextran on the rate of insulin aggregation is evident from the rate constant of insulin alone which was $(1.76 \pm 0.051) \times 10^{-2} \text{ min}^{-1}$, while in the presence of 10% w/v dextran it increased to $(7.75 \pm 0.41) \times 10^{-2} \text{ min}^{-1}$.

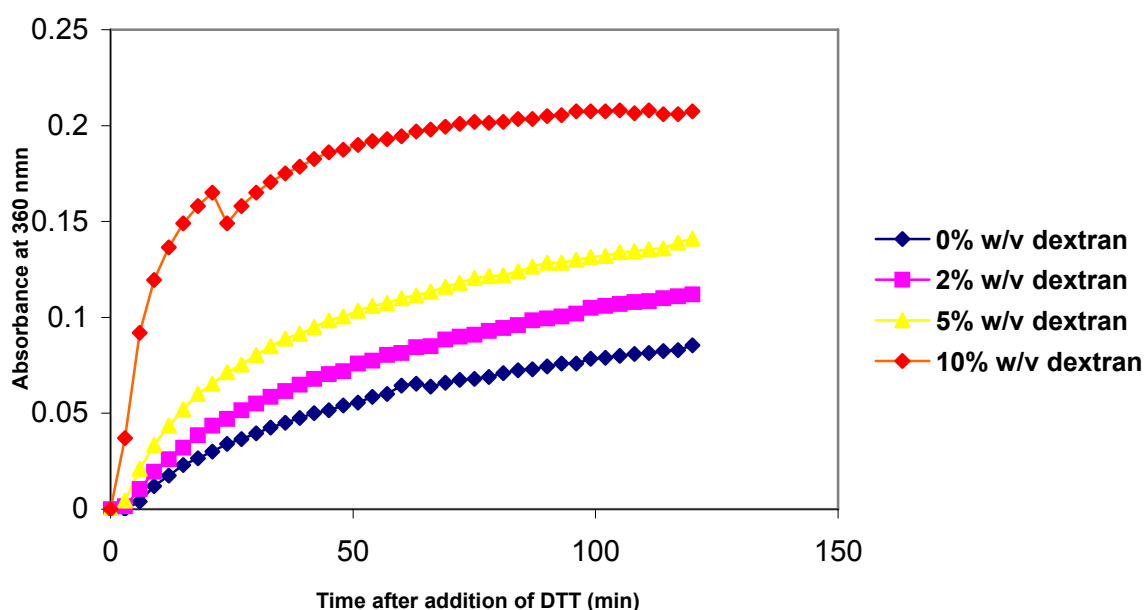


Figure 3.4: Insulin (0.42 mg/mL) aggregation in the presence of dextran (w/v). The protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , 1 mM EDTA, and pH 7.4, at 37°C . The aggregation of the insulin B chain was initiated by the addition of 20 mM DTT. Note that the experiment was done in duplicate.

3.2.4 The effect of dextran on the chaperone action of α -crystallin with aggregating reduced insulin

The chaperone-like action of α -crystallin in preventing the aggregation of insulin was examined in the absence of dextran. Reduced insulin aggregated immediately in the absence of dextran and this aggregation was prevented by a 2-fold molar excess of α -crystallin as shown in Figure 3.5 A. In the presence of 10% w/v dextran, the time to the onset of aggregation remained essentially the same as in its absence i.e. in the absence of 10% w/v dextran aggregation started after approximately 3 minutes, compared to zero minutes in the presence of 10% w/v dextran. Also, dextran had little effect on the extent of aggregation of insulin and the effectiveness of α -crystallin to prevent it (Figures 3.5A and B) (98% efficiency vs 100% in the absence of dextran with 2-fold molar excess of α -crystallin).

3.2.5 The effect of dextran on the chaperone action of α -crystallin with aggregating reduced α -lactalbumin

Bovine α -lactalbumin, with molecular mass of 14,200 KDa (Acharya et al. 1991), is a globular protein found in milk (Ren et al. 1993). It contains four disulfide bonds and chelates calcium and zinc ions, thus modulating their absorption (Kelleher et al. 2003). A partly folded state of α -lactalbumin can be induced by removing the calcium ion at neutral pH (Laureto et al. 2002, Engel et al. 2002),

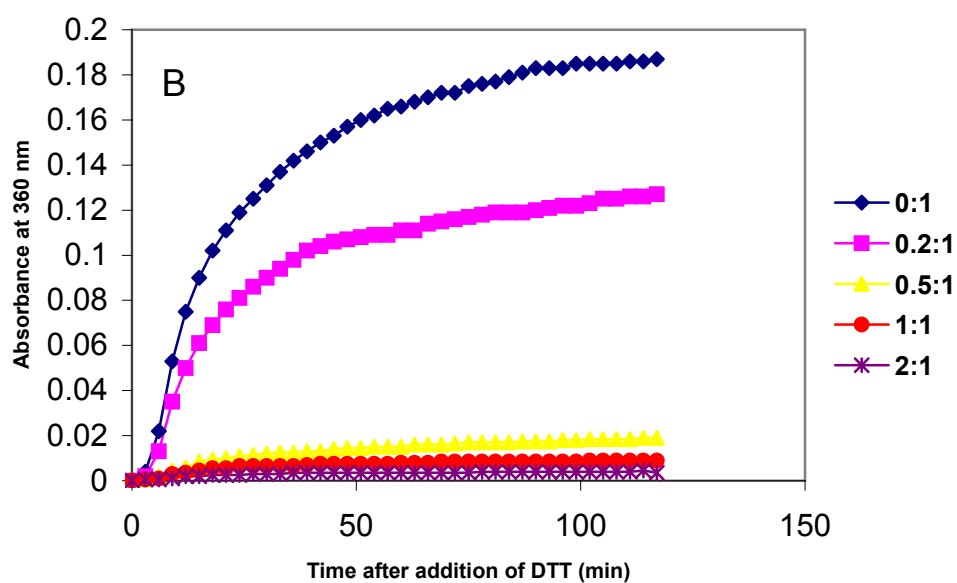
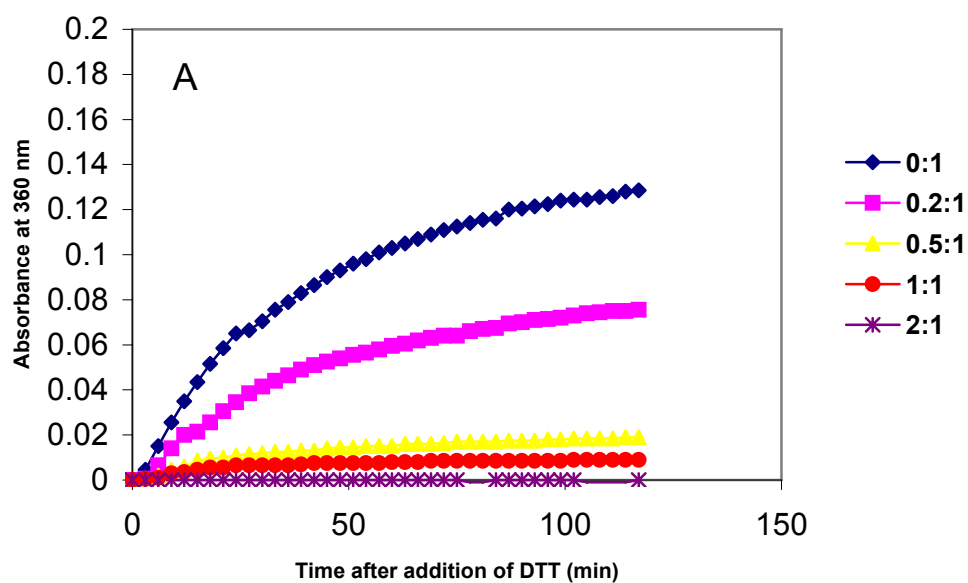


Figure 3.5: Insulin (0.42 mg/mL) aggregation in the presence of (A) α -crystallin and (B) α -crystallin and 10% w/v dextran. Protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , 1 mM EDTA, pH 7.4, and the incubation temperature was 37°C. The aggregation of insulin was initiated by the addition of 20 mM DTT. The molar ratios of α -crystallin:insulin are indicated. Note that the experiment was done in duplicate.

which makes the disulfides prone to reduction and leads to protein unfolding (Carver et al. 2002). This form of α -lactalbumin, called the 'molten globule' state, has little tertiary structure but retains most elements of its secondary structure (Carver et al. 1995). The molten globule state of α -lactalbumin is thought to be a target for interacting with sHsps (Kuwajima et al. 1995, Lindner et al. 1997, Lindner et al. 2001, Carver et al. 2002). Being an unstable state, it can aggregate and precipitate from solution. This aggregation is suppressed by molecular chaperones such as sHsps (Lindner et al. 1997, Carver et al. 2002).

Previous study (Lindner et al. 1997, Carver et al. 2002) showed that α -lactalbumin interacts with α -crystallin following the reduction of α -lactalbumin with DTT. Figure 3.6 shows the action of α -crystallin to prevent aggregation and precipitation of reduced α -lactalbumin (in 50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 0.05% (w/v) NaN_3 , at 37°C). Aggregation of α -lactalbumin was suppressed by α -crystallin in a concentration-dependent manner, e.g. 1:1 w:w ratio of α -crystallin: α -lactalbumin provided a 91% protection against aggregation and precipitation at time point of 123 minutes. This is in agreement with Carver et al. (2002) who found 97% protection under the same experimental conditions. Dextran increased the rate of aggregation of reduced α -lactalbumin and the time to the onset of aggregation significantly decreased from 36 min in the absence of dextran to 3 min (Figures 3.6 and 3.7).

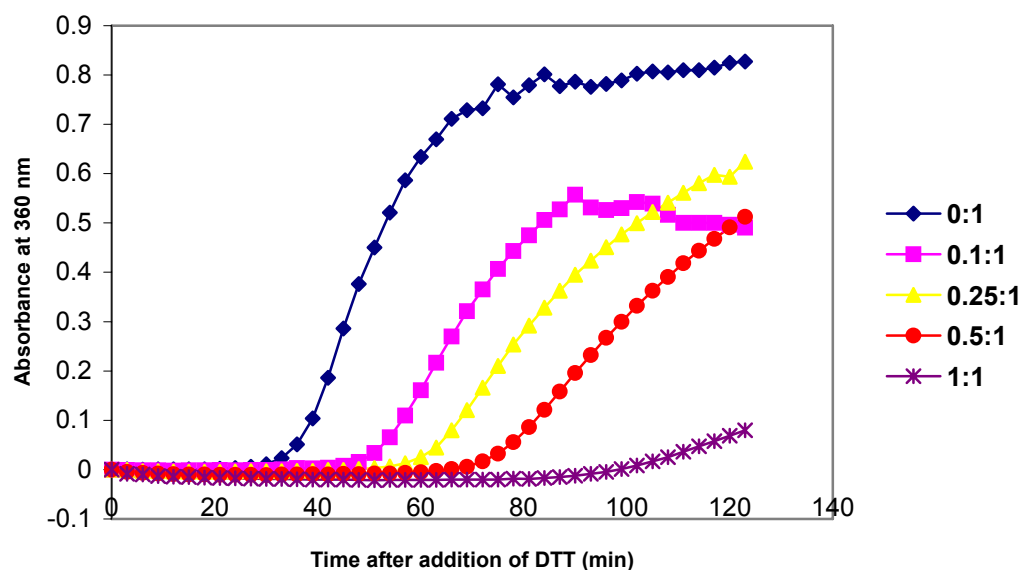


Figure 3.6: α -Lactalbumin (2 mg/mL) aggregation in the presence of α -crystallin. The protein was in 50 mM phosphate buffer, 1 mM EDTD, 0.1 M NaCl, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C. The induced aggregation of α -lactalbumin was initiated by the addition of 20 mM DTT. The ratios indicated are of α -crystallin: α -lactalbumin, in w:w ratio. Note that the experiment was done in duplicate.

In the absence of dextran, α -crystallin at 1:1 w:w ratio provided a 91% protection at the end point (123 minutes), while in the presence of dextran, α -crystallin prevented only 70% of α -lactalbumin aggregation (Figure 3.6 and 3.7). This is also evident from rate constants of α -lactalbumin and α -crystallin in 1:1 w:w ratio, which in the absence of dextran was $(4.43 \pm 0.26) \text{ min}^{-1}$, while in the presence of 10% w/v dextran it increased to $(9.76 \pm 0.68) \text{ min}^{-1}$. Thus in the presence of dextran α -crystallin is a less effective chaperone towards reduced α -lactalbumin.

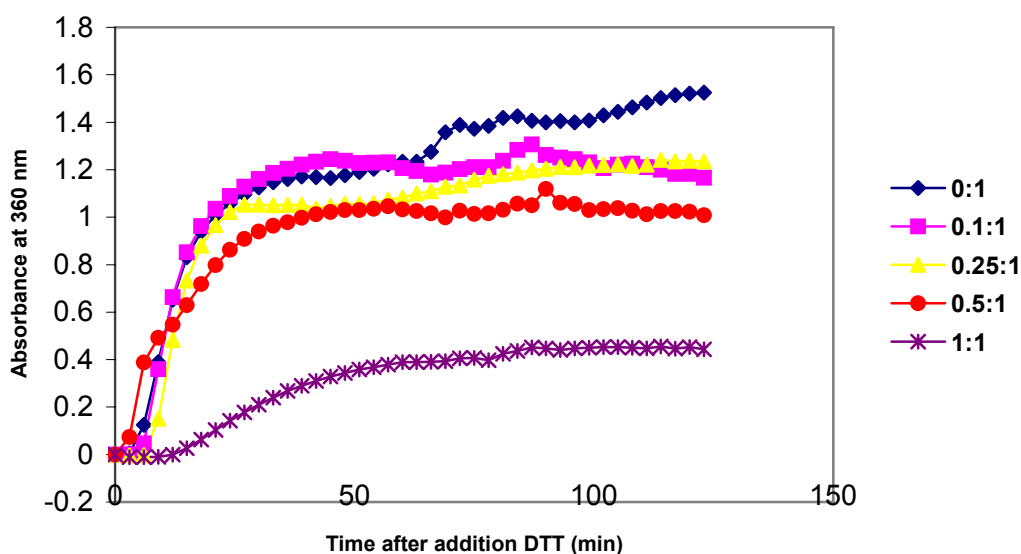


Figure 3.7: α -Lactalbumin (2 mg/mL) aggregation in the presence of 10% w/v dextran and α -crystallin. The protein was in 50 mM phosphate buffer, 1 mM EDTA, 0.1 M NaCl, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C. Aggregation of α -lactalbumin was initiated by the addition of 20 mM DTT. The ratios indicated are of α -crystallin: α -lactalbumin, in w:w ratio. Note that the experiment was done in duplicate.

Real-time ^1H NMR spectroscopy was performed to analyze in more detail the effect of dextran on the chaperone action of α -crystallin towards reduced α -lactalbumin (Section 3.5).

3.2.6 The effect of dextran on the aggregation of heated β_{L} -crystallin

β_{L} -Crystallin is a chromatographic fraction of lens proteins composed of various β -crystallin subunits e.g. basic βB1 , βB2 , βB3 , and acidic βA1 , βA2 , βA3 , βA4 -crystallin (Jaenicke et al. 1994, Slingsby et al. 1994). These water-soluble proteins play a structural role in the eye lens and are responsible for its transparency (Groenen et al. 1994, Carver et al. 1996). β_{L} -Crystallin is a natural

target protein for chaperone action of α -crystallin in the aging eye lens, and thus often used *in vitro* as a model of a heat-stressed protein (Datta et al. 1999, Derham et al. 2001, Putilina et al. 2003).

The effect of the macromolecular crowding on the aggregation of heated β_L -crystallin, was studied in an analogous fashion to previously described experiments with ovotransferrin, insulin and α -lactalbumin (Sections 3.2.1, 3.2.3, 3.2.5) except that aggregation was induced by heating the sample to 60°C. Similar to the reduction assays described above, dextran increased the rate of aggregation of β_L -crystallin (Figure 3.8) and decreased the time to the onset of aggregation in a concentration-dependent manner.

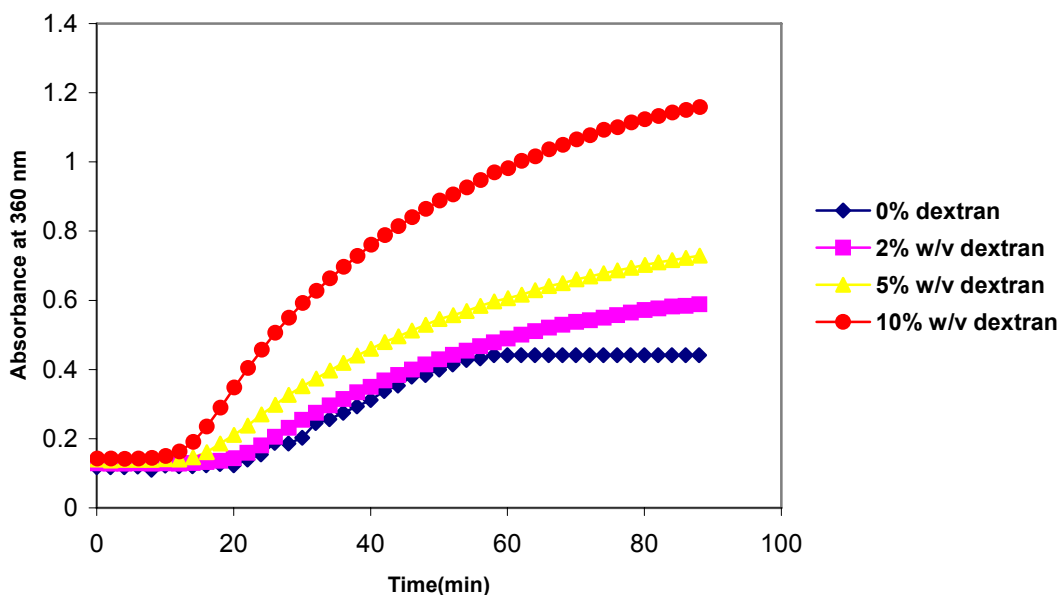


Figure 3.8: β_L -crystallin (0.3 mg/mL) aggregation in the presence of dextran (w/v). The protein was in total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , 1 mM EDTA, pH 7.4, and the incubation temperature was 60°C. Note that the experiment was done in duplicate.

3.2.7 The effect of dextran on the chaperone action of α -crystallin with heated β_L -crystallin

Horwitz et al. (1992) demonstrated that α -crystallin could suppress the heat-induced aggregation of proteins and hence exhibited chaperone activity. To confirm this with a natural target protein of α -crystallin, β_L -crystallin was incubated at 60°C in 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 0.05% (w/v) NaN_3 with increasing w:w ratios of α -crystallin: β_L -crystallin. Under these conditions, a 0.25:1.0 w:w ratio of α -crystallin: β_L -crystallin provided complete protection against aggregation and precipitation of β_L -crystallin (97%) (Figure 3.9A). In the presence of 10% w/v dextran, the rate of aggregation and precipitation of β_L -crystallin more than doubled and the time to the start of aggregation decreased to 14 min compared to 20 min in the absence of dextran (Figures 3.9A and B). Under these conditions and at a w:w ratio of about 1:1 α -crystallin: β_L -crystallin, complete protection of β_L -crystallin from aggregation and precipitation was not achieved and the 0.25:1.0 ratio of α -crystallin: β_L -crystallin only suppressed aggregation by about 73% (compared to 97% in the absence of dextran).

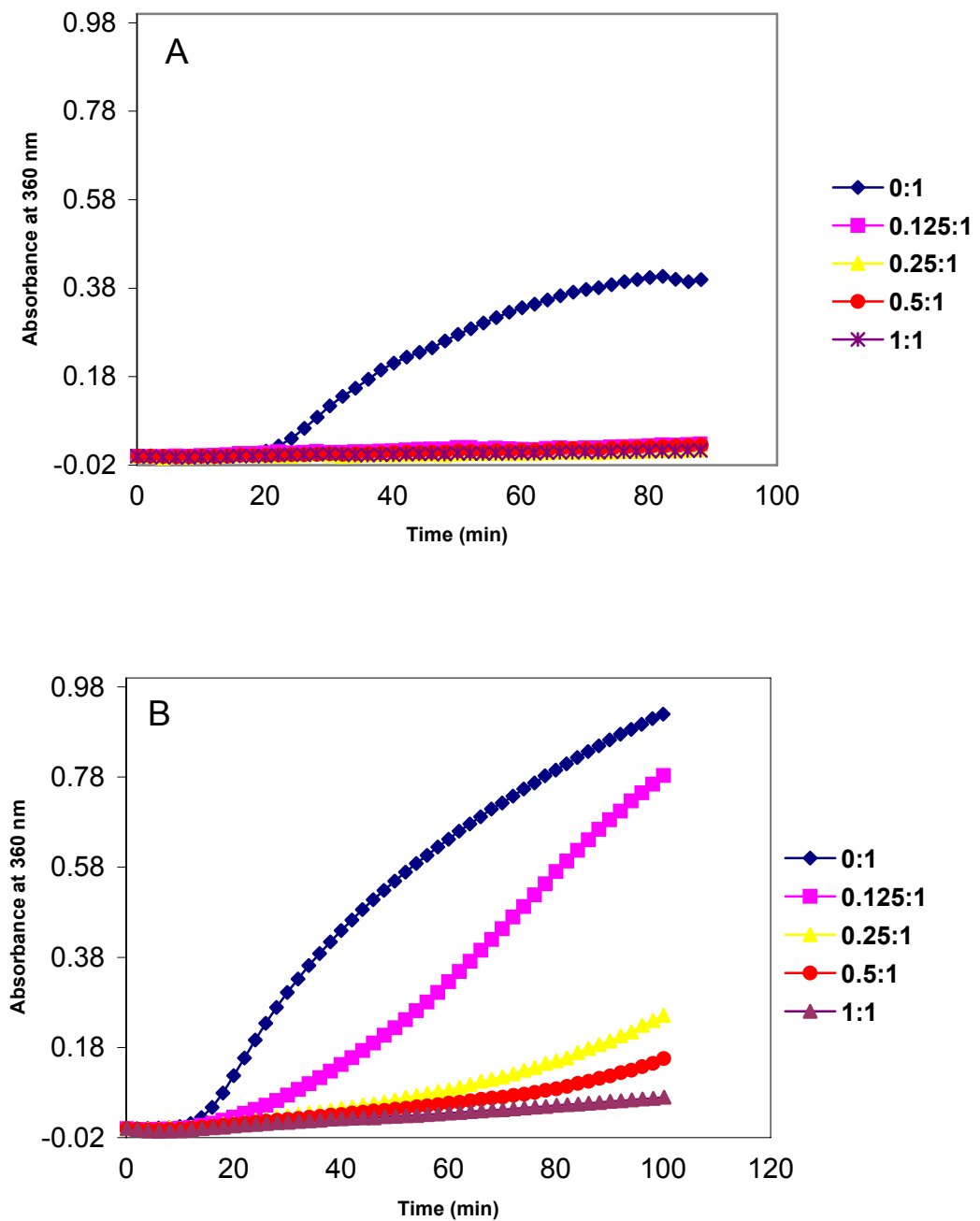


Figure 3.9: β_L -crystallin (0.3 mg/mL) aggregation in the presence of (A) α -crystallin and (B) α -crystallin and 10% w/v dextran. The protein was in total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , 1 mM EDTA, pH 7.4, and the incubation temperature was 60°C. Heating induced the aggregation of β_L -crystallin. The ratios indicated are of α -crystallin: β_L -crystallin, in w:w subunit ratio. Note that the experiment was done in duplicate.

3.3 Intrinsic tryptophan fluorescence spectroscopy

Monitoring tryptophan fluorescence is a useful method to detect a change in the structure of proteins under different conditions. The intrinsic fluorescence of a protein comes from aromatic residues; tryptophan, and to a lesser degree, tyrosine and phenylalanine residues; and a change in fluorescence properties of a protein (λ_{\max} and intensity) reflects changes in the environments of these residues. The λ_{\max} of a tryptophan residue varies from 308 to 353 nm, depending on the polarity of its environment (Ladokhin et al. 2000, Reshetnyak et al. 2001). At the extremes, there are two classes of tryptophan residues in proteins: one inside the protein in a hydrophobic environment which has a low λ_{\max} of around 330 nm and the other is an exposed residue in a polar environment with a higher λ_{\max} of around 350 nm and a lower intensity of fluorescence than the tryptophan in a hydrophobic environment (Ladokhin et al. 2000, Reshetnyak et al. 2001).

3.3.1 Changes in intrinsic tryptophan fluorescence of α -crystallin upon interaction with reduced insulin in the presence of 10% w/v dextran

To investigate the effect of dextran on the environment of the tryptophan residues of α -crystallin, the intrinsic fluorescence of α -crystallin in the presence and absence of a target protein (insulin) and/or 10% w/v dextran was measured. All samples with α -crystallin had their intrinsic fluorescence maximum around 340 nm as shown in Figure 3.10. A comparison of maximum

fluorescence wavelength (λ_{\max}) and fluorescence intensity at λ_{\max} of various combinations of α -crystallin, insulin and dextran is shown in Figures 3.14A and B, respectively.

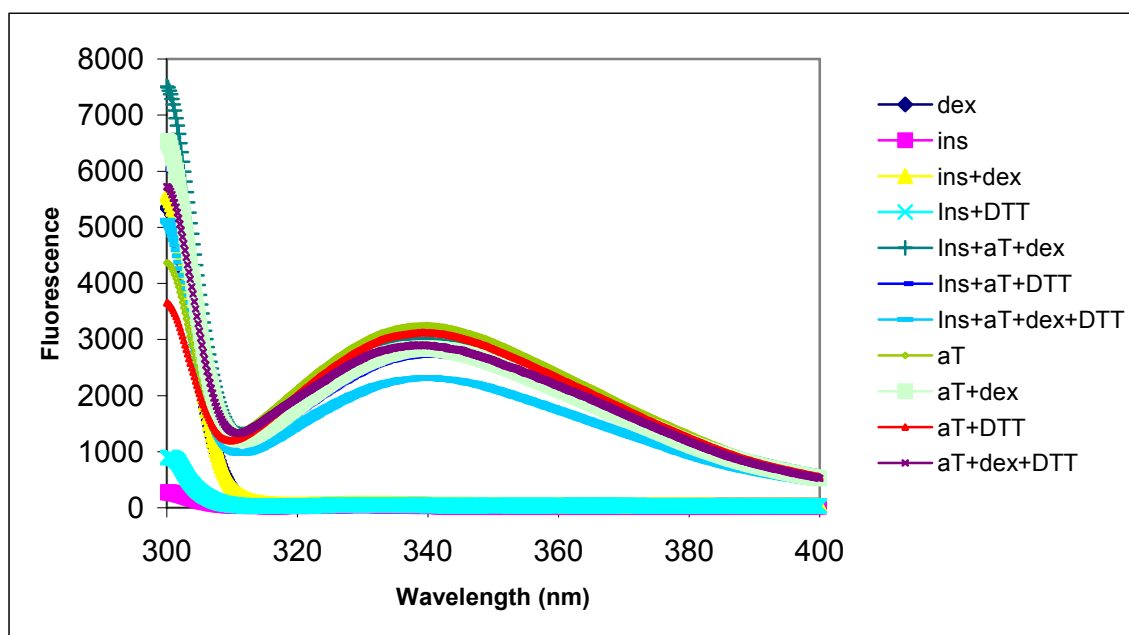


Figure 3.10: The intrinsic tryptophan fluorescence spectra of insulin (10 μ M), α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran. The fluorescence spectra were measured at excitation and emission wavelength of 300 and 400 nm respectively. All experiments were conducted in 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , and pH 7.4 at 37°C for 1 hr.

A very low fluorescence intensity of insulin (Figure 3.11A) comes from its tyrosine and phenylalanine as the only aromatic residues i.e. it contains no tryptophan residues (Lindner et al. 1998, Ladokhin et al. 2000). Compared to insulin, α -crystallin showed a high fluorescence emission from its tryptophan residues: one in α A, (W9), and two in the α B subunit, (W9, W60). Adding DTT to insulin, the small fluorescence intensity increased by almost 16% indicating that unfolding of insulin exposed its aromatic residues to the solvent. On addition of DTT to α -crystallin, the fluorescence intensity decreased only 4%. Adding reduced insulin to α -crystallin decreased the total fluorescence intensity

by 13% and increased the wavelength of maximum fluorescence (λ_{\max}) from 339.3 nm to 340.1 nm (Figures 3.11A and B). These changes indicate an increase in the polarity of the environment around the tryptophan residues located in the N-terminal domain of α -crystallin upon complexation with insulin and are consistent with the results of Lindner et al. (1998). Because DTT only had a small effect on the fluorescence of α -crystallin alone and the contribution of insulin fluorescence to the fluorescence of the mixture of insulin and α -crystallin is negligible relative to that of α -crystallin, these changes must be due to the conformational effect of reduced insulin interacting with α -crystallin. The addition of dextran to the mixture of α -crystallin and reduced insulin decreased fluorescence intensity (by 15%) and increased the λ_{\max} (from 339.3 nm to 340.7 nm) compared to measurements in the absence of dextran. Are these changes due to the effect of dextran on the insulin B chain or on the α -crystallin? Control experiments revealed that intrinsic fluorescence of reduced insulin and α -crystallin in the presence of dextran was 23% lower than the sums of the individual proteins in the presence of DTT and dextran. Thus it is concluded that the decrease in fluorescence observed most likely arises from more interaction of reduced insulin with α -crystallin which causes the environment of tryptophan to become more polar upon chaperone action i.e. increased the polarity of the environment of tryptophan 9 and tryptophan 60 in the N-terminal region of α -crystallin when it interacted with the insulin B chain during chaperone action.

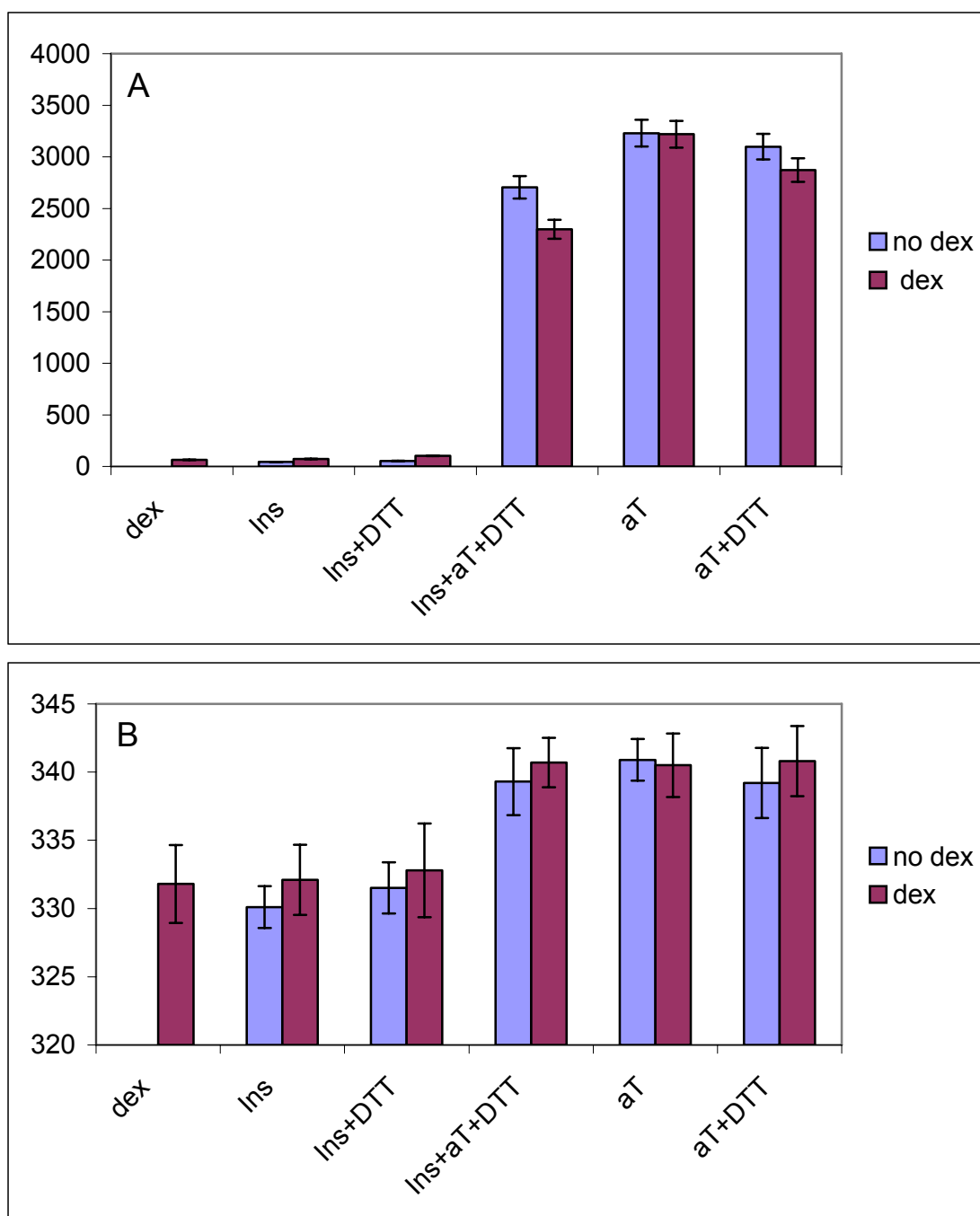


Figure 3.11: (A): The maximum intrinsic fluorescence of insulin (10 μ M), α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran, and (B): The average λ_{max} of insulin, α -crystallin and dextran. All experiments were conducted in 50 mM phosphate buffer, 0.05% (w/v) NaN₃, pH 7.4 and 37°C. The error bar indicate standard. The error bars are absolute values of maximum calculated errors.

3.4 ANS fluorescence studies: Interaction of insulin with α -crystallin in the presence of dextran

1-Anilino-8-Naphthalene Sulfonate (ANS) is a hydrophobic fluorescent molecular probe which has been used for examining the nonpolar character of proteins and membranes (Kirk et al. 1994, Matulis et al. 1998a. and b). It binds to nonpolar sites on protein and its fluorescence intensity corresponds to the polarity and pH of the environment (Matulis et al. 1998a and b).

Figure 3.12A shows the level of ANS binding by α -crystallin, insulin, and dextran in various combinations to obtain information on the exposed hydrophobicity and hence conformation of these proteins in the presence of dextran. An increase in the fluorescence of ANS in the mixture of insulin, α -crystallin and dextran resulted from the sequential addition of ANS (10 μ M), followed by the plateau region. The maximum ANS fluorescence of samples is shown in Figure 3.12B. Insulin alone showed very little ANS binding. Upon the addition of DTT to insulin, the ANS fluorescence intensity increased 15%, which is indicative of the exposure of hydrophobic regions to solution. α -Crystallin alone at the same concentration showed ANS binding approximately 16 times greater than that for insulin, indicative of large exposed hydrophobic areas in this protein (Carver et al. 1995). Since α -crystallin has no disulfide bonds, little change in ANS-binding

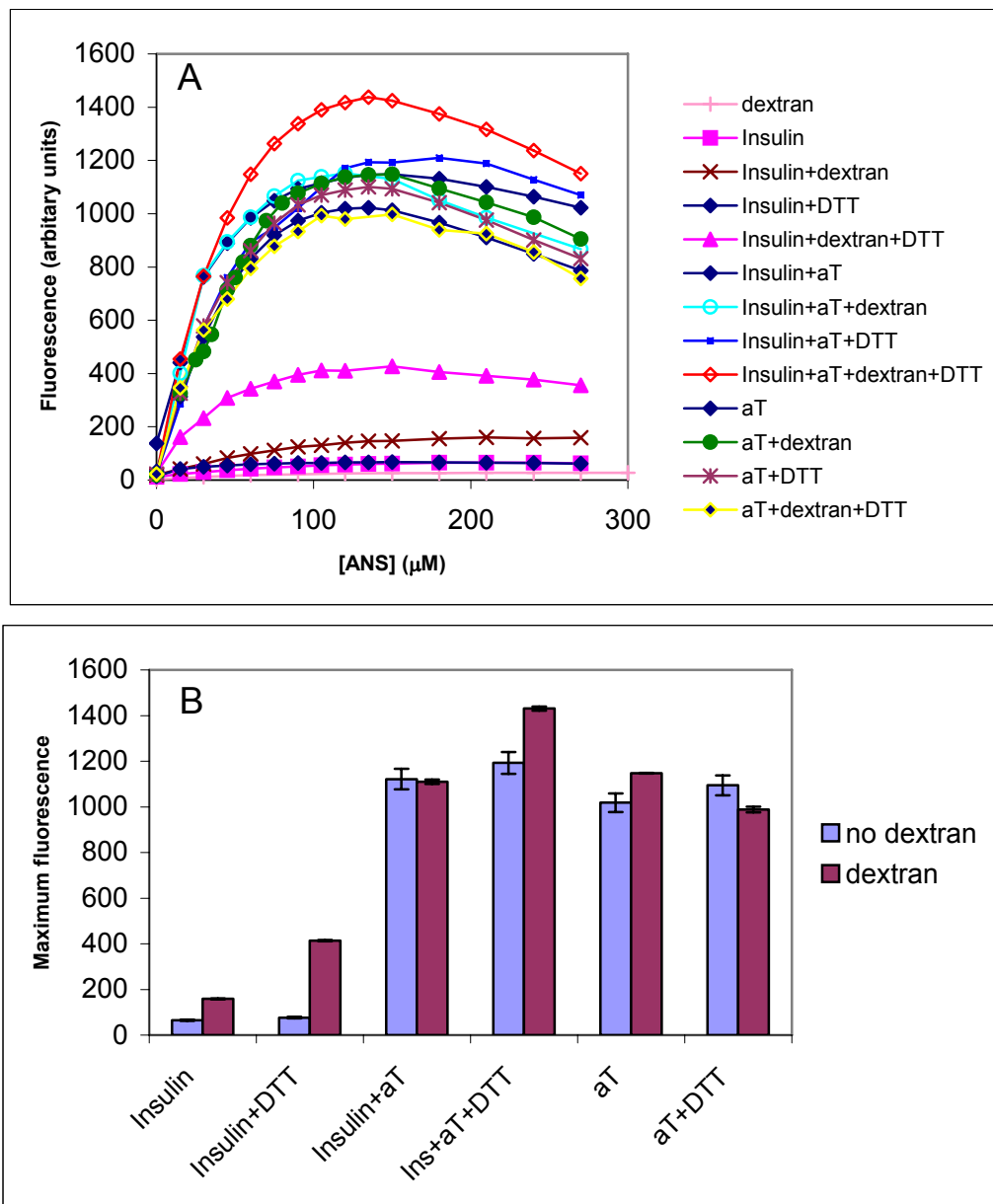


Figure 3.12: (A): Fluorescence emission results for the binding of the hydrophobic probe ANS to insulin (10 μ M), α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran in the presence and absence of DTT, (B): Average maximal fluorescence for insulin, α -crystallin and dextran, alone and in the presence of each other, and 20 mM DTT. Note the plateau region, which arises from the saturation of ANS binding sites on the protein. All experiments were conducted in 50 mM phosphate buffer, pH 7.4 and 37°C. The error bars are absolute values of maximum calculated errors.

resulted from the addition of DTT to it. The ANS fluorescence value of a mixture of insulin and α -crystallin in the absence of DTT were similar to the sum of those of individual proteins, implying that there was no interaction between

them. The levels of ANS fluorescence of the insulin and α -crystallin mixtures in the presence of DTT were very similar to the sum of the individual proteins in the presence of DTT similar to the result of Lindner et al. (1998).

Adding dextran to the reduced insulin and α -crystallin mixture increased the ANS binding by 19% relative to the absence of dextran. Control experiments revealed that after adding dextran to reduced insulin, the ANS binding increased 5 times compared to non-crowding conditions, consistent with insulin exposing more hydrophobic groups to the solution. Dextran decreased the ANS fluorescence intensity in reduced α -crystallin by 10% indicating slightly less hydrophobic exposure. It is concluded, therefore, that the increase in fluorescence was observed mostly upon formation of the complex between reduced insulin and α -crystallin after addition of dextran. This most likely arises from the exposure of more hydrophobic groups of the reduced insulin to the solution rather than conformational changes in α -crystallin, which led to an alteration in the exposed hydrophobicity of the protein.

In summary, dextran had an effect on both reduced insulin and α -crystallin with more effect on insulin. It slightly decreased the exposed hydrophobicity of α -crystallin, however it had more of an effect on the unfolding of reduced insulin thereby enhancing formation of a HMM complex with α -crystallin.

3.5 Changes in intrinsic tryptophan fluorescence of α -lactalbumin upon interaction with α -crystallin in the presence and absence of 10% w/v dextran

The intrinsic fluorescence of α -lactalbumin was compared in the presence and absence of α -crystallin, DTT and dextran to investigate the effect of dextran on native and reduced α -lactalbumin. All samples of α -lactalbumin had their intrinsic fluorescence maximum from 330 nm to 340 nm as shown in Figure 3.13A. Comparison of maximum fluorescence wavelength (λ_{\max}) and fluorescence intensity at λ_{\max} of various combinations of α -lactalbumin, α -crystallin, and dextran are shown in Figures 3.14A and B, respectively. Figure 3.14 shows that adding DTT to α -lactalbumin led to an increase in the maximum fluorescence intensity about 28% which indicated partial unfolding of α -lactalbumin. Adding DTT to α -crystallin, which has a very large intrinsic fluorescence intensity, showed a slight decrease in fluorescence intensity. Intrinsic fluorescence values of α -crystallin and α -lactalbumin mixture after addition of DTT was 19% lower compared to the sum of the individual proteins in the presence of DTT. This shows the formation of complex between α -lactalbumin and α -crystallin after mixing implying conformational changes in the environment of tryptophan residue in the two proteins during chaperone action (Lindner et al. 1998).

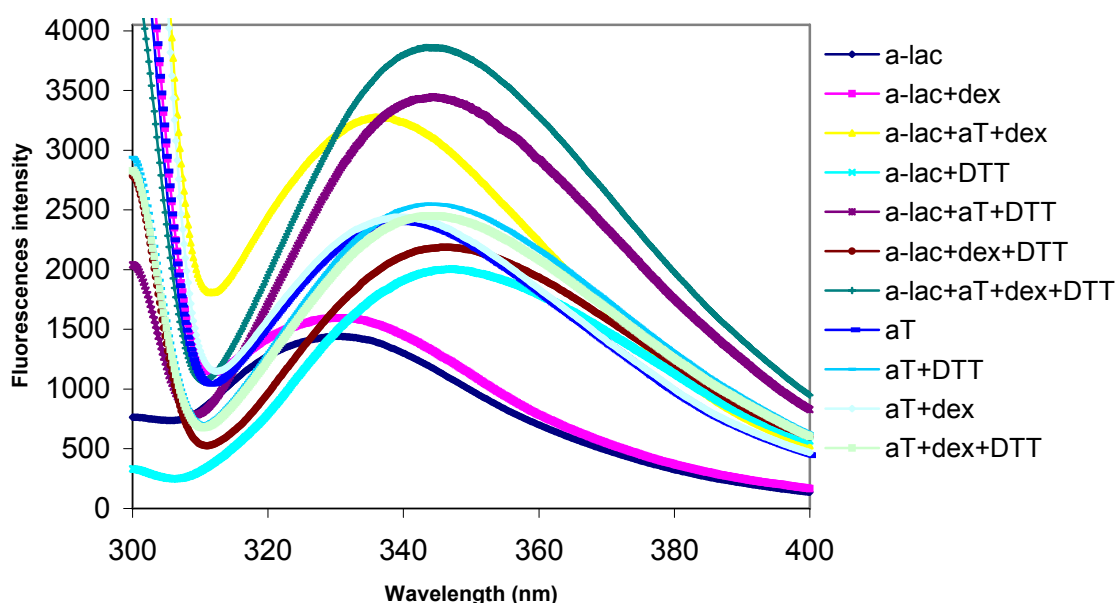


Figure 3.13: The intrinsic tryptophan fluorescence spectra of α -lactalbumin (10 μ M), α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran. The fluorescence spectra were measured at excitation and emission wavelength of 300 and 400 nm respectively. All experiments were conducted in 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , and pH 7.4 at 37°C.

The addition of dextran to the reduced α -lactalbumin increased the fluorescence intensity around 28%, and decreased the fluorescence intensity (from 347.1 nm to 346.2 nm) compared to reduced α -lactalbumin alone. In addition dextran increased the fluorescence intensity in reduced α -lactalbumin and α -crystallin mixture (by 12%). Since dextran had small effects on α -crystallin + DTT (4% decrease in the presence of dextran) compare to α -lactalbumin thus it should be considered that the effect of dextran on the mixture of reduced α -lactalbumin and α -crystallin is more due to the effect of dextran on the α -lactalbumin since it increased the fluorescence intensity in it.

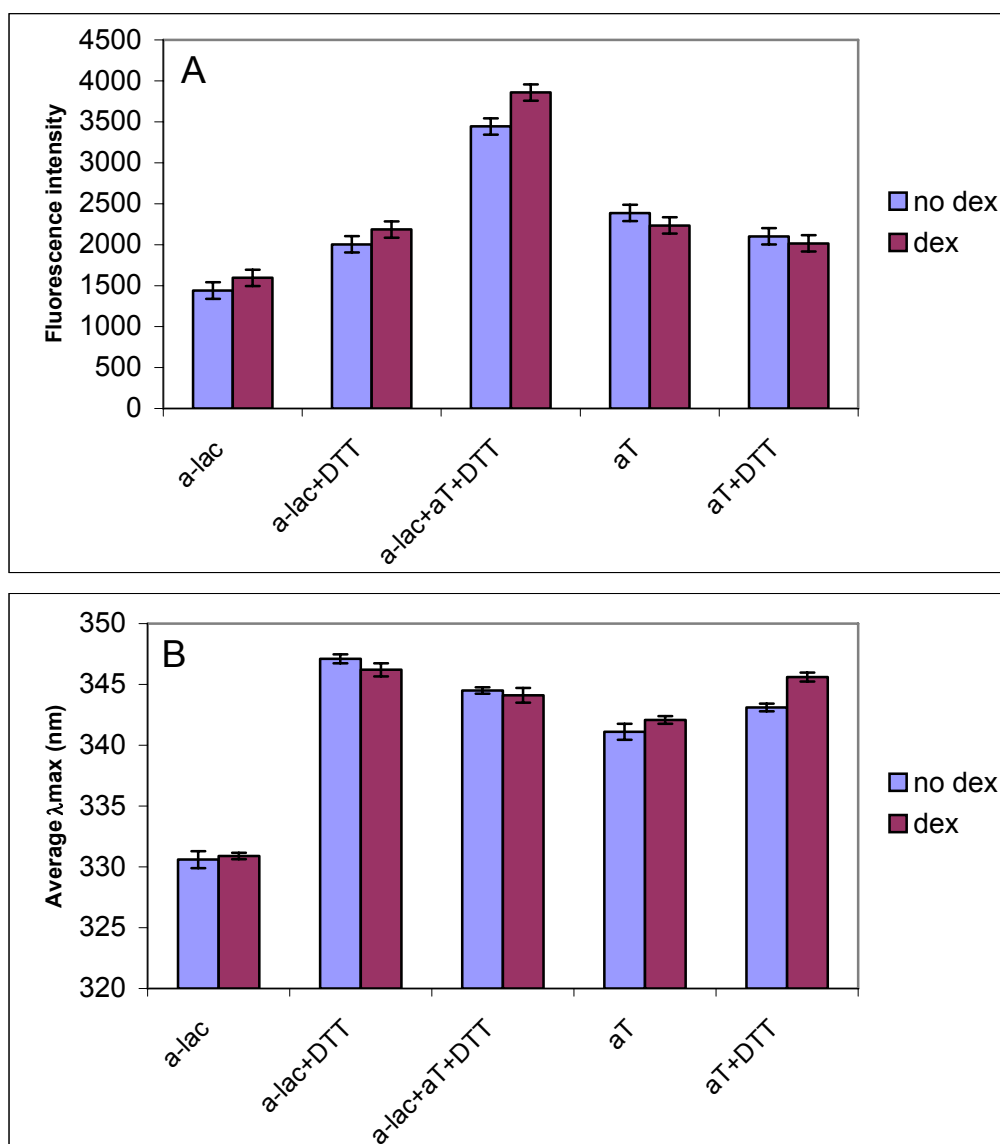


Figure 3.14: (A): The maximum intrinsic fluorescence of α -lactalbumin (10 μ M), α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran, and (B): The average λ_{max} of α -lactalbumin, α -crystallin and dextran. All experiments were conducted in 50 mM phosphate buffer, 0.05% (w/v) NaN₃, and pH 7.4 at 37°C. The error bars are absolute values of maximum calculated errors.

Therefore dextran caused the environment of tryptophan residues in α -lactalbumin to become more hydrophobic.

3.6 ANS fluorescence studies: Interaction of α -lactalbumin with α -crystallin in the presence and absence of dextran

Figure 3.15A shows the level of ANS binding by α -crystallin, α -lactalbumin, and dextran in various combinations. An increase in the fluorescence of ANS in the mixture of α -lactalbumin, α -crystallin and dextran resulted from the sequential addition of ANS (10 μ M), followed by the plateau region. The maximum ANS fluorescence of samples is shown in Figure 3.15B. According to these data, α -lactalbumin alone shows very little ANS binding (as expected since it is a natively folded protein). Upon the addition of DTT, the ANS fluorescence intensity increased 5.2 times, which is indicative of the exposure of hydrophobic regions of α -lactalbumin to the solution. α -Crystallin alone at the same concentration showed ANS binding approximately 10 times greater than that for native α -lactalbumin (Figure 3.15). This is indicative of large exposed hydrophobic areas of α -crystallin (Carver et al. 1995). Since α -crystallin has no disulfides, very little increase (3%) in ANS-binding resulted from the addition of DTT. The ANS fluorescence values of mixtures of α -lactalbumin and α -crystallin in the absence of DTT were very close to the sum of those of the individual proteins, implying that there was no interaction between the two proteins in their

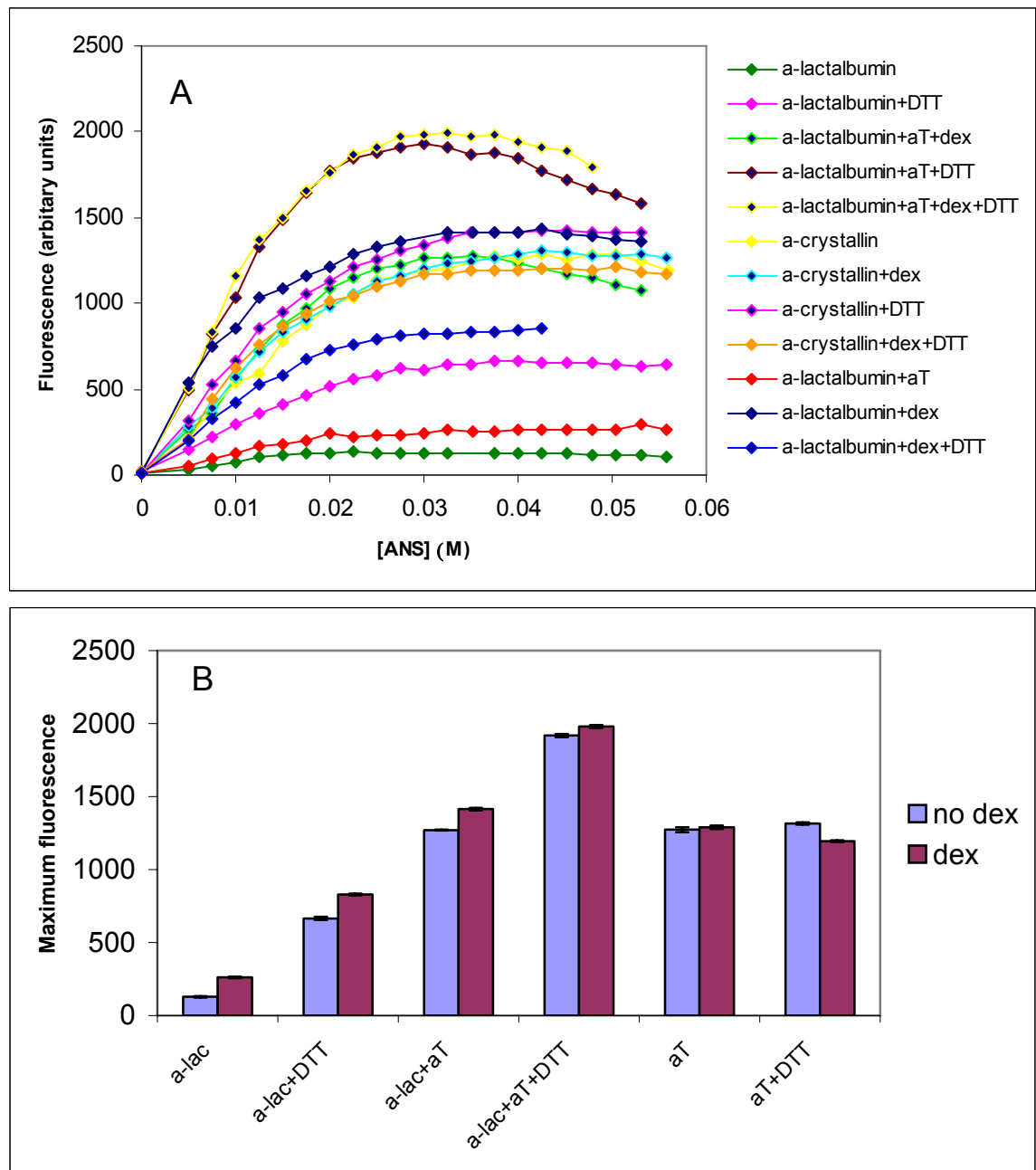


Figure 3.15: (A): Fluorescence emission results for the binding of the hydrophobic probe ANS to α -lactalbumin (10 μ M), α -crystallin (1:1) molar ratio and 10% w/v dextran in the presence and absence of DTT, (B): Average maximal fluorescence for α -lactalbumin, α -crystallin and dextran, alone and in the presence of each other, and 20 mM DTT. Note the plateau region, which arises from the saturation of ANS binding sites on the protein. All experiments were conducted in 50 mM phosphate buffer, pH 7.4 and 37°C. The error bars are absolute values of maximum calculated errors.

native state (i.e. non-stressed conditions) (Lindner et al. 1998). The levels of ANS fluorescence of the α -lactalbumin and α -crystallin mixtures in the presence of DTT were very similar to the sum of individual proteins in the presence of DTT. However the level of ANS fluorescence values of reduced α -lactalbumin and α -crystallin increased by 34% compared to the non reducing condition implying that the exposed hydrophobicity of reduced α -lactalbumin interacted with α -crystallin. After adding dextran to reduced α -lactalbumin, ANS fluorescence increased 20% compared to non-crowding conditions which indicates alteration in exposed hydrophobicity to solution. Adding dextran to α -crystallin decreased the fluorescence intensity by 9%, which indicates little change in exposed of the hydrophobic areas. The ANS binding of the reduced α -lactalbumin and α -crystallin mixture increased by 3% with the addition of dextran. Compared to the control experiments (α -lactalbumin and dextran mixture and α -crystallin and dextran mixture) this small increase is not significant within experimental error.

3.7 Unfolding and aggregation of reduced α -lactalbumin in the presence and in the absence of 10% w/v dextran (as monitored by real-time 1D ^1H NMR spectroscopy)

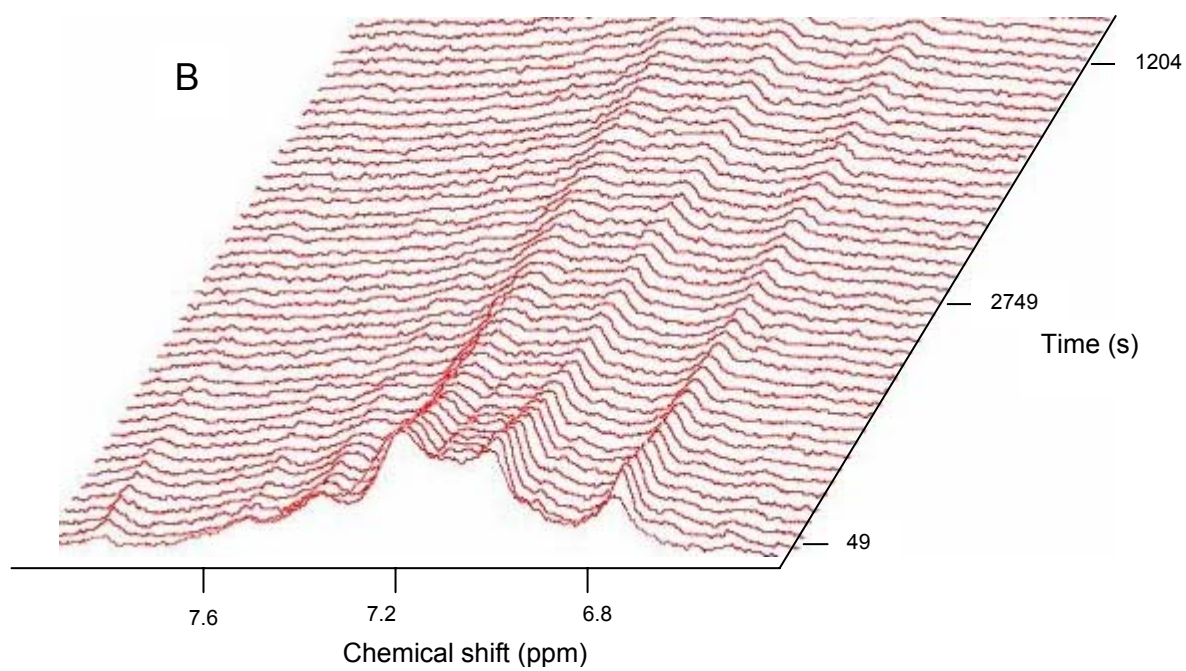
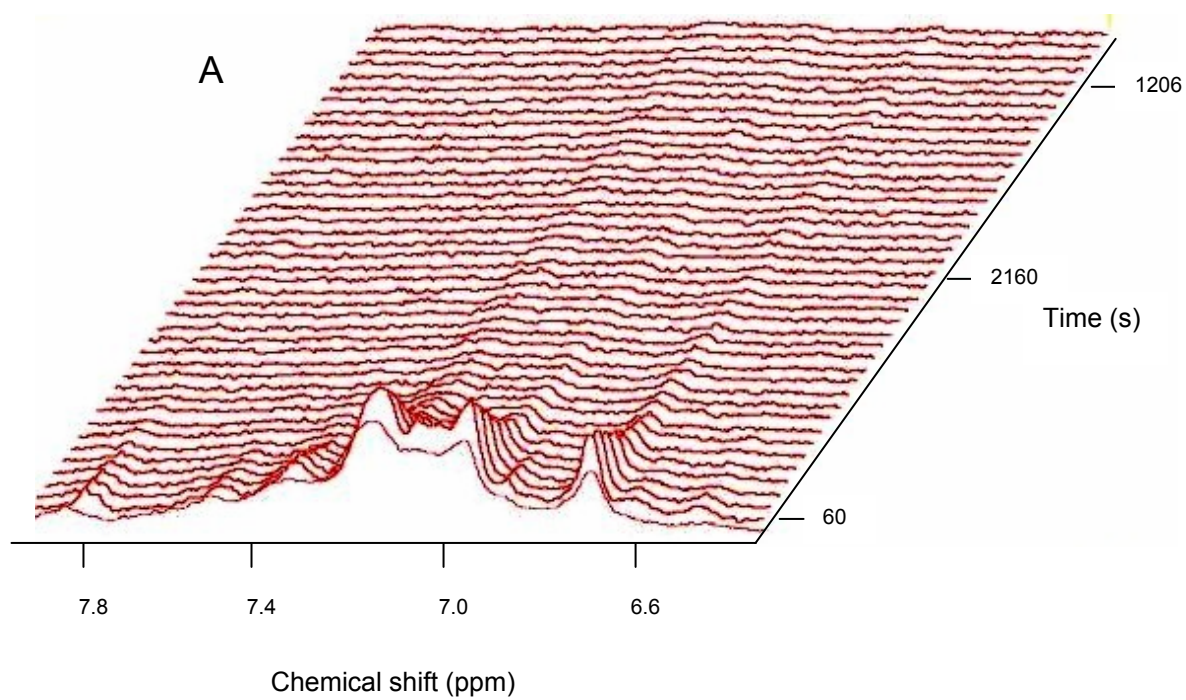
To investigate the effect of dextran on α -lactalbumin and the chaperone action of α -crystallin, a series of ^1H 1D spectra was obtained for α -lactalbumin with time following reduction with DTT, both in the presence and absence of dextran and α -crystallin (Figure 3.16). α -Crystallin does not contain any aromatic

residues in its flexible C-terminal extensions, therefore, one-dimensional ^1H spectra of the mixture of α -crystallin and a substrate protein can be used to ascertain information about the conformational changes in the substrate protein. The aggregation and precipitation of α -lactalbumin was induced by the addition of concentrated deuterated DTT to a final concentration of 20 mM. Figure 3.16 shows the change in the aromatic region of ^1H 1D spectra obtained for α -lactalbumin with time, following the addition of DTT. An initial increase in signal occurred after addition of DTT due to unfolding of the α -lactalbumin polypeptide chain as a result of its four disulfide bonds being reduced (Carver et al. 2002). The rate of reduction of disulfide bonds is not affected by the presence of α -crystallin (Lindner et al. 1997, Carver et al. 2002). A steady decrease in signed intensity occurred after reaching a maximum, implying that after unfolding the protein aggregated. Figure 3.17 illustrates the changes over time in the intensity of the resolved tyrosine (3,5) proton resonance of α -lactalbumin at 6.8 ppm, which is characteristic of α -lactalbumin in the molten globule state (Balbach et al. 1995, Lindner et al. 1997, Carver et al. 2002). After maximum unfolding to form the molten globule state, the resonance intensity decreased as the aggregation, and eventually the precipitation, of partially unfolded α -lactalbumin occurred (Carver et al. 2002). In the absence of dextran and α -crystallin (Figures 3.16A and 3.17), the maximum intensity of the tyrosine (3,5) resonance of α -lactalbumin occurred at ~ 338 s when the fully reduced form of the protein was present. This is consistent with the time of 320 s reported by Carver et al. (2002). The rate of the loss of the 6.8 ppm resonance intensity for reduced α -lactalbumin was found to be $(4.08 \pm 0.16) \times 10^{-2} \text{ (s}^{-1}\text{)}$

from a simple first order kinetic decay (Figure 3.17). The complete loss of signal occurred at 50 minutes, consistent with the onset of precipitation as determined by light scattering (Figure 3.16A).

In the presence of α -crystallin at a 1:1 (w:w) ratio (Figures. 3.16B and 3.17) the time to maximum resonance intensity increased slightly to 348 s and the rate of decay decreased to $(2.87 \pm 0.23) \times 10^{-2} \text{ (s}^{-1}\text{)}$ (Figure 8). Thus, in the presence of α -crystallin the rate constant was 1.4-fold lower compare to in its absence. The ability of α -crystallin to stabilize the molten globule state of α -lactalbumin is similar to that of Treweek et al. (2005) who found for α B-crystallin but slightly lower (~ 1.4 -fold) compared with that found for α -crystallin by Carver et al. (2002). The different rate constant in the present study could be due to differences in various buffer component concentration and pH which affected the rate of aggregation of reduced α -lactalbumin.

Adding 10% w/v dextran to reduced α -lactalbumin and the mixture of reduced α -lactalbumin and α -crystallin (Figures 3.16C, 3.16D and 3.17) gave the time of maximum unfolding of ~ 324 s and ~ 334 s respectively and gave rates of signal decay to $(6.09 \pm 0.34) \times 10^{-2} \text{ (s}^{-1}\text{)}$ and $(3.55 \pm 0.25) \times 10^{-2} \text{ (s}^{-1}\text{)}$, respectively (Figure 3.17). In all cases, the observed times of maximum resonance intensity are 336 ± 10 s, which is consistent with α -crystallin and dextran not affecting disulphide bond reduction of α -lactalbumin (Carver et al. 2002). However, dextran increased the rate of aggregation of α -lactalbumin, and in this situation α -crystallin was a less efficient chaperone to prevent aggregation (Figure 3.16).



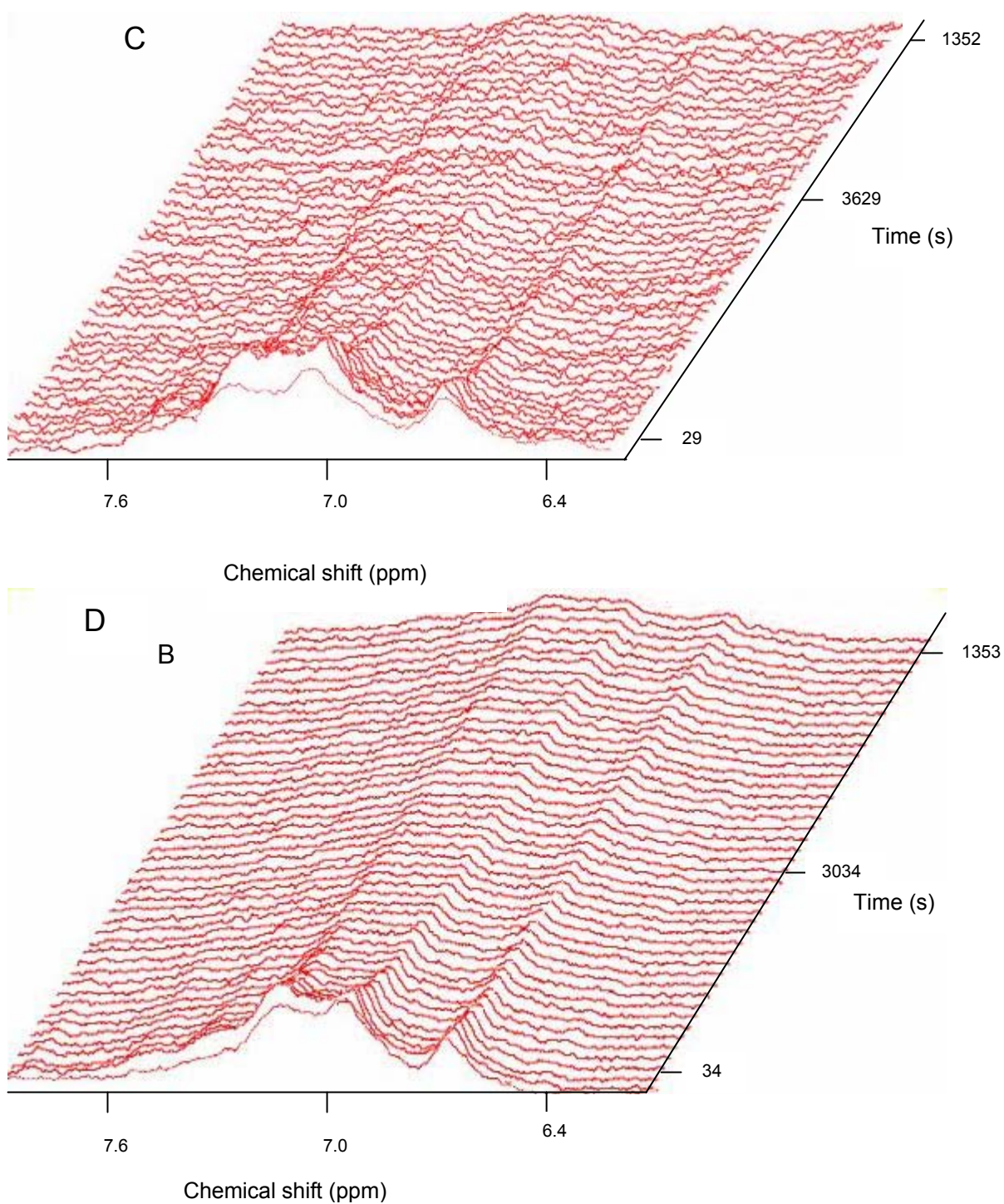


Figure 3.16: The change in aromatic region of real-time ^1H 1D NMR of apo α -lactalbumin (2.0 mg/mL) (A): α -lactalbumin. (B): 1:1 w:w ratio of α -lactalbumin: α -crystallin. (C): α -lactalbumin and 10% w/v dextran (D): 1:1 w:w ratio of α -lactalbumin: α -crystallin and 10% w/v dextran. All experiments were conducted in 50 mM phosphate buffer, 100% D_2O , pD 7.4, 0.1 mM NaCl, 1 mM EDTA, at 37°C .

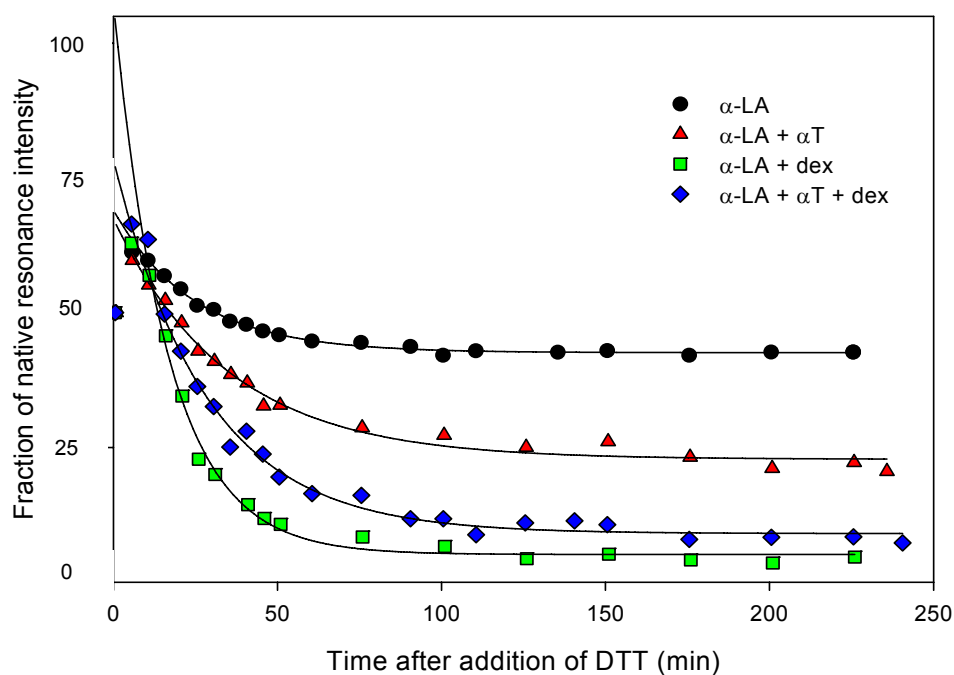


Figure 3.17: Decay in the Tyr (3,5) resonance at 6.8 ppm of reduced α -lactalbumin (2 mg/mL) in the presence and absence of α -crystallin and 10% w/v dextran. All experiments were conducted in 50 mM phosphate buffer, 100% D_2O , pD 7.4, 0.1 mM NaCl, 1 mM EDTA, at 37°C.

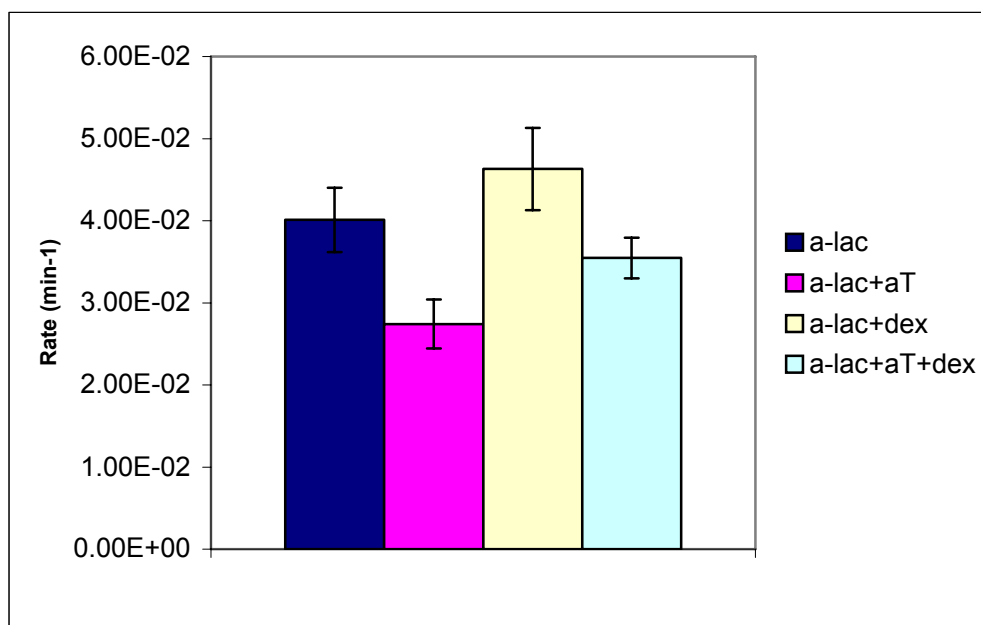


Figure 3.18: The apparent first order rate constants of signal decay at 6.8 ppm of reduced α -lactalbumin.

3.8 ^1H NMR spectroscopy of α -lactalbumin in the presence and absence of dextran

The 1D and 2D ^1H NMR spectra of α -lactalbumin in the presence and absence of dextran were monitored over a time period of 16 hrs to observe if dextran led to any alterations in the structure of the native protein. The aromatic and NH region of 1D ^1H NMR spectra of α -lactalbumin in the presence and absence of dextran (Figure 3.19) revealed obvious differences. The spectrum of α -lactalbumin (Figure 3.19A) shows the chemical shift dispersion characteristic of well folded protein (Alexandrescu et al. (1992). While this remained in the presence of dextran, (Figure 3.19 B) a marked over-all resonance broadening was observed, implying a slower rate of tumbling of the protein due to interaction with the dextran.

Two-dimensional ^1H - ^1H total correlation spectroscopy (TOCSY) can be used to elucidate multiple through-bond couplings in isolated spin systems, i.e. individual amino acids in a polypeptide chain. To further investigate changes in the tertiary structure of α -lactalbumin in the presence of dextran, 2D ^1H - ^1H TOCSY experiments were performed on α -lactalbumin in the presence and absence of 5% w/v dextran. 10% w/v dextran was not used because of the large dextran resonance which obscured part of the α -lactalbumin spectrum. Figure 3.20A shows the TOCSY spectrum of α -lactalbumin in the absence of dextran. It shows a large population of well-resolved cross-peaks, arising from couplings between ^1H resonances of tryptophan 26, 60, 118, 103 and other aromatic residues. The cross-peaks were assigned, according to Alexandrescu

et al. (1992). Upon the addition of 5% w/v dextran a significant loss of cross-peak intensity was observed (Figure 3.20B). It is apparent from Figures 3.20A and B that in the presence of dextran, there is a little alteration in the structure of α -lactalbumin, just a general broadening and corresponding loss of cross-peak intensity. Thus, dextran has little or no effect on the conformation of α -lactalbumin. α -Lactalbumin does, however, interact with dextran and/or itself (leading its aggregation) via a mechanism that is slow on the NMR timescale, which results in broadening of α -lactalbumin resonances.

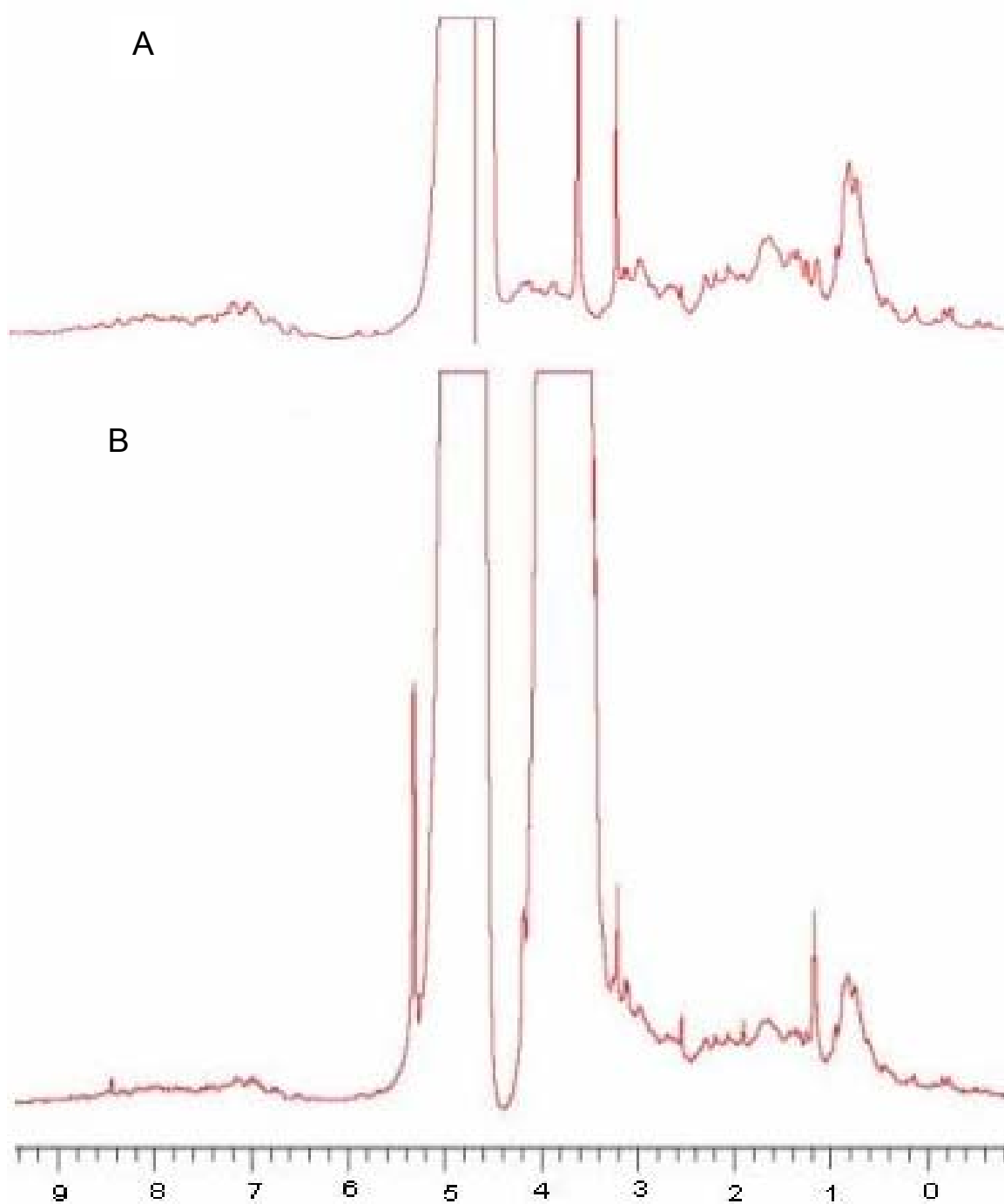


Figure 3.19: 1D ^1H NMR of α -lactalbumin in (A): no dextran and (B): 5% w/v dextran. All experiments were conducted in 50 mM phosphate buffer, 90% H_2O , 10% D_2O , pH 7.4, 0.1 mM NaCl, 1 mM EDTA, at 37°C. Peak intensities were referenced to the resonance at 0.9 ppm. Spectra plots were scaled to the height of the resonance at 0.9 ppm NaCl, 1 mM EDTA, at 37°C.

Figure 3.20: The aromatic region of a TOCSY spectrum of α -lactalbumin (4.2 mg/mL) in the absence (A) and presence of (B) 5% w/v dextran. Both experiments were conducted in 50 mM phosphate buffer, 90% H₂O, 10% D₂O, pH 7.4, 0.1 mM NaCl, 1 mM EDTA, at 37°C. Contour plots were generated in spectra with the same vs2d. The residues were assigned according to Alexandrescu et al. (1992).

3.9 Discussion

In vitro experiments with proteins are usually conducted in dilute solution in order to minimize non-specific protein interactions (Dobson et al. 1999). These conditions are substantially different from the intracellular environment, where there is a high concentration of macromolecules such as carbohydrates, proteins and nucleic acids interacting with each other. In an attempt to understand what happens *in vivo*, the living cell interior was mimicked by the addition of dextran as an inert macromolecular crowding agent (Ganea et al. 2001, Sasahara et al. 2003). The effects of a molecular chaperone (α -crystallin) on the aggregation of target proteins in the presence and absence of dextran were also investigated.

The chaperone action of α -crystallin in preventing aggregation of a variety of target proteins has been previously demonstrated e.g. a 10-fold molar excess of α -crystallin prevented aggregation of ovotransferrin, and a 1:1 w:w ratio of α -crystallin: α -lactalbumin prevented aggregation of reduced α -lactalbumin (Horwitz et al. 1992, Lindner et al. 1998, Carver et al. 2002). In this study, visible absorption spectroscopy revealed an increase in light-scattering with time of stressed ovotransferrin, insulin, α -lactalbumin and β_L -crystallin due to their aggregation and precipitation.

α -Crystallin effectively prevented aggregation of ovotransferrin and α -lactalbumin in the absence of dextran. This result is in agreement with that obtained by Lindner et al. (1998) and Carver et al. (2002), considering that in

the previous study with reduced ovotransferrin used 100 mM NaCl which stimulated protein aggregation and limited the efficiency of α -crystallin to suppress its precipitation (Lindner et al. 1998). Dextran had a significant effect on the rate, as well as on the time to the onset of aggregation of reduced ovotransferrin and α -lactalbumin both in the presence and absence of α -crystallin. As dextran increased the rate of aggregation, α -crystallin was found less efficient in preventing it. This is consistent with α -crystallin being more effective as a chaperone for slowly aggregating proteins (Lindner et al. 1998).

α -Crystallin effectively suppressed aggregation of reduced insulin. This is consistent with Farahbakhsh et al. (1995) who found that at 25°C a 1:6 w:w ratio of insulin: α -crystallin completely suppressed aggregation of reduced insulin. It was observed that dextran accelerated insulin aggregation but it had little effect on the time to the onset of insulin aggregation. α -Crystallin was found equally effective in preventing aggregation of insulin both in the presence and absence of dextran.

The effect of α -crystallin on thermally induced aggregation of β_L -crystallin was demonstrated in previous studies (e.g. Horwitz et al. 1992), whereby α -crystallin effectively suppressed thermally induced aggregation of β_L -crystallin at 55°C. At these temperatures, α -crystallin undergoes a structural change, which results in an increase in exposed surface area (Horwitz et al. 1992, Das et al. 1997). In this study herein, α -crystallin effectively prevented aggregation and precipitation of β_L -crystallin when a solution containing both proteins was heated to 60°C. There was a significant effect of dextran on β_L -crystallin, i.e. dextran increased

the rate of aggregation and precipitation of heated β_L -crystallin and reduced the time of the onset of aggregation. In the presence of dextran, α -crystallin was less efficient chaperone in preventing aggregation of heated β_L -crystallin.

These data are consistent with other studies (Lindner et al. (2001), Carver et al. (2002) and Ren et al. (2003)). Lindner et al. (2001) and Carver et al. (2002) showed that the presence of various salts (e.g. SO_4^{2-}) led to more rapid aggregation of target proteins. Under such conditions, α -crystallin's chaperone activity decreased, which parallels observations of more rapid aggregation in the presence of dextran. Ren et al. (2003) also proposed that adding polyethylene glycol 20,000 (PEG 20k) or dextran 70 increased the rate of aggregation of D-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). They also reported that the rate and extent of aggregation increased by increasing concentration of crowding agent which is comparable to results of this study. In the cases of enhanced aggregation in the presence of dextran, α -crystallin's chaperone activity decreased. Two possible causes are that dextran either increased the association and aggregation of target proteins and/or had a structural effect on α -crystallin.

Our data showed that dextran enhances and accelerates aggregation of many proteins to various extents. The different effect of dextran on different proteins can be explained by the theory of macromolecular crowding as described in detail previously (see Section 1.4). Crowding agents show thermodynamic and kinetic effects on protein self-assembly. Crowding ordinarily affects equilibria by destabilizing either reactants or products because it alters their association or

dissociation constants (Minton et al. 2000a, Ganea et al. 2002, Dobson et al. 2001, Sasahara et al. 2003). Macromolecular crowding is also called 'the excluded volume effect' (Section 1.4). If we consider a single molecule being introduced into a solution of another macromolecule, the amount of intracellular volume, which is available to the introduced molecule, depends on the number, size and shape of the molecules of the dissolved substance. If an introduced molecule is relatively small, it can access virtually all the spaces between the macromolecules, however if it is large, the volume available to it is limited, and steric repulsion occurs between molecules that affects reaction equilibria and reaction rates (Ellis et al. 1997, Ellis et al. 2001). In the work described here, it can be hypothesized that for ovotransferrin (78 kDa), α -lactalbumin (14 kDa) and β_L -crystallin (23.137 kDa), the association constant was significantly increased by higher concentrations of the macromolecular agent (2%, 5% and 10% w/v dextran (68 kDa)) compared to the non-crowded conditions (0% dextran). By comparison, the insulin B chain (3.4 kDa) is very small, so it can access the free space between macromolecules; thus, dextran did not have a significant effect on insulin's association or the ability of α -crystallin to suppress its aggregation. In addition, this discrepancy of the effect of dextran can be related to the increasing amount of exposed surface area on a protein molecule which occurs in the presence of the crowding agent. As a result of increasing amount of exposed surface area, the molecule tries to reduce the amount of exposed surface area, for example by intermolecular association (Minton et al. 2000b). The increasing aggregation of protein in the presence of dextran indicates that aggregation results in a greater loss of surface area compared to

association of unfolding species with sHsps. As a consequence, the fact that dextran had less impact on insulin is possibly a reflection of the smaller exposed surface area on this protein compared to ovotransferrin, α -lactalbumin and β_L -crystallin.

Studies of other molecular chaperones have shown the effect of crowding agents on its chaperone action. Martin et al. (1996) reported the effect of a crowding agent on the chaperone action of GroEL. GroEL is a cylindrical structure with two heptameric rings of 57 kDa subunits which are stacked on top of each other (Braig et al. 1990). The single substrate protein is bound in hydrophobic binding region of the internal cavity in GroEL (where the refolding of protein occurs). Martin et al. (1996) proposed that by adding a crowding agent such as Ficoll or dextran, the release of refolded polypeptide from GroEL into the bulk solution was significantly reduced. They proposed that under crowding conditions, the hydrophobic binding regions in GroEL increase. This increases the capacity of GroEL to capture nonnative protein and prevents their release into the bulk solution. sen Zhang et al. (2001) and Ren et al. (2003) reported similar results. They proposed that GroEL enhances the refolding of GAPDH and Glucose-6-phosphate Dehydrogenase (G6PDH) even under crowding conditions. However, in the presence of crowding agent the refolding rate decreases because the dissociation of the GroEL-protein complex could be prevented by the crowding agents.

A previous study by real-time ^1H 1D NMR spectroscopy found that α -crystallin was an efficient chaperone for the slow aggregation of apo- and holo

α -lactalbumin (Carver et al. 2002). Likewise in the current study, adding α -crystallin to reduced apo α -lactalbumin prevented the aggregation of the stressed protein. This is consistent with previous reports (Lindner et al. 1997, Lindner et al. 2001, Carver et al. 2002, Engel et al. 2002) showing that under stress conditions, α -crystallin stabilizes α -lactalbumin in its molten globule state and then complexes with it. This is also in agreement with light scattering data showing that the amorphous aggregation rate of α -lactalbumin decreased in the presence of α -crystallin, although light scattering provides no specific information about the nature of the interaction between α -crystallin and the target protein.

Dextran promoted the maximal formation of the molten globule state of α -lactalbumin (which is due to complete reduction of the four disulfide bonds in the presence and absence of dextran and α -crystallin). It was evident from a decrease in the time to maximal unfolding and an increase in the rate of the NMR signal loss compared to the dextran-free conditions. This is also evident from the light scattering data showing that dextran promoted the interaction of the molten globule state of α -lactalbumin aggregation and decreased the time to the onset of aggregation.

Also, consistent with the light scattering data in the presence of dextran, α -crystallin had less effect on the rate as well as on the time to the onset of aggregation of reduced α -lactalbumin. The lower efficiency of the chaperone action of α -crystallin was found by previous research (Lindner et al. 2001) when adding salts like sulphate ions also accelerated the aggregation of

α -lactalbumin. The NMR data indicate that the kinetics of aggregation of the target protein is a crucial factor for α -crystallin chaperone activity.

The effect of dextran on α -crystallin is possibly preventing the structural change (in the C-terminal region at higher temperature (more than 37°C) that leads to the exposure of greater hydrophobicity to solution), which facilitates chaperone action (Raman et al. 1995, Lindner et al. 1998). Regarding this point, intrinsic fluorescence and near-UV CD spectra of α -crystallin in the presence and absence of 10% w/v dextran at different temperature (Sections 5.4, 5.5 and 5.6) showed that in the absence of dextran the environment of the tryptophan residues (the N-terminal domain of the protein at positions 9 in α A and 9 and 60 in α B) and the phenylalanine residues, were altered significantly with increasing temperature in parallel with an increase in chaperone activity. However, in the presence of dextran these environments were not altered significantly in contrast to the situation in the absence of dextran. Another possibility is a direct effect of macromolecular crowding on α -crystallin that was explored using fluorescence spectroscopy of insulin and α -crystallin in the presence and absence of dextran. Another factor is the effect of dextran on the subunit exchange rate of α -crystallin (Chapter 5, Section 5.3.2.2.) i.e. to slow it down and reduced efficiency of its chaperone action (Bova et al. 1997).

The effect of dextran on α -crystallin's structure was investigated by intrinsic fluorescence spectroscopy. A previous study of α -crystallin and insulin showed that no large structural change occurred within α -crystallin under reducing conditions during chaperone action (Lindner et al. 1998). In this study, dextran

slightly increased polarity of α -crystallin tryptophan residues in the absence of a target protein. In the presence of reduced insulin, dextran stimulated the exposure of α -crystallin's tryptophan residues to the solvent indicating interaction of α -crystallin with reduced insulin. In particular, from the intrinsic fluorescence measurements, the relatively hydrophobic N-terminal domain does not undergo any gross conformational change in the presence of dextran.

Intrinsic fluorescence of α -lactalbumin also showed that a large structural change occurred within α -lactalbumin under reducing conditions that reflected the partial unfolding of α -lactalbumin. In addition, upon adding dextran to the reduced α -lactalbumin the intrinsic fluorescence intensity increased both in the presence and absence of α -crystallin consistent with α -crystallin being a poorer chaperone in the presence of dextran.

Previous ANS-binding studies (Lindner et al. 1998) showed that the structure of α -crystallin was not greatly affected during its chaperone action, in particular during the formation of HMM complex with reduced insulin (Lindner et al. 1998). In this study, dextran slightly decreased the exposure of the hydrophobic regions of α -crystallin. This is consistent with intrinsic fluorescence results, implying that there is a little alteration in the hydrophobicity of α -crystallin in the presence of dextran. The increase in ANS binding in the reduced insulin and α -crystallin mixture in the presence of dextran, compared to its absence, was probably due to stabilization of a partially unfolded conformation of monomeric insulin B chain by α -crystallin.

The ANS binding assay of α -lactalbumin and α -crystallin also showed an increase in fluorescence intensity of α -lactalbumin and α -crystallin mixture following by reduction with DTT with more increase in the presence of dextran as was observed for insulin. This is probably due to stabilization of a partially unfolded conformation of reduced α -lactalbumin by α -crystallin.

Near-UV CD and intrinsic fluorescence spectroscopy of α -crystallin in the presence and absence of dextran at different temperature (Sections 5.4 and 5.5) showed that α -crystallin did not undergo significant structural alteration with temperature in the presence of dextran.

Dextran had little effect on the tertiary structure of α -lactalbumin as observed by ^1H NMR spectroscopy. Reduction in resonance and cross-peak intensities implied that more structure of α -lactalbumin was exposed to the solvent due to interaction with dextran.

3.10 Conclusions

This study reveals that a competition exists between the rate of aggregation of unfolding proteins and the interaction of partially unfolded protein with α -crystallin in the presence and absence of a macromolecular crowding agent. As shown by light scattering profiles and NMR spectroscopy, the presence of dextran leads to more rapid aggregation and precipitation of most partially-folded target proteins (ovotransferrin, α -lactalbumin and β_L -crystallin). α -Crystallin was more efficient in preventing the aggregation of reduced

ovotransferrin, α -lactalbumin and heated β_L -crystallin in the absence of dextran than in its presence. α -Crystallin stabilized the partially folded state of proteins i.e. α -lactalbumin in the presence and absence of dextran.

The possible mechanism by which dextran interferes with chaperone action of α -crystallin were investigated by intrinsic fluorescence spectroscopy, ANS-binding fluorescence and NMR spectroscopy.

Intrinsic fluorescence spectroscopy of α -crystallin indicated that dextran caused some structural rearrangements in its N-terminal domain, which may affect the binding of target proteins. α -Crystallin's chaperone binding site is believed to be in the C-terminal domain. However, because the N-terminal domain is hydrophobic, it has a crucial role in self-assembly and capture of target proteins, and is thus implicated in chaperone activity (Smulders et al. 1997).

ANS binding assays showed that the presence of a macromolecular crowding agent (dextran) resulted in the exposure of hydrophobic regions in the target protein (insulin), and decreased slightly their exposure in α -crystallin. Thus, it can be concluded that dextran accelerated destabilization of target proteins and somewhat structurally affected α -crystallin to prevent its interaction with target proteins, and both these effects are responsible for the poor chaperone performance of α -crystallin in the presence of a crowding agent.

NMR results show that dextran promoted, while α -crystallin decreased, rate of aggregation of α -lactalbumin. Furthermore, dextran increased the rate of

aggregation and of precipitation of reduced α -lactalbumin, more than in the presence of α -crystallin.

In summary, dextran had an effect both on the destabilization of target proteins and conformation of α -crystallin (shown by intrinsic fluorescence intensity, ANS binding assay and NMR spectroscopy). α -Crystallin suppresses the amorphous aggregation, but not as well as in the absence of dextran. Dextran has an effect on the conformation of α -crystallin. Thus, kinetic factors and structural changes of α -crystallin may be determinants in the reduced efficiency of α -crystallin as a molecular chaperone under molecular crowding conditions.

Chapter 4

**The effect of α -crystallin on the fibril
formation of target protein in the presence
and absence of dextran**

Many studies have shown that stress conditions like adding DTT or decreasing pH causes proteins to aggregate and form amyloid fibrils (Groves et al. 1998, Goers et al. 2002, Hatters et al. 2002a). However, molecular chaperones can prevent amyloid fibril formation of proteins (Hatters et al. 2001, Hatters et al. 2002b, Thorn, et al. 2005).

Crowding agents (e.g. dextran) increase the rate and extent of amyloid formation (Hatters et al. 2002a). α -Crystallin acts as a molecular chaperone interacting with partly structured amyloidogenic precursors, inhibiting amyloid formation in a nucleation dependent manner (Hatters et al. 2001). The chaperone action of α -crystallin in preventing amyloid fibril formation has, until now, been investigated in the absence of crowding agents. Thus, to mimic the chaperone action of α -crystallin *in vivo*, this study explored the effects of dextran (a crowding agent) on protein fibril formation and α -crystallin's ability to suppress it.

Bovine milk contains of 3.0-3.5% (w:w) protein, of which 80% is casein (Bobe et al. 1998). The bovine caseins exist in milk as a unique colloidal complex containing inorganic phosphate and calcium termed the casein micelle. This colloidal complex is composed of submicelles, consisting of α s₁-, α s₂-, β - and κ -casein molecules (Rasmussen et al. 1992, Farrell et al. 2003). Among these molecules, only α s₂-casein and κ -casein contain cysteines, with two residues in each polypeptide chain. α s₂-Casein is found both as a monomer and a dimer. In the α s₂-casein dimer both cysteines form interchain disulfide bonds linking the

two chains of each α_s -casein to each other. On the contrary, κ -casein is a disulfide-linked polymer with a wide distribution of sizes (Kruif et al. 1991). The monomeric form of κ -casein has a molecular weight of 19 kDa and contains two cysteine residues, which join together by interchain disulfide bond (Rasmussen et al. 1994, Fox and McSweeney, 2003). These cysteines are also capable of forming polymers by participating in interchain disulfide linkages (Rasmussen et al. 1994, Fox and McSweeney, 2003). According to Groves et al. (1998), the reduction of disulfide bonds in κ -casein occurs in the presence of urea or SDS with moderate concentration of DTT, or in the presence of higher concentration of DTT in the absence of urea or SDS. Thorn et al. (2005) induced fibril formation in α_s -casein and κ -casein with the addition of 20 mM DTT at 37°C. In the present study, DTT was added to reduce the disulfide bonds in α_s - and κ -casein.

α -Lactalbumin is a milk protein containing four disulphide bonds, which adopts a partially folded conformation under denaturing conditions. Goers et al. (2002) showed that α -lactalbumin forms fibrils under the conditions of low pH or by partial disulfide reduction at neutral pH. At low pH values, α -lactalbumin forms the so-called 'A' state with characteristics of a molten globule state (i.e. most of the secondary structure is in place but little tertiary structure is present), which makes it prone to amyloid fibril formation (Carver et al. 2002, Geors et al. 2002). In this study, low pH was used as a trigger for fibril formation by α -lactalbumin.

4.1 Thioflavin T binding assays with α -lactalbumin, α_s - and κ -casein

Thioflavin T binding assay of α -lactalbumin, α_s - and κ -casein was utilized to gain further insight into the effects of crowding agents on amyloid fibril formation of these proteins. Thioflavin T is a fluorescent dye that interacts with amyloid fibrils by binding specifically to stacked β -sheets characteristic of amyloid fibril, leading to a major increase in fluorescence intensity in the wavelength region from 480 to 490 nm (Hatters et al. 2002a). Amyloid fibril formation was monitored using thioflavin T binding assay in a spectrofluorimeter and a fluorescence plate reader and detected by the increase in ThT fluorescence. The ability of α -crystallin to prevent amyloid formation of α_s - and κ -casein (but not α -lactalbumin due to the extreme pH utilized) was also monitored using the same technique.

4.1.1 Thioflavin T binding assays with α -lactalbumin

The effect of dextran, as a macromolecular crowding agent, on amyloid fibril formation was demonstrated for apolipoprotein C-II by Hatters et al. (2002a) who demonstrated that addition of an inert polymer, such as dextran, greatly increased the rate of amyloid fibril formation. Furthermore, they confirmed that this effect is mostly due to macromolecular crowding. By comparison, it would be expected that dextran would increase amyloid fibril formation of α -lactalbumin as monitored by increasing in ThT fluorescence intensity. In order to confirm this hypothesis, 1.75 mg/mL of α -lactalbumin was incubated in 100

mM NaCl, pH 2.0, at 37°C in a shaker incubator. An enhancement in thioflavin T fluorescence and a reduction of the lag time, with an increase in the concentration of dextran, were observed (Figure 4.1), implying that dextran increased the rate of amyloid fibril formation. This was also evident from first-order rate constant from i.e. rate constant in the absence of dextran was $(2.23 \pm 0.93) \times 10^{-2} \text{ min}^{-1}$ while in the presence of 10% w/v dextran, it increased to $(10.7 \pm 1.1) \times 10^{-2} \text{ min}^{-1}$.

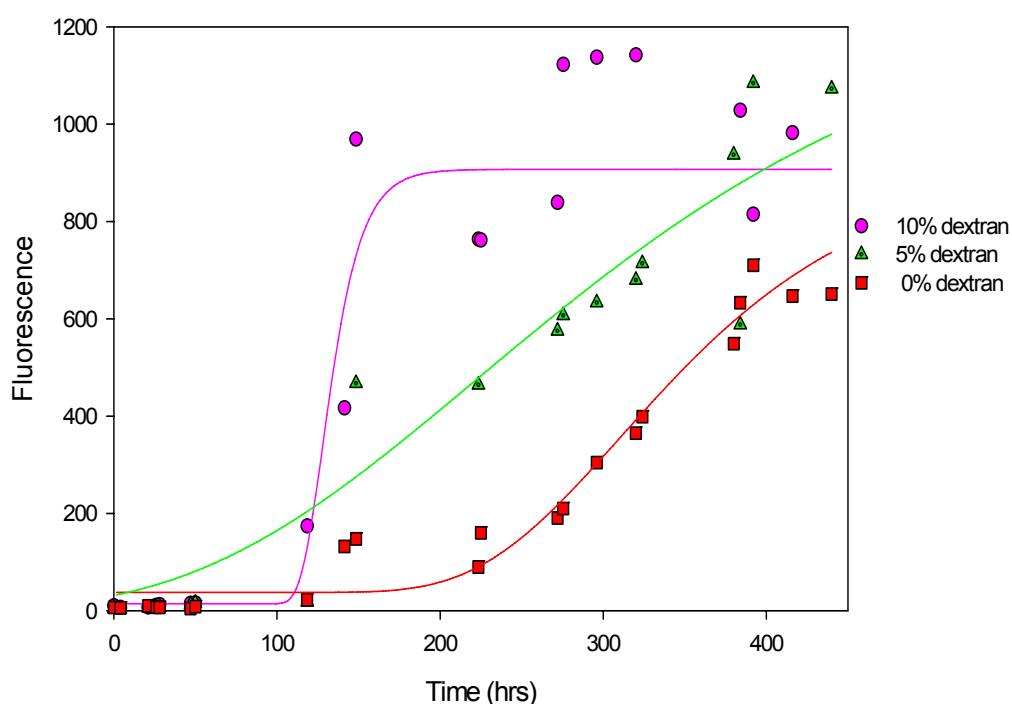


Figure 4.1: α -Lactalbumin amyloid formation (1.75 mg/mL) in the presence and absence of dextran as observed from the enhancement of thioflavin T fluorescence. The protein was in 100 mM NaCl, 0.05% (w/v) NaN_3 , pH 2 and the incubation temperature was 37°C with shaking. The experiment was done once and fitted using sigmaplot software.

4.1.2 Thioflavin T binding assays with α_s -casein

To compare the fibril formation of α_s -casein under reducing and non-reducing conditions, and the effect of dextran on amyloid formation, 2.5 mg/mL of α_s -casein was incubated at 37°C. In the absence of DTT, the increase in fluorescence intensity was minimal for the entire incubation period, with slightly elevated ThT binding at higher concentrations of dextran (Figure 4.2). Fibril formation was much more evident after repeating the experiment in the presence of 20 mM DTT, suggesting that reducing conditions are prerequisites for α_s -casein amyloid formation (Figure 4.3). These results in the absence of dextran are consistent with those of Thorn et al. (2005).

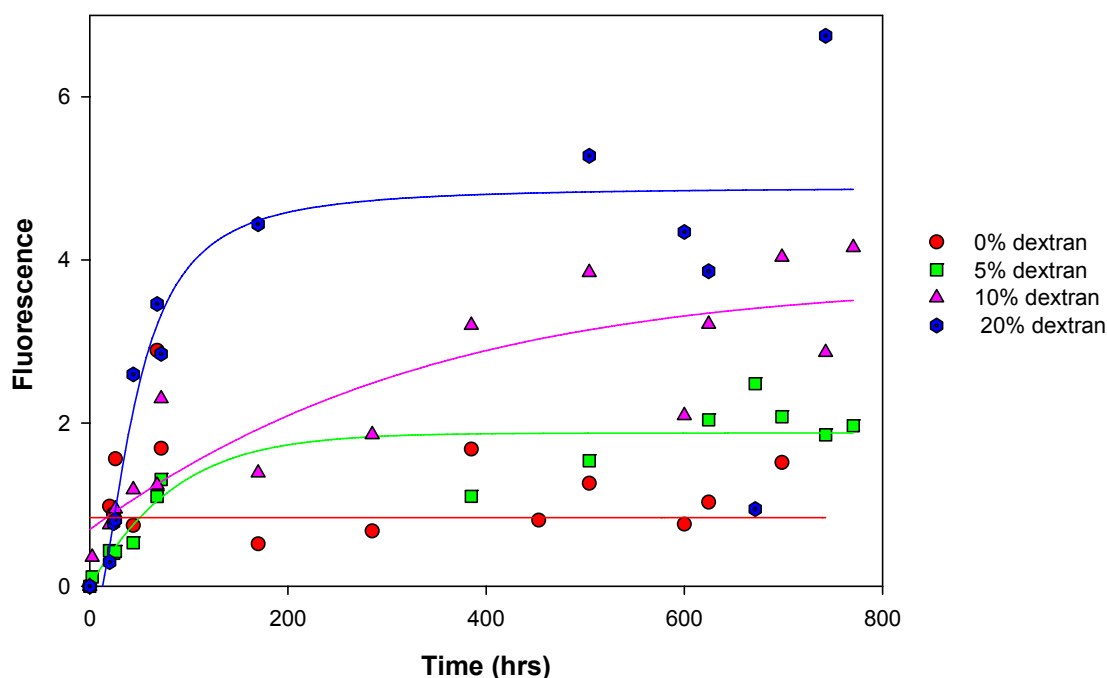


Figure 4.2: α_s -casein (2.5 mg/mL) amyloid formation in the presence of dextran and no DTT. Protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C. The experiment was done once and fitted using sigmaplot software.

Furthermore, fibril formation was promoted by dextran, as shown by an increase in ThT fluorescence intensity with the increasing concentrations of dextran (Figure 4.3). This was also evident from the first-order rate constants i.e. in the absence of dextran this value was $(3.57 \pm 0.73) \times 10^{-2} \text{ min}^{-1}$ while in the presence of 10% w/v dextran it increased to $(4.30 \pm 0.68) \times 10^{-2} \text{ min}^{-1}$. In general, the increase in the extent and rate of fibrillation was much greater in the presence of DTT than in its absence (compare Figures 4.2 and 4.3).

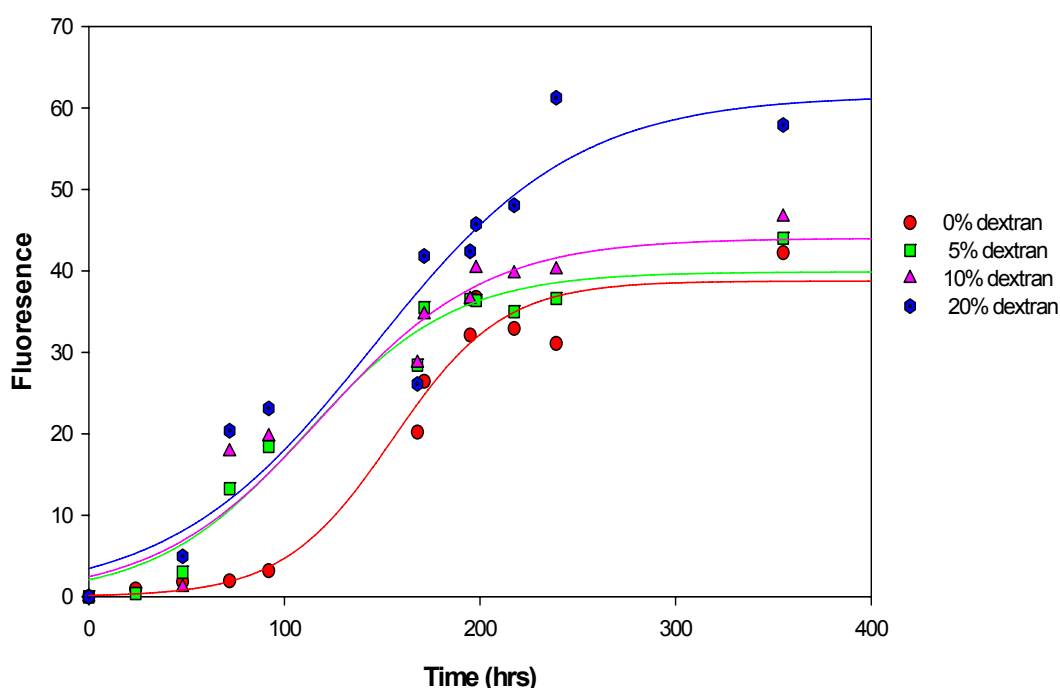


Figure 4.3: Amyloid fibril formation of reduced α_s -casein (2.5 mg/mL) in the presence and absence of different concentration of dextran (w/v). Protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C . The amyloid fibril formation of α_s -casein was initiated by the addition of 20 mM DTT. The experiment was done once and fitted using sigmaplot software.

4.1.3 Thioflavin T binding assay of α_s -casein in the presence of α -crystallin and 10% w/v dextran

It is well known that molecular chaperones (e.g. α -crystallin) can prevent the amorphous aggregation and precipitation of protein (Hatters et al. 2001, Thorn. et al. 2005). The effect of α -crystallin on amyloid formation by α_s -casein in the presence and absence of dextran was studied by incubation of α_s -casein with and without 10% w/v dextran and an equivalent amount of α -crystallin. α -Crystallin decreased amyloid formation of α_s -casein as shown in Figure 4.4. However, in the presence of dextran α -crystallin was a much poorer chaperone than in the absence of dextran in preventing amyloid fibril formation. In the

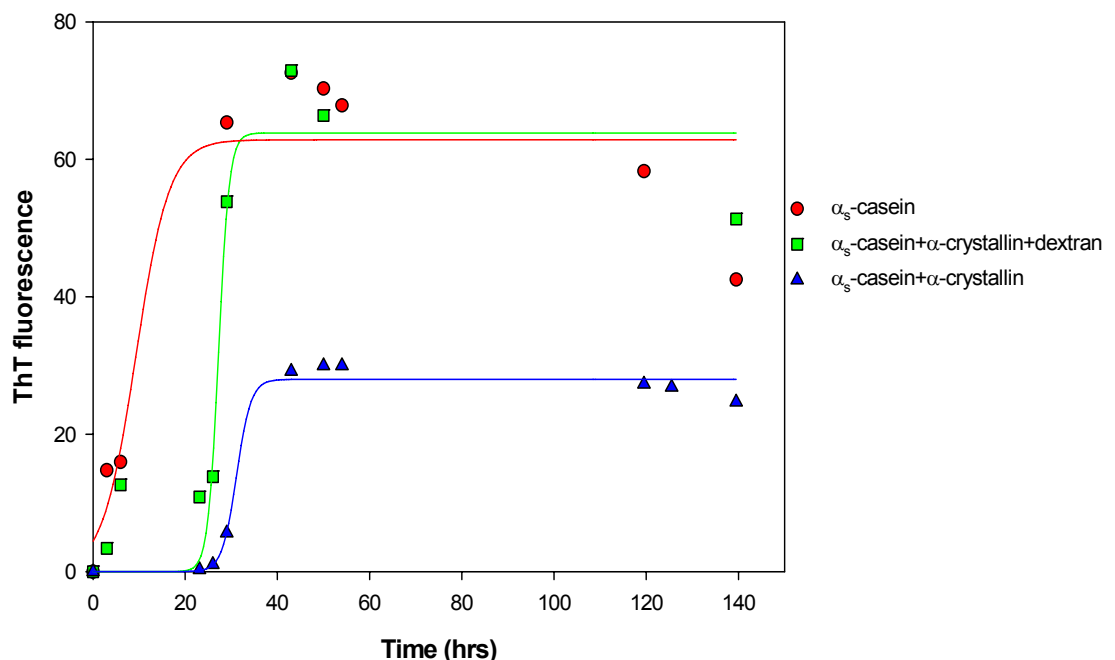


Figure 4.4: Amyloid fibril formation of reduced α_s -casein (2.5 mg/mL) in the presence and absence of α -crystallin (1:1 molar ratio) and 10% w/v dextran. Protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C. Amyloid fibril formation of α_s -casein was initiated by the addition of 20 mM DTT. The experiment was done once and fitted using sigmaplot software.

absence of dextran, the rate of aggregation of reduced α_s -casein with α -crystallin was $(4.05 \pm 1.0) \times 10^{-1} \text{ min}^{-1}$ while in the presence of dextran it increased to $(8.60 \pm 5.5) \times 10^{-1} \text{ min}^{-1}$.

4.1.3.1 Transmission electron microscopy (TEM) of reduced α_s -casein in the presence and absence of dextran and α -crystallin

Amyloid fibril formation of reduced α_s -casein, both in the presence and absence of dextran, was not detected by TEM. After 175 hours incubation, no fibrils of reduced α_s -casein (2.5 mg/mL) were observed by TEM (Figure 4.5A). Chains of globular particles were observed, as found by Thorn (2003). In addition, there were not noticeable differences in the morphology of aggregates with or without dextran (compare Figures 4.5A and B). However, in the presence of dextran more pronounced aggregated forms of α_s -casein were observed.

α_s -Casein contains two subunits named α_{s1} - and α_{s2} - with little sequence homology but similar physiochemical properties (Fox and McSweeney, 1998). The S1 subunit is the predominant subunit in α_s -casein and it does not contain any cysteine residues. The S2 subunit, which comprises approximately 20% of whole α_s -casein, contains two cysteine residues, which form dimers under non-reducing conditions by participating in intermolecular disulfide binding (Fox and McSweeney, 1998). Therefore, the predominant subunit, S1, does not form amyloid fibril and enhanced ThT binding arises from the reduction of disulphide-

linked dimers of α_{s2} -casein, which represent very limited fibril formation (Thorn. 2003). The spherical particles observed in TEM may arise

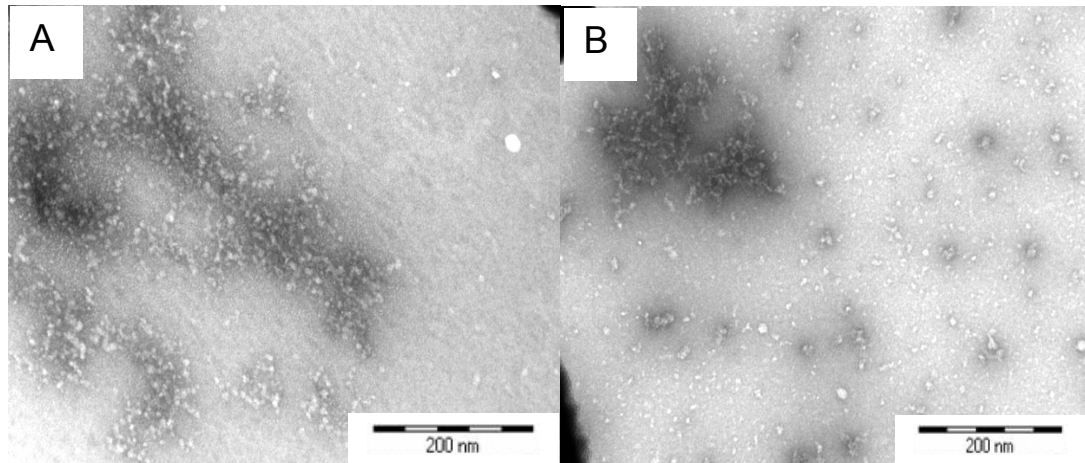


Figure 4.5: Negatively stained electron micrograph of reduced α_s -casein formed in the (A): absence and (B): presence of 10% w/v dextran. α_s -Casein (2.5 mg/mL) was incubated with 50 mM phosphate buffer, pH 7.5 and 20 mM DTT at 37°C for 60 hrs, before being analyzed by TEM. Scale bar represent 200 nm.

from aggregation upon heating or reducing condition which is non-fibrillar in character (Thorn. 2003).

4.1.4 Thioflavin T binding assay of κ -casein in presence of α -crystallin and 10% w/v dextran

κ -Casein was incubated in 50 mM phosphate buffer, pH 7.4 and 20 mM DTT at 37°C with constant shaking in the presence and absence of equivalent amount of α -crystallin to investigate α -crystallin ability to suppress amyloid fibril formation of this protein. κ -Casein, which in its native state contains extensive intermolecular disulfide bridging (Rasmussen et al. 1992), revealed a stronger tendency to form amyloid fibrils than α_s -casein. κ -Casein is classified as an

unfolded protein (Farrell et al., 2002) and hence there was no lag phase for increase in thioflavin T fluorescence. ThT binding assays showed that α -crystallin decreased the amyloid formation of κ -casein (Figure 4.6). This was also evident from the first-order rate constant which decreased from $(2.27 \pm 0.67) \times 10^{-1} \text{ min}^{-1}$ in the absence of α -crystallin to $(1.07 \pm 0.64) \times 10^{-1} \text{ min}^{-1}$ in the presence of α -crystallin.

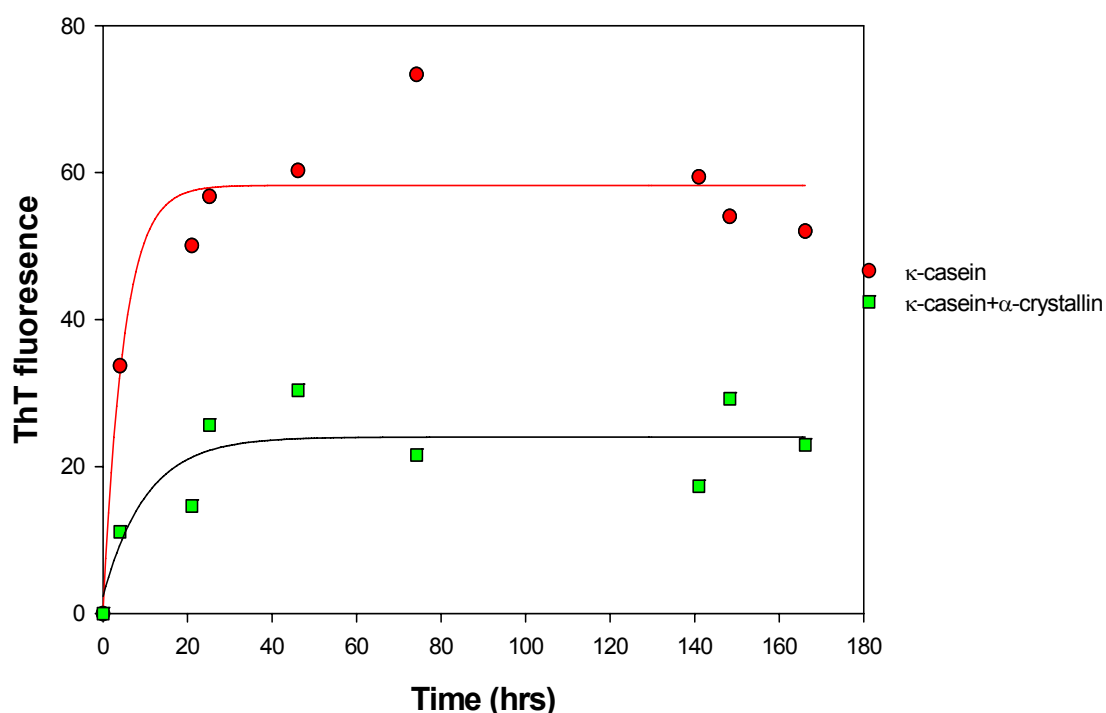


Figure 4.6: κ -casein (2.5 mg/mL) amyloid formation in the presence and absence of α -crystallin (1:1 molar ratio). Protein was in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C in shaker. The induced amyloid formation of κ -casein was initiated by addition of 20 mM DTT. The experiment was done once and fitted using sigmaplot software.

This experiment was repeated by a real-time measurement on a fluorescence plate reader. As shown in Figure 4.7, α -crystallin similarly decreased the amyloid formation of κ -casein. Furthermore, the reaction between α -crystallin

and κ -casein completed in less than 24 hrs, which is comparable to the timescale seen in Figure 4.6. In addition, rate constants for reduced κ -casein in the absence and presence of α -crystallin were $(3.73 \pm 0.10) \times 10^{-1} \text{ min}^{-1}$ and

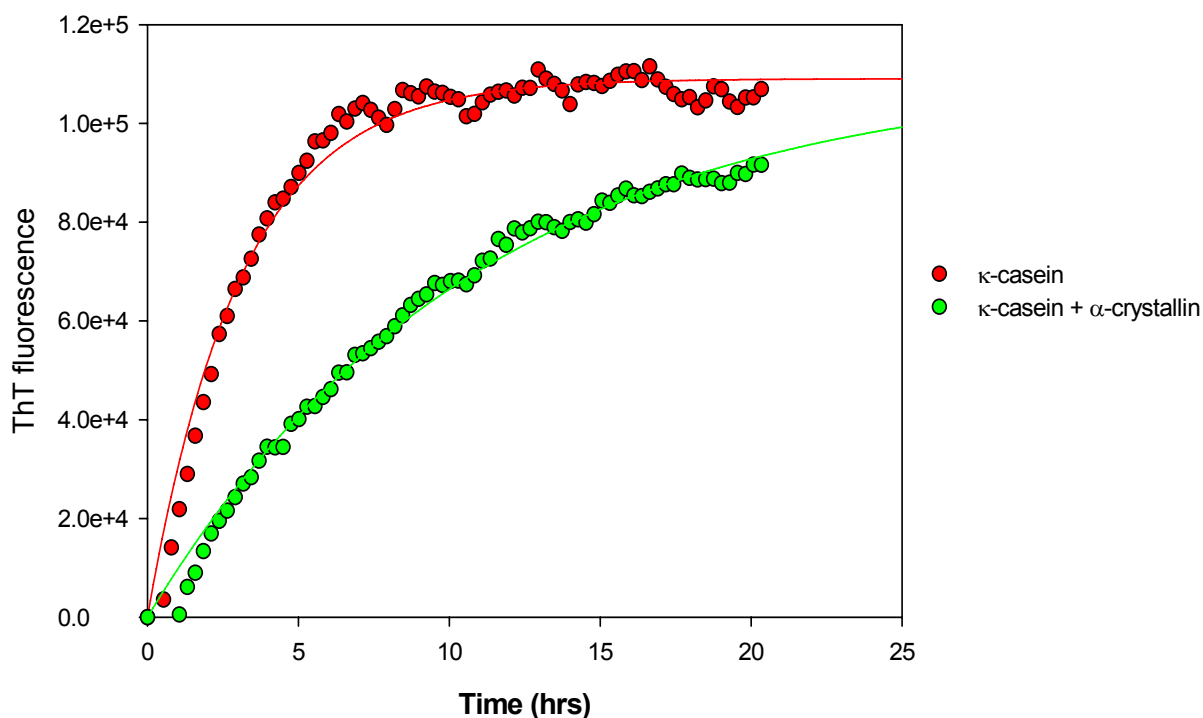


Figure 4.7: κ -casein (2.5 mg/mL) amyloid formation in the presence and absence of α -crystallin (1:1 molar ratio). Protein was in sodium phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 37°C in plate reader. The induced amyloid formation of κ -casein was initiated by addition of 20 mM DTT. The experiment was done once and fitted using sigmaplot software.

$(1.24 \pm 0.04) \times 10^{-1} \text{ min}^{-1}$, respectively, which are comparable to the rate constants obtained from Figure 4.6.

Under the same conditions but in presence and absence of α -crystallin and dextran, Figure 4.8 shows that dextran considerably accelerated the rate of

amyloid formation of κ -casein. This is evident from the rate constants, which increased from $(3.89 \pm 0.17) \times 10^{-1} \text{ min}^{-1}$ to $(7.09 \pm 1.14) \times 10^{-1} \text{ min}^{-1}$ in the presence of dextran. α -Crystallin decreased the rate of amyloid formation by reduced κ -casein, as the first-order rate constant decreased from $(3.89 \pm 0.17) \times 10^{-1} \text{ min}^{-1}$ in the absence to $(1.10 \pm 0.49) \times 10^{-1} \text{ min}^{-1}$ in the presence of α -crystallin, while dextran considerably accelerated the rate of amyloid formation by κ -casein (Figure 4.8). In the presence of dextran, α -crystallin was less able to prevent amyloid formation of κ -casein (rate constants $(1.10 \pm 0.49) \times 10^{-1} \text{ min}^{-1}$ vs $(1.54 \pm 0.17) \times 10^{-1} \text{ min}^{-1}$ in the absence

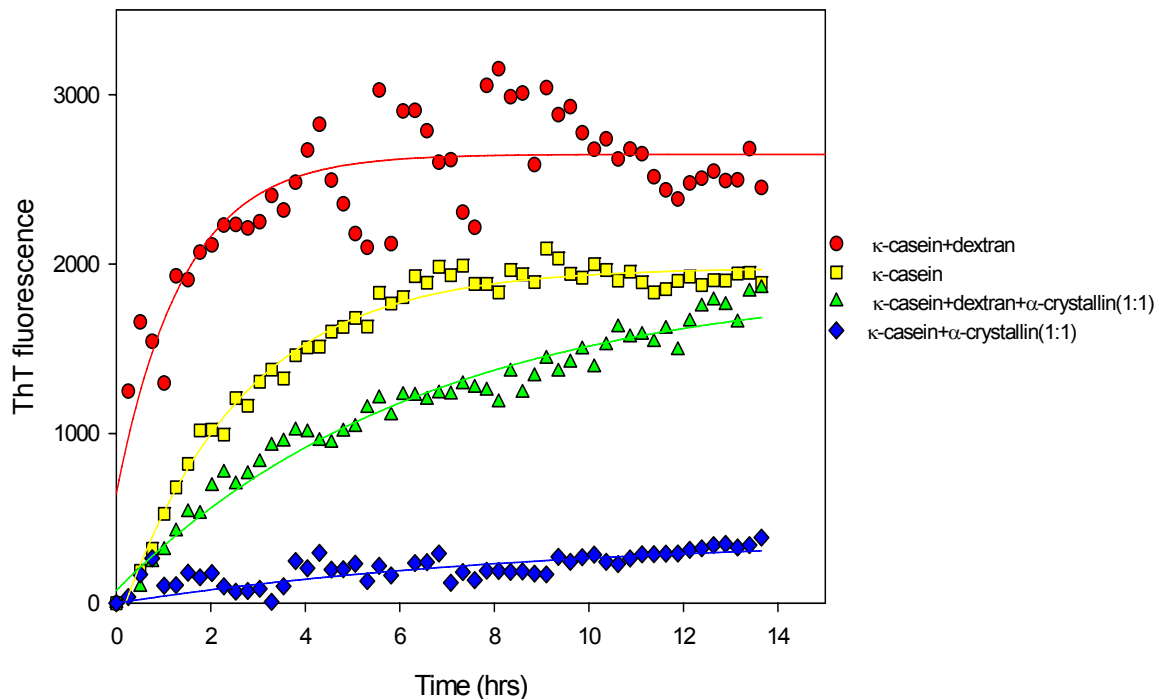


Figure 4.8: κ -casein (2.5 mg/mL) amyloid formation in the presence and absence of α -crystallin (1:1 molar ratio) and 10% w/v dextran. Protein was in 50 mM sodium phosphate buffers, 0.05% (w/v) NaN_3 ; pH 7.4, 4 μM ThT and the incubation temperature was 37°C in plate reader. The amyloid fibril formation of κ -casein was initiated by the addition of 20 mM DTT. The experiment was done once and fitted using sigmaplot software.

and presence of dextran, respectively). The rate constants for fibril formation by κ -casein measured under different conditions are summarised in Table 3.1.

Table 4.1: Summary of rate constants for κ -casein in thioflavinT binding assays. Rate constants were calculated by fitting an exponential function to thioflavinT binding data using Sigmaplot software (version 8.0). The standard deviation obtained from Sigmaplot upon regeneration calculation.

Sample components	Rate constant $\times 10^{-1}$ (min ⁻¹)
κ -casein	3.89 \pm 0.17
κ -casein + 10% w/v dextran	7.10 \pm 1.14
κ -casein + α -crystallin (1:1 molar ratio)	1.10 \pm 0.49
κ -casein + 10% w/v dextran + α -crystallin (1:1 molar ratio)	1.54 \pm 0.17

In summary, as assessed by thioflavin T binding, dextran accelerated the rate of amyloid fibril formation of α -lactalbumin, α_s - and κ -casein and reduced the chaperone efficiency of α -crystallin towards fibril-forming species. TEM experiments, intrinsic fluorescence, ANS binding and CD spectroscopy were performed on κ -casein under these conditions to confirm the change in the structure of κ -casein in the presence of dextran and α -crystallin.

4.1.5 Transmission electron microscopy (TEM) of reduced κ -casein in the presence and absence of dextran and α -crystallin

κ -Casein formed amyloid fibrils in the presence of 20 mM DTT, which was verified by TEM analysis as indicated in Figure 4.9. After an incubation period of 60 hours of reduced κ -casein at 2.5 mg/mL an abundance of fibrils was seen with an average length of 155.7 ± 60.2 nm (Figure 4.9A) agreement with Thorn et al. (2005). Amorphous aggregates were also present but only occasionally. In the presence of α -crystallin, fibrils were still observed but average fibril length was significantly reduced to approximately 40.6 ± 14.3 nm (Figure 4.9C). Amorphous aggregates were more prevalent in the samples compared to κ -casein in absence of α -crystallin. In the presence of dextran (Figure 4.9B), an increase in the number and length of fibrils was observed with an average length of 206.5 ± 83.7 nm. In the presence of dextran, α -crystallin appeared marginally less effective at inhibiting fibril formation than in the absence of dextran (Figure 14.9D) with 42 ± 11.0 nm being the average length. Amorphous aggregates were also present but the morphology of non-fibrillar aggregates were noticeably different compared to κ -casein with α -crystallin in the absence of dextran. The presence of amorphous aggregates of κ -casein in the presence of α -crystallin and in an α -crystallin and dextran mixture is agreement with Rekas et al (2005) who reported more amorphous aggregates of α -synuclein in the presence of α B-crystallin. In addition, it also fits with the notion that molecular

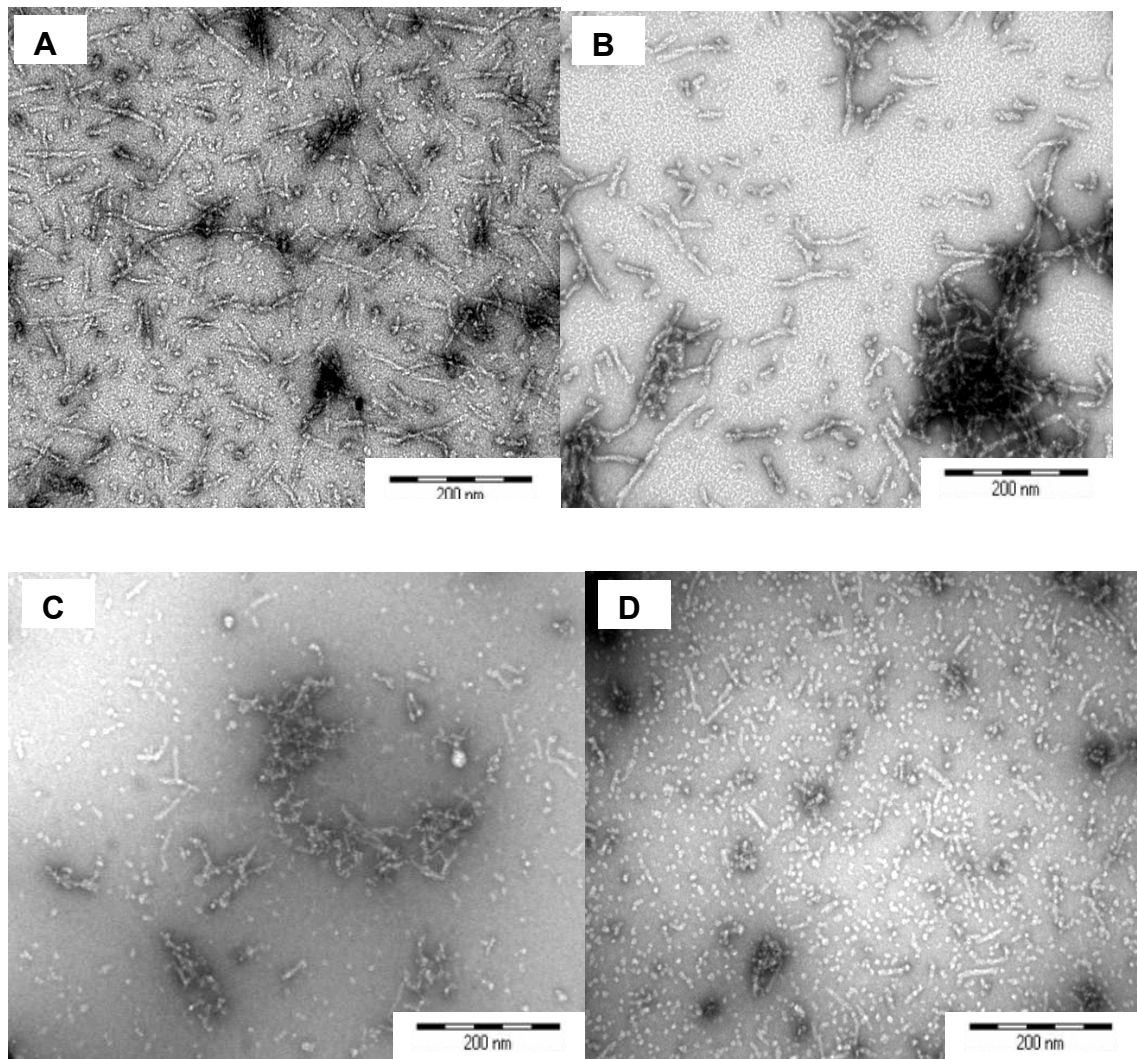


Figure 4.9: Negatively stained electron micrograph of (A): reduced κ -casein, (B): reduced κ -casein and 10% w/v dextran (C): reduced κ -casein and α -crystallin, (D): reduced κ -casein, 10% w/v dextran and α -crystallin. κ -Casein (2.5 mg/mL) was incubated in 50 mM phosphate buffer, pH 7.5 and 20 mM DTT at 37°C for 60 hrs, before being analyzed by TEM. Scale bars represent 200 nm. Note that the average length of fibril was calculated by measuring all fibrils within a selected representative area using Image J software.

chaperones redirect fibril forming species from the fibril-forming aggregation pathway to the amorphous one (Muchowski et al. 2002). These results supported the formation of amyloid fibrils by reduced κ -casein in the presence and absence of α -crystallin and dextran and showed that α -crystallin is effective in suppressing fibril formation, if only by reducing their size.

4.2 Intrinsic tryptophan fluorescence spectroscopy of κ -casein with α -crystallin and 10% w/v dextran

Further investigations of the effect of dextran on the amyloid formation of κ -casein and α -crystallin were undertaken via measurements of the intrinsic fluorescence of κ -casein in the presence or absence of α -crystallin and dextran. κ -Casein showed a high fluorescence emission from its single tryptophan residue at position 76 (Fox and McSweeney, 2003). After 3 hours of incubation of κ -casein with DTT at 37°C, the intrinsic tryptophan fluorescence decreased

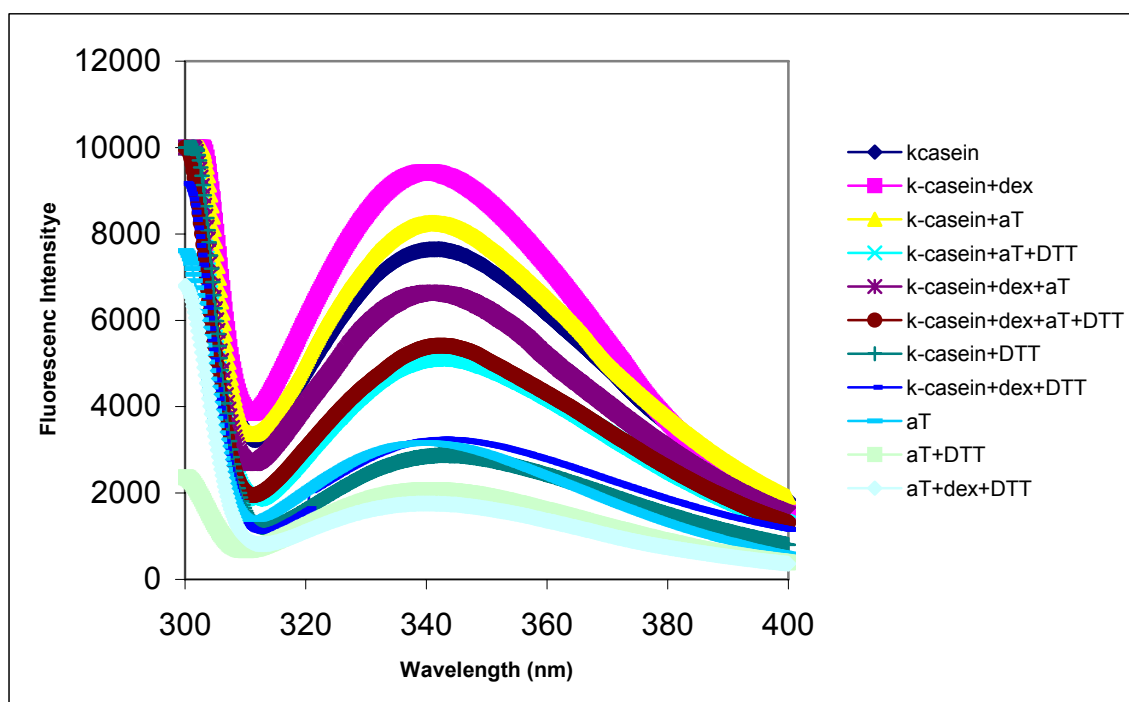


Figure 4.10: Intrinsic tryptophan fluorescence spectra of κ -casein, α -crystallin (α T) and dextran. The fluorescence spectra were measured at excitation and emission wavelengths of 300 and 400 nm respectively. All experiments were conducted in 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4 and 37°C.

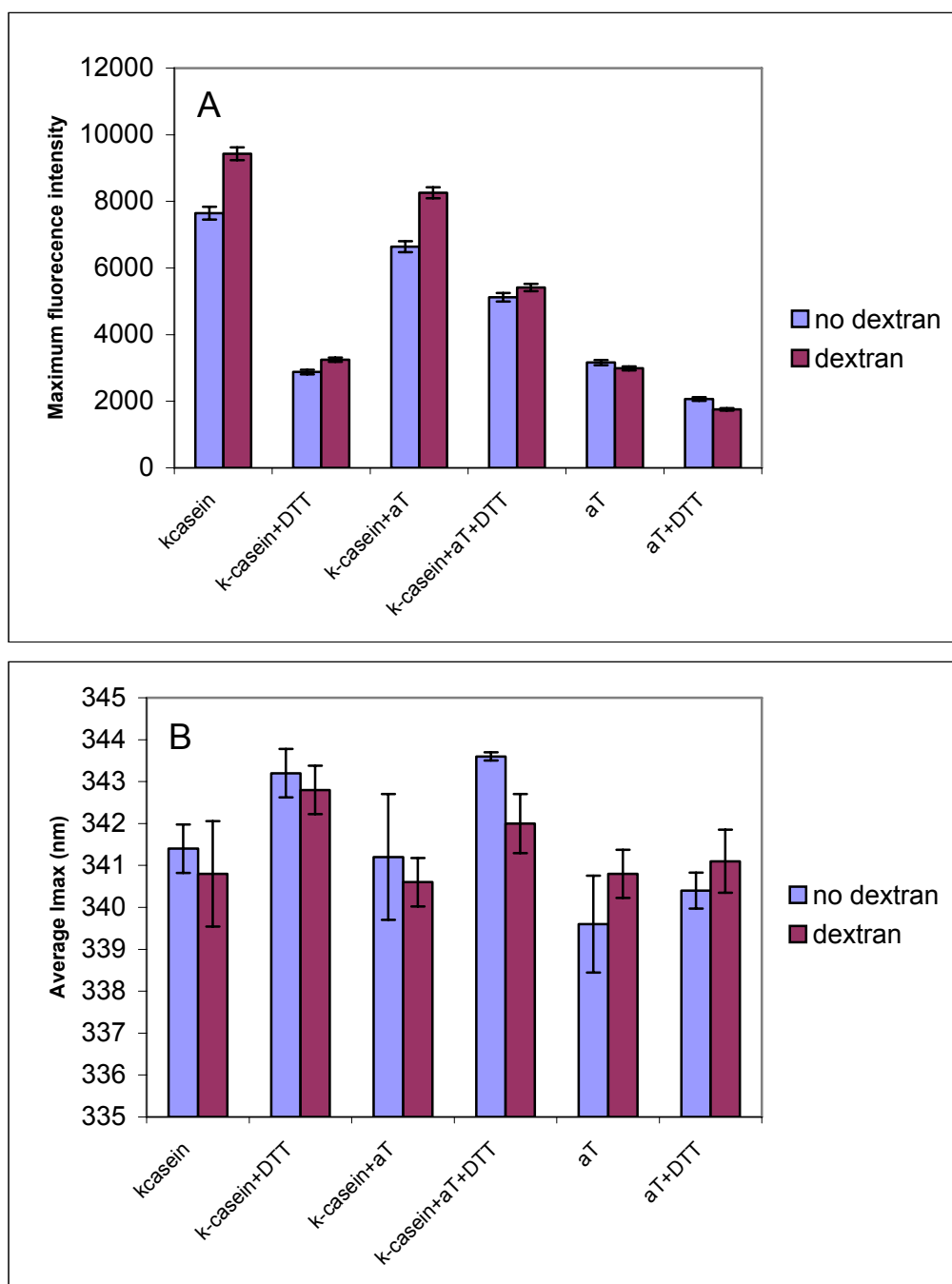


Figure 4.11: (A): The maximum intrinsic fluorescence of κ -casein (1 mg/mL) α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran and **(B):** The average λ_{\max} of κ -casein (1 mg/mL), α -crystallin (1:1) molar ratio and 10% w/v dextran. All experiments were conducted in a 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4 and 37°C. The error bars are absolute values of maximum calculated errors.

approximately 3-fold suggesting a major change in the conformation of κ -casein in the environment of its tryptophan residue (Figure 4.11). However, dextran

caused an increase in the fluorescence intensity of both native and reduced κ -casein and a decrease in λ_{\max} , indicating a conformational change that leads to less solvent exposure of tryptophan residues or more further away from charged groups in the structure of κ -casein. In both native and reduced κ -casein, the fluorescence intensity of the κ -casein and α -crystallin mixture was higher in the presence of dextran compared to in the absence of dextran. However, the effect of dextran was greater for the native κ -casein and α -crystallin than the reduced one.

4.3 ANS fluorescence studies: Interaction of κ -casein with α -crystallin in the presence and absence of 10% w/v dextran

4.3.1 κ -Casein in the presence of dextran and α -crystallin

ANS binding was used to detect changes in exposed hydrophobicity of reduced κ -casein in the presence or absence of dextran and α -crystallin. An increase in fluorescence intensity was observed by increasing the concentration of ANS. A plateau region was observed after the addition of ANS at around 10 μ M. This plateau region represents the amount of ANS bound to the exposed clustered hydrophobic regions of κ -casein, dextran and α -crystallin saturated with ANS. κ -Casein has a very large capacity for binding ANS (Figure 4.12), which indicates large exposed hydrophobic areas (Kumosinski et al. 1993). By contrast, at the same subunit concentration, α -crystallin's ANS fluorescence is approximately 9 times lower than that of κ -casein because of its lower exposed

hydrophobicity compared to κ -casein. The ANS fluorescence values of mixtures of κ -casein and α -crystallin in the absence of DTT were 13% less than the sum of those of the individual proteins. In the presence of dextran, the fluorescence intensity decreased about 29% compared to native κ -casein. By the addition of α -crystallin to the κ -casein and dextran mixture, the fluorescence intensity decreases by about 12% compared to κ -casein and dextran mixture. This latter of κ -casein and α -crystallin and dextran mixture is about 59% lower than the fluorescence intensity of the κ -casein and dextran mixture. These data suggest that no interaction occurs between κ -casein and α -crystallin in their respective native states. Furthermore, the decrease in the signal of the κ -casein, 12% of which is monomeric at 25°C (Groves et al. 1992), indicates less ANS binding to the monomeric protein in the presence of dextran. This implies that at 37°C, κ -casein is less monomeric which means, in turn, that κ -casein is aggregated (Figure 4.12. B). This is in agreement with the idea that native κ -casein polymerizes at 37°C (Vreeman et al. 1986, Groves et al. 1998, Farrell et al. 2003).

4.3.2 Reduced κ -casein in the presence of dextran and α -crystallin

The above experiments were carried out with the addition of DTT to κ -casein in the presence and absence of dextran and α -crystallin. After 3 hours of incubation of κ -casein with DTT, the ANS fluorescence intensity decreased (by approximately 32%) (Figure 4.12), indicating that aggregation of reduced

κ -casein decreased the exposure of its clustered hydrophobic residues. In both native

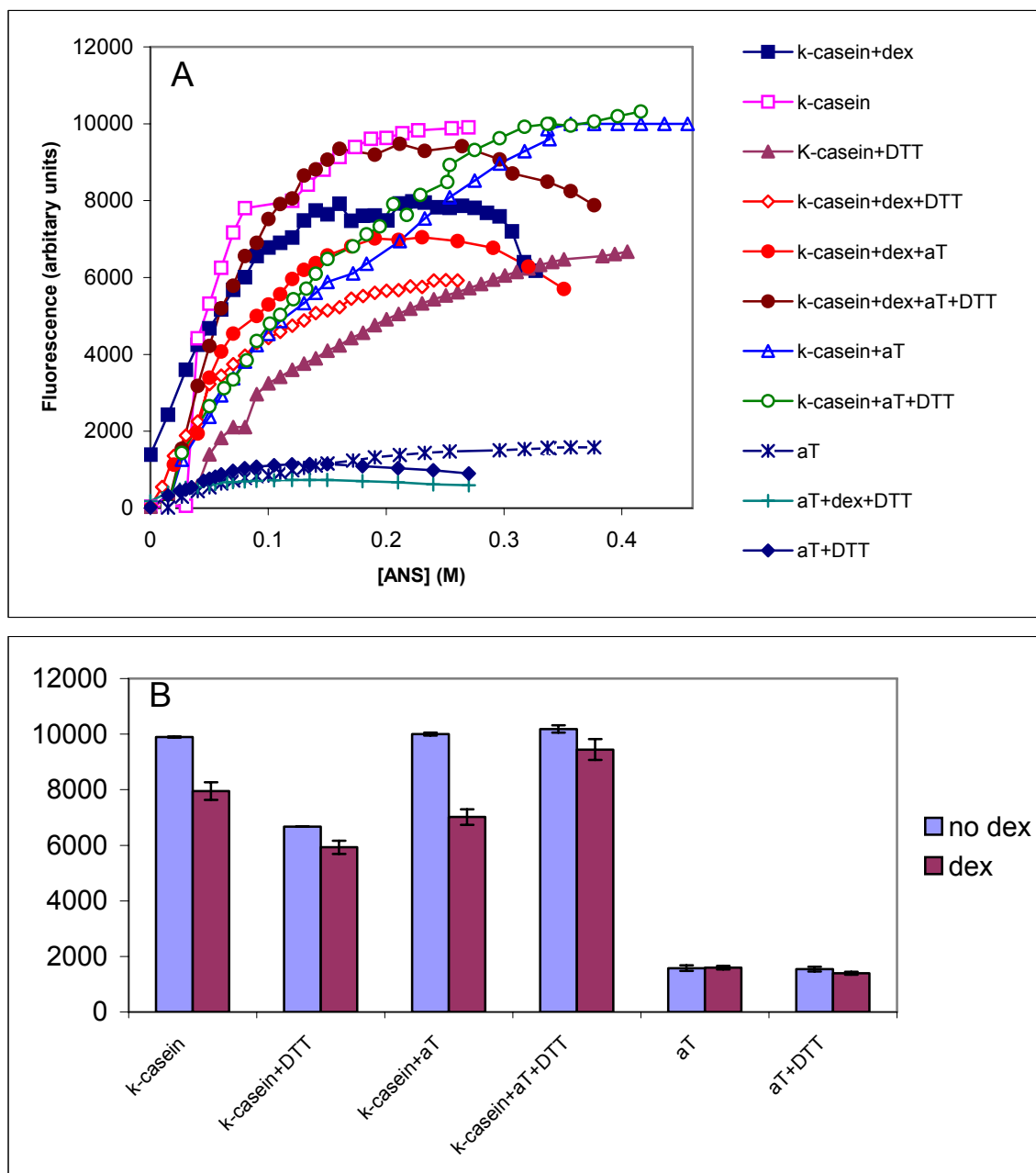


Figure 4.12: (A): Fluorescence emission results for the binding of the hydrophobic probe ANS to κ -casein (1 mg/mL), α -crystallin (α T) (1:1 molar ratio) and 10% w/v dextran in the presence and absence of DTT. **(B):** Average maximal fluorescence (180-420 μ L) for κ -casein (1 mg/mL), α -crystallin (1:1 molar ratio) and 10% w/v dextran alone and in the presence of each other, and 20 mM DTT. Note the plateau region, which arises from the saturation of ANS binding sites on the protein. All experiment was conducted in 50 mM phosphate buffer, pH 7.4 and 37°C. The error bars are absolute values of maximum calculated errors.

and reduced κ -casein, dextran decreased the fluorescence intensity (by ~29% and 12% respectively) (Figure 4.12) consistent with enhanced aggregation and/or conformational change (Farrell et al. 2003). Upon the addition of an equimolar amount of α -crystallin to reduced κ -casein, the ANS fluorescence intensity increased by approximately 35% compared to approximately 10% under non-reducing conditions, showing an interaction between the two proteins along the aggregation pathway of κ -casein (Bobe et al. 1998). The addition of dextran to the mixture of reduced κ -casein and α -crystallin caused a decrease in fluorescence intensity of about 37%, compared to that in the absence of dextran, implying that dextran increases aggregation of κ -casein and decreases the interaction between κ -casein and α -crystallin.

4.4 Circular Dichroism (CD) spectroscopy of κ -casein

CD provides information about the overall secondary and tertiary structure of proteins. The overall secondary structural content can be determined by CD spectroscopy in the far-UV spectral region, i.e. the CD signal reflects an average of secondary structure of the entire molecular population. The CD spectrum of a protein in the near-UV spectral region is sensitive to the tertiary structure of a protein, especially the environment of aromatic, (e.g. tryptophan) residues. The change in the near-UV spectrum results from protein-protein interactions or from changes in solvent conditions (Ewbank et al. 1993). Figure 4.13 shows the near-UV CD spectra of the native state, the reduced state and the dextran-induced species of κ -casein. The near-UV spectrum of native

κ -casein comes from the aromatic residues and disulphide bonds, which are scattered through the protein molecule (Figure 4.13). It shows a negative ellipticity at around 270 nm and 275 nm and a positive peak at 290-297 nm due to the nine tyrosine and one tryptophan residues, respectively (Technical report. E.C.S. 2001). A spectrum recorded 1 minute after the reduction of κ -casein showed an increase in Trp signal at around 280 nm indicating some change in the environment of the single tryptophan residue (Trp 76) correlating with the intrinsic fluorescence results. The spectrum also shifted to lower intensity. This effect could also be caused by the cleavage of the disulfide bonds, as was reported for the reduction of α -lactalbumin in a previous study (Ewbank et al. 1993) indicative of a decrease in the amount of tertiary structure due to unfolding of the protein. Adding dextran to native κ -casein shifted the signal of tyrosine residues from 268.2 nm to 277.6 nm and caused some shift in tryptophan residue signal as indicated by Figure 4.13. However, dextran had more effect on reduced κ -casein than on native κ -casein. Reduction of κ -casein in the presence of dextran decreased the signal from tryptophan and tyrosine residues even more than under non-crowding conditions. Thus, dextran had an effect on the tertiary structure of κ -casein especially in the region of the tryptophan residue (N-terminus).

The far-UV spectrum of κ -casein could not be acquired due to high absorbance of dextran in this region.

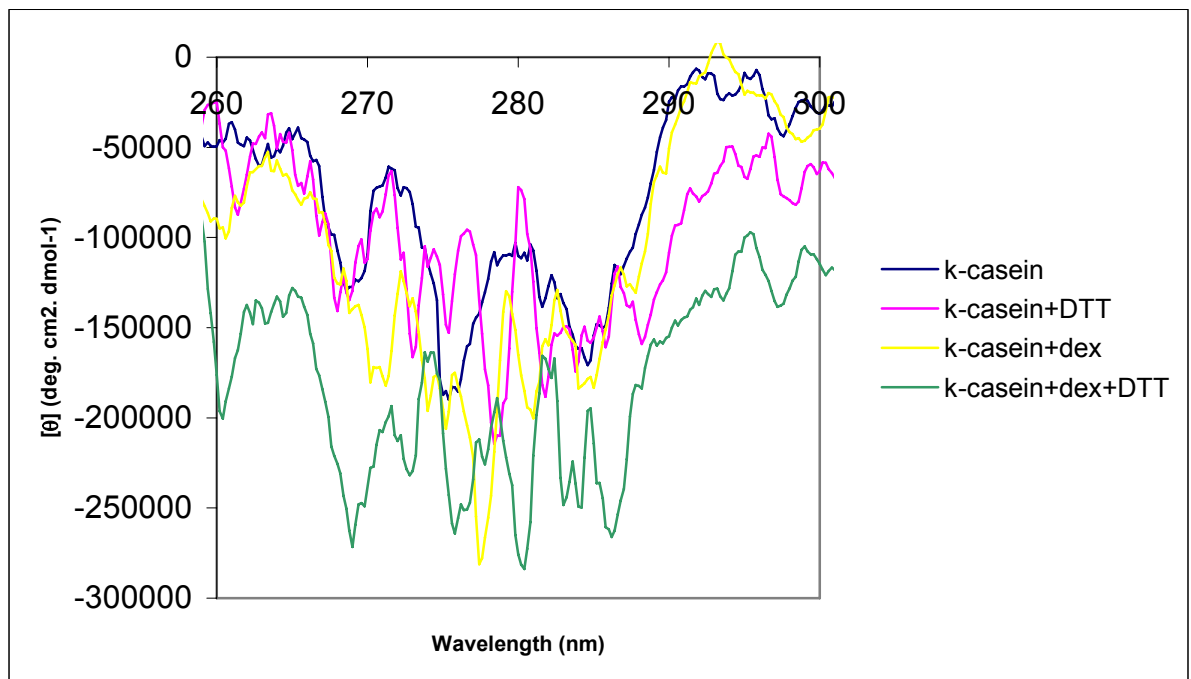


Figure 4.13: Near-UV CD spectra of the native state, the reduced state and the dextran-induced species of κ -casein. Protein concentration was 4 mg/mL in 10 mM phosphate buffer, pH 7.0, and 37°C in a J-810 spectropolarimeter with a 1 cm pathlength cell.

4.5 ^1H NMR spectra of κ -casein aggregation in the presence and absence of 10% w/v dextran after the addition of DTT

As mentioned in Section 4.1, κ -casein has disulphide bonds, which can be reduced by the addition of DTT (Kruif et al. 1991, Thorn. 2003). This reduced state of κ -casein is unstable and aggregates to form amyloid fibrils in less than 24 hrs at 37°C (Thorn. 2003), which was accelerated in the presence of dextran. This process was monitored by real time ^1H NMR spectroscopy. Figures 4.14A and B show the changes in the aromatic region of the 1D ^1H NMR spectra obtained for κ -casein in the presence and absence of dextran, respectively, at various times following the addition of DTT.

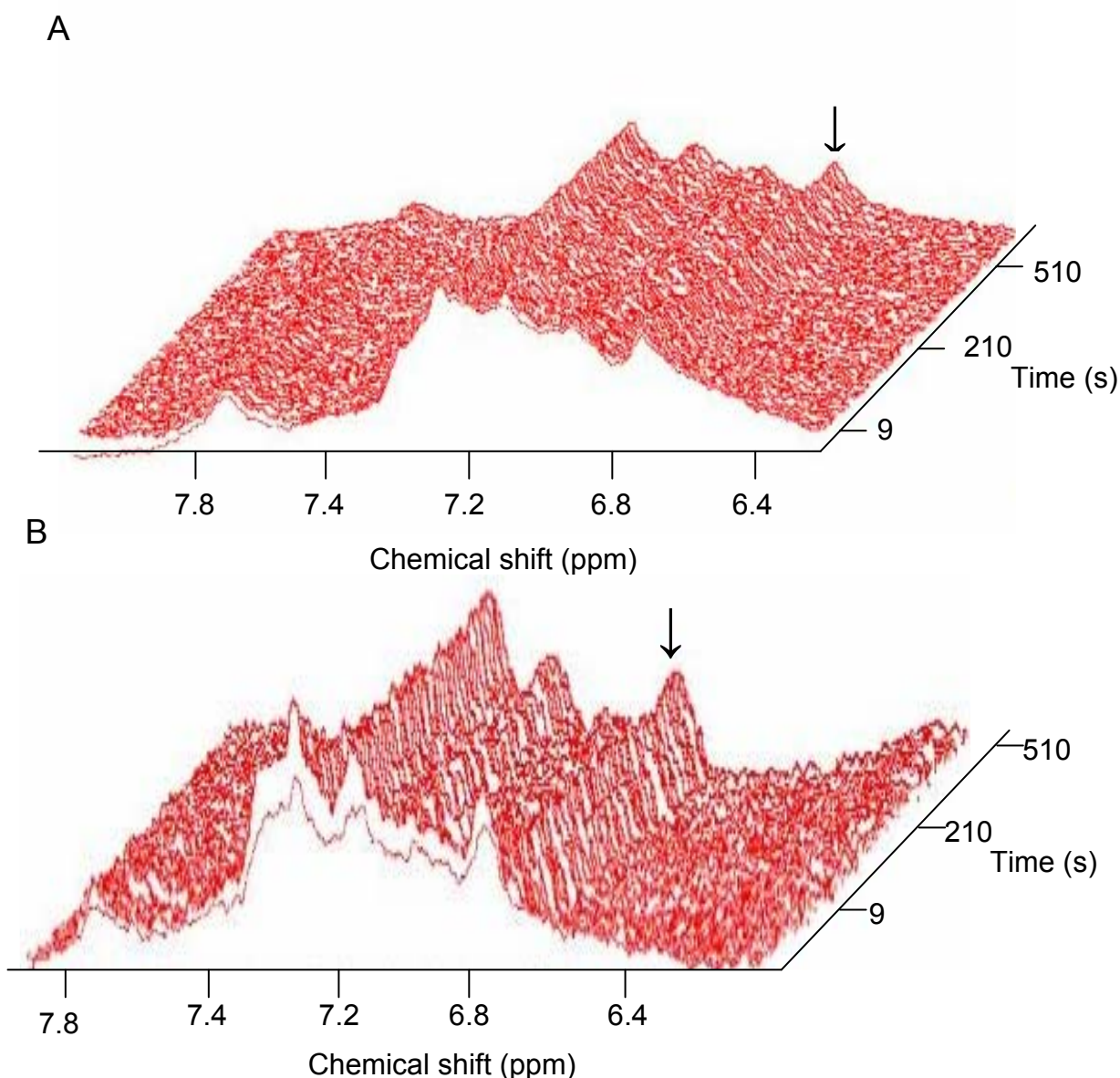


Figure 4.14: The aromatic region of series 1D ¹H NMR spectra of κ -casein in (A): reduced κ -casein and in (B): reduced κ -casein and 10% w/v dextran mixture. All experiments were conducted in 50 mM phosphate buffer, 100% D₂O, 1 mM EDTA, pH 7.4 and 37°C.

The increase in intensity of 6.86 ppm peak to a maximum level was due to κ -casein undergoing maximum reduction of its disulphide bonds and adoption of an intermediately folded conformation that is different to its native one. This is most likely, by analogy with what occurs for α -lactalbumin upon reduction of its four disulphide bonds (Lindner et al. 1997, Carver et al. 2002). It is generally agreed that the structure of κ -casein is natively unfolded (Rasmussen et al.

1994, Fox and McSweeney, 2003) and what happens to it upon reduction is not known. A decrease in the height of this peak is due to the aggregation and eventual precipitation of κ -casein out of solution.

The reduction and aggregation of κ -casein in the presence and absence of dextran was analyzed by monitoring with time the intensity of the Tyr (3,5) resonance at 6.88 ppm in κ -casein (Fig 4.15). For both reduced κ -casein and the mixture of reduced κ -casein and dextran the intensity of the resonance initially increased reaching a maximum at ~ 45 min and ~ 15 min, respectively. These times corresponded to the maximum unfolding of the polypeptide chain due to disulfide reduction, which was faster for κ -casein in the presence of dextran. After these times, the resonance intensity decreased due to aggregation of fully reduced κ -casein.

The decrease in the intensity of the resonance at 6.88 ppm was fitted to a simple first-order kinetic decay. The rate of aggregation of reduced κ -casein was calculated from the first-order decay of the signal as being $(2.16 \pm 0.45) \times 10^{-1} \text{ min}^{-1}$. In the presence of 10% w/v dextran, the rate of signal decay increased approximately four fold $(8.45 \pm 0.29) \times 10^{-1} \text{ min}^{-1}$. The complete loss of signal was seen at 625 min and 350 min for reduced κ -casein and the reduced κ -casein and dextran mixture, respectively. In the ThT binding assay of κ -casein (Figure 4.8) these times correspond to complete amyloid formation of κ -casein. Thus, the aggregation rate of κ -casein is faster in the presence of dextran compared to the absence of dextran.

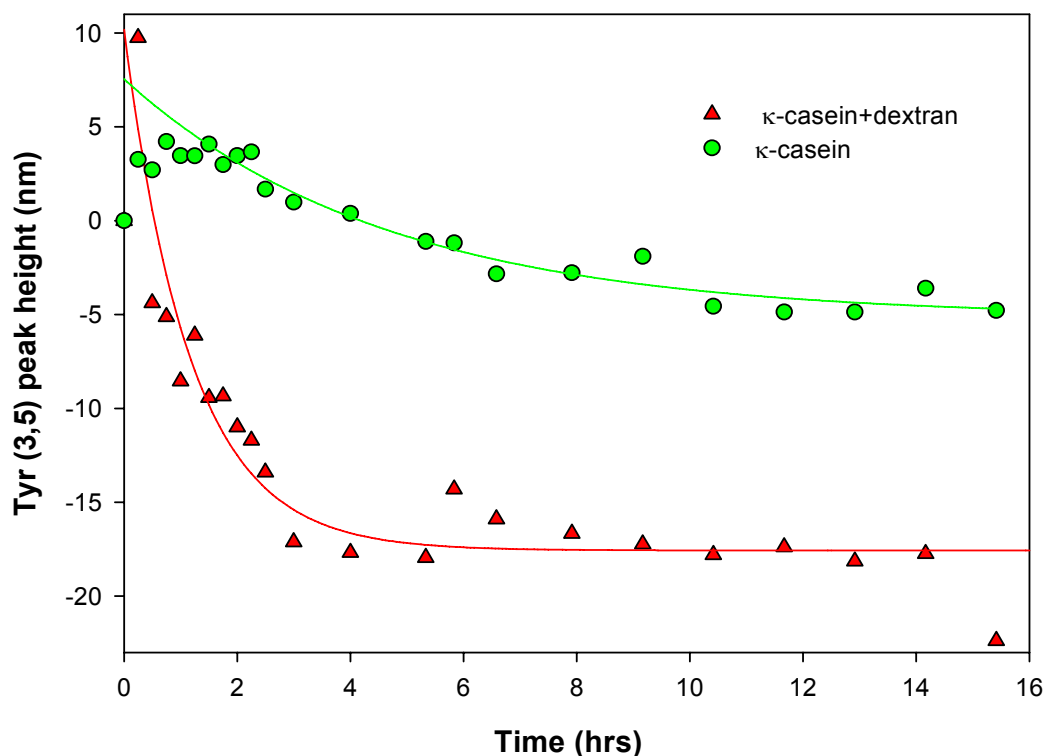


Figure 4.15: First order decay plots of reduced κ -casein (2.5 mg/mL) tyrosine (3,5) ring proton resonance with time in the absence and presence of 10% w/v dextran. Experiments were conducted in 50 mM phosphate buffer, pH 7.4, 100% D₂O, 1mM EDTA, and 37°C.

4.6 1D and 2D ¹H NMR spectroscopy of κ -casein in the presence and absence of dextran.

To further investigate changes in the tertiary structure of κ -casein in the presence of dextran, 2D ¹H-¹H TOCSY experiments were performed on κ -casein in the presence and absence of 5% w/v dextran. A 10% w/v level of dextran was not used since the high intensity of its resonances obscured those of κ -casein. The aromatic and aliphatic regions of 1D ¹H NMR of κ -casein in the presence and absence of dextran (Figure 4.16) revealed obvious differences.

Resonance intensities were measured at discrete frequencies from a baseline connecting the baselines at 0.0 ppm and 8.0 ppm after phasing of the spectra. Spectral plots were scaled to the height of the resonance at 1.94 ppm. The spectrum of κ -casein (Figure 4.16A) shows, as expected, little chemical shift dispersion characteristic of a relatively unstructured (naturally unfolded) protein (Rasmussen et al. 1994, Fox and McSweeney, 2003). While the spectrum was similar in the presence of dextran, (Figure 4.16B) a marked overall resonance broadening (loss of signal) occurred implying that the structure of κ -casein was not affected significantly by the presence of dextran and that the correlation time of the protein was increased leading to broader resonances.

Figure 4.17A shows the TOCSY spectrum of κ -casein in the absence of dextran with a large population of well-resolved cross-peaks, arising from the aliphatic region. Upon the addition of 5% w/v dextran, a significant loss of cross-peak intensity was observed (Figure 4.17B). Thus, the cross-peaks from the aliphatic region were altered significantly by the presence of dextran i.e. cross peak arising in the region of 0.9 to 3.0 ppm showed a significant loss of intensity. This could be due to the increased correlation time of κ -casein in the presence of dextran similar to what was observed with α -lactalbumin and dextran (Section 3.7). In addition cross peaks in the area between 3.5 and 4.5. ppm were not observed in the presence of dextran. In addition it is apparent from Figures 4.16A and B that there is a significant loss of cross-peak intensity in the aliphatic area in the presence of dextran. In summary, these result show

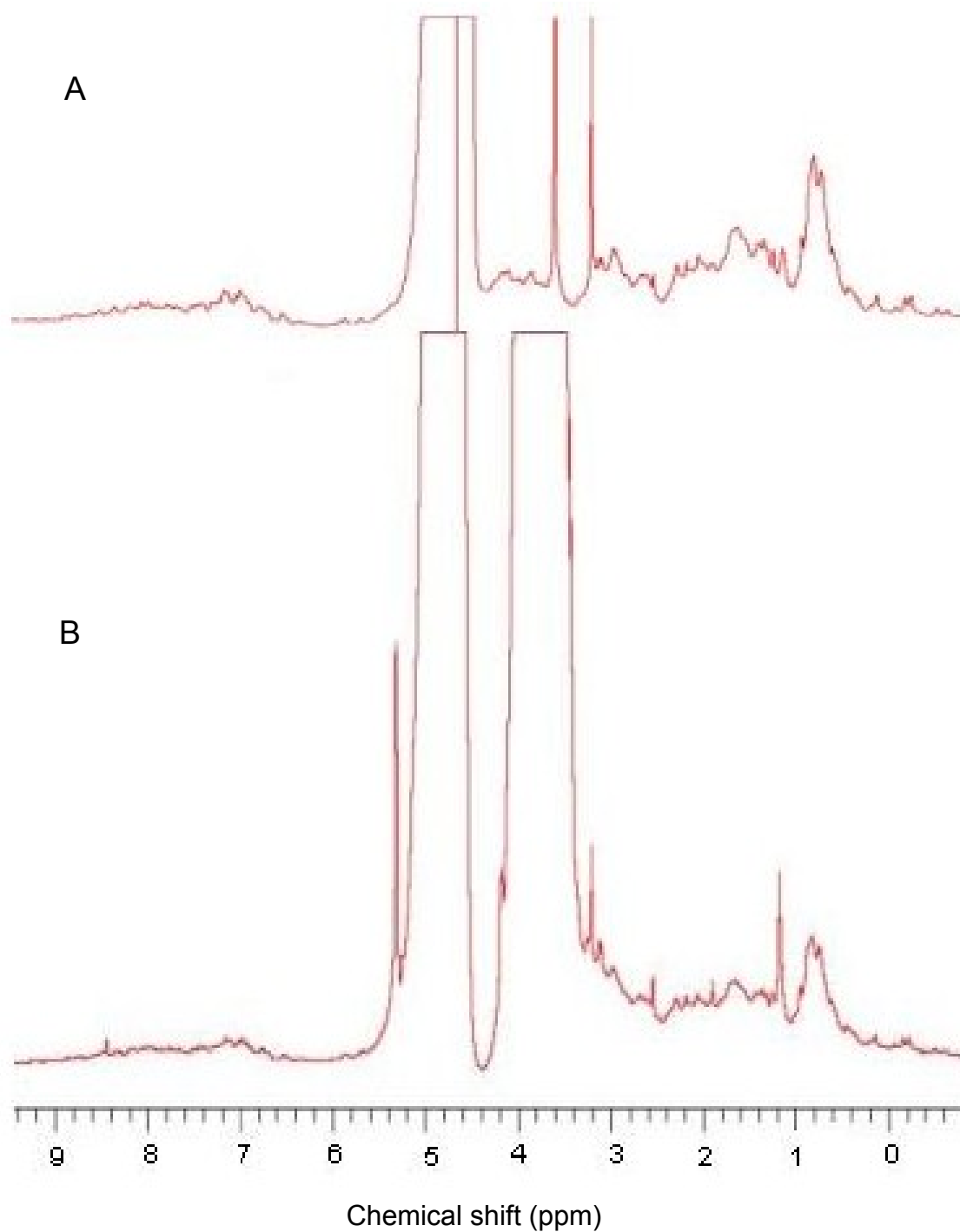
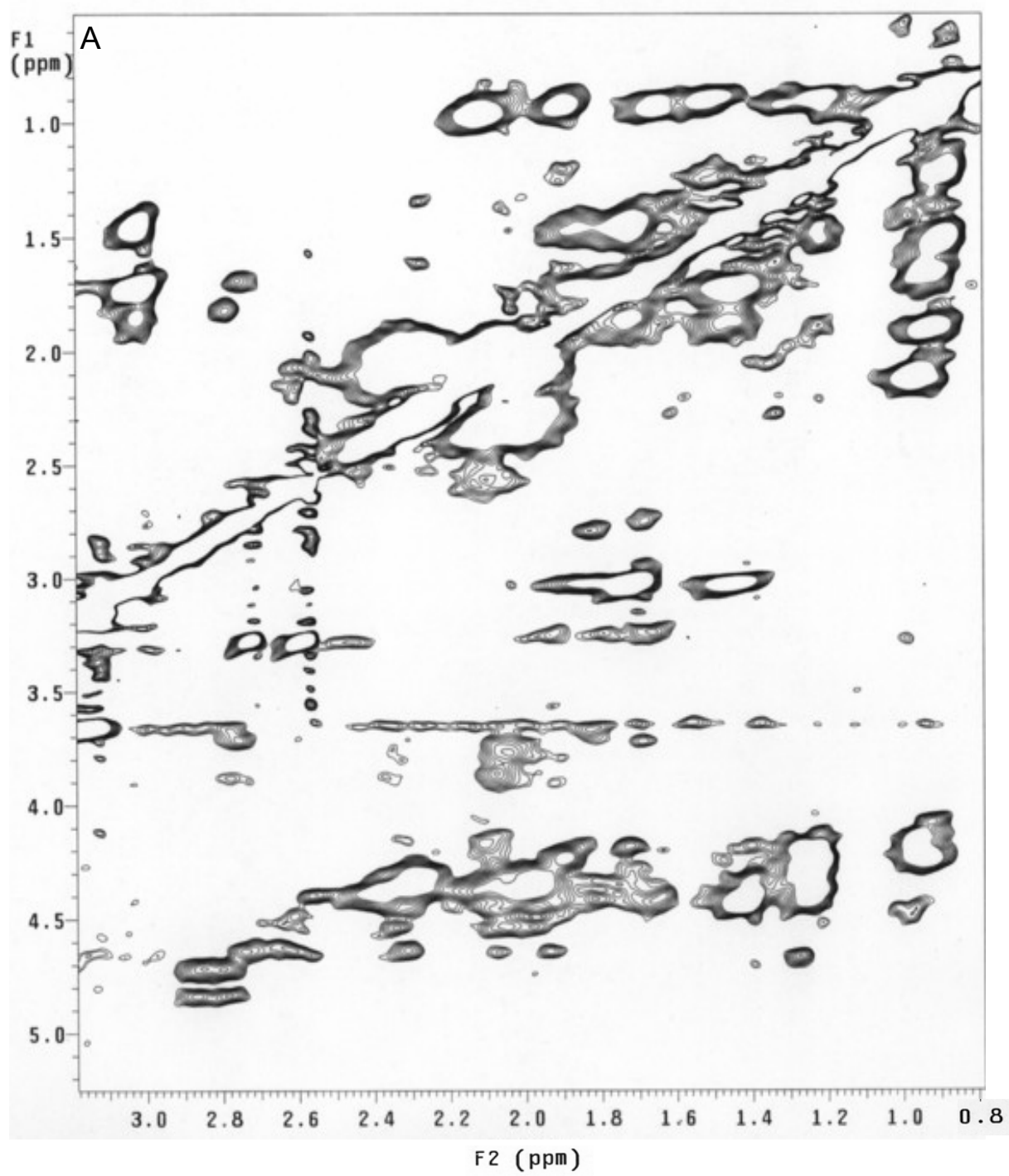


Figure 4.16: ^1H 1D NMR spectra of κ -casein in (A): no dextran and (B): 5% w/v dextran. All experiments were conducted in 50 mM phosphate buffer, 100% D_2O , pH 7.4, 1 mM EDTA, at 37°C . The spectral region between 3.0–4.5 ppm arises from the sugar resonances of dextran. Peak intensities were referenced to the resonance at 1.94 ppm, which arises from aromatic residues.



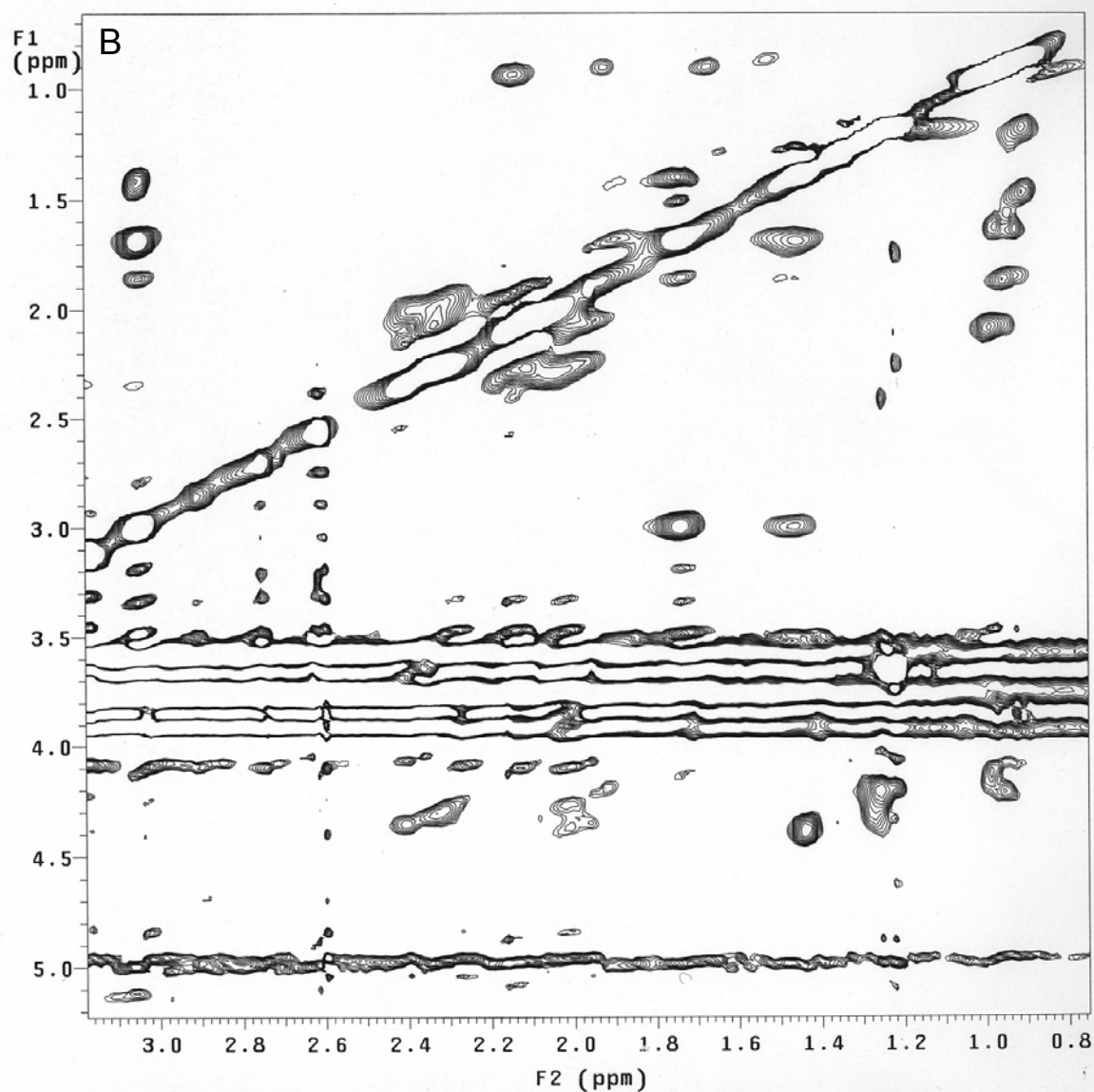


Figure 4.17: The aromatic region of a TOCSY spectrum of κ -casein (2.5 mg/mL) in the (A) absence and (B) presence of 5% w/v dextran. Both experiments were conducted in 50 mM phosphate buffer, 100% D₂O, and pH 7.4, 1 mM EDTA, at 37°C. Note that the vs2d levels of these spectra are different and contour plots were generated with the lowest level set close to the noise level. The band of noise on the F1 axis between 3.4-4.0 in Figure (B) arises from the sugar resonances of dextran.

that dextran has little effect on the overall structure of κ -casein which in its natural state is that of a predominately unfolded protein.

4.7 Size exclusion HPLC

Size exclusion HPLC was employed to investigate and compare the size of κ -casein molecules in the absence and in the presence of different concentrations of dextran. These experiments were performed with a Phenomenex® BioSep SEC4000S column which is capable of separating proteins over a mass range of 78-2000 kDa. Standard samples were prepared in the same buffer and with the same concentration of κ -casein (10 mg/mL) and dextran to determine their mass ranges. These standard samples included blue dextran (2.0 MDa), bovine thyroglobulin (670 kDa), catalase (250 kDa), ovotransferrin (78 kDa), and bovine serum albumin (BSA) (67 kDa). All protein standards were detected at 280 nm except in the case of blue dextran, which was detected at 600 nm.

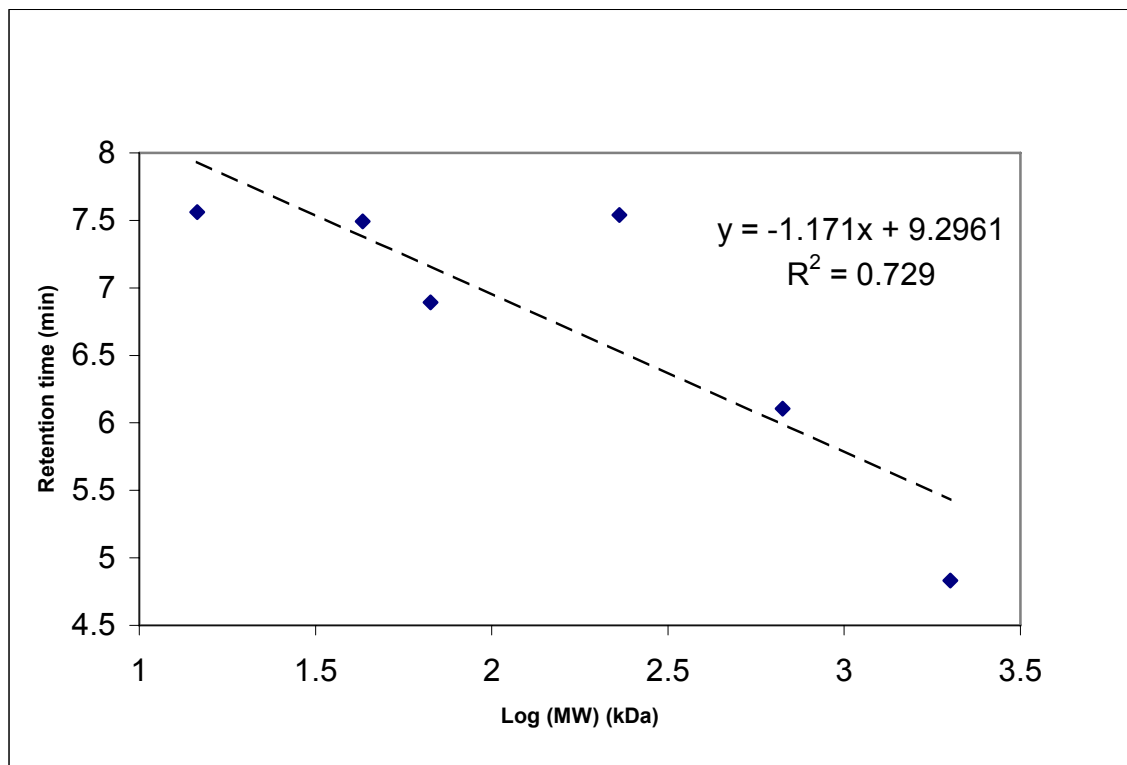


Figure 4.18: Size exclusion HPLC standard curve of retention time (min) versus log molecular mass (kDa) for ovotransferrin (78 kDa), bovine serum albumin (BSA) (67 kDa), catalase (230 kDa), bovine thyroglobulin (669 kDa) and blue dextran (2.0 MDa).

As shown in Figure 4.19, all samples of κ -casein loaded onto the column eluted as symmetrical peaks. κ -Casein alone eluted as a major peak at approximately 5.42 min, which corresponded to an average aggregate mass of slightly greater than 2 MDa (Figure 4.18). κ -Casein in the presence of 10%, 5% and 2% w/v dextran gave similar profiles to those obtained in the absence of dextran with almost the same elution times (5.39 min for 2% and 5% dextran and 5.38 min for 10% dextran) than in the absence of dextran. This indicated a slightly greater average aggregate mass of κ -casein in the presence of dextran.

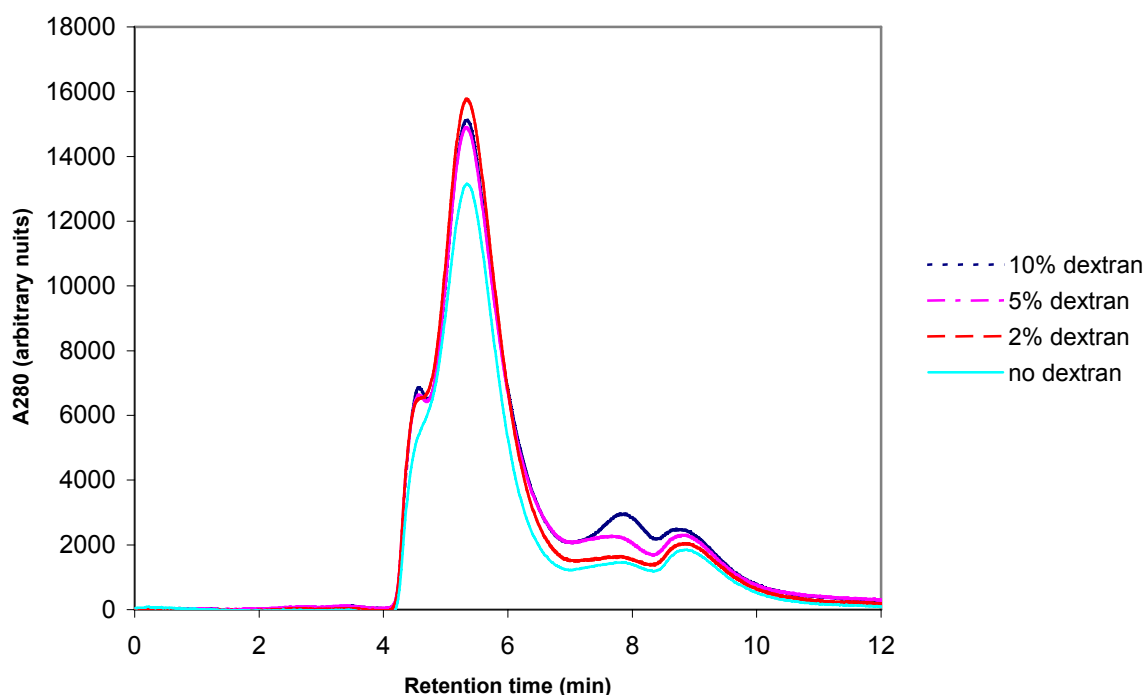


Figure 4.19: Size exclusion HPLC traces for κ -casein (10 mg/mL) aggregates in the absence and presence of different concentrations of dextran. 25 μ L aliquots of samples in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 and pH 7.4 were injected onto the column.

κ -Casein also formed high molecular weight (HMW) species eluting at approximately 4.0 minutes, with an average mass of bigger than 2 MDa. It also showed peaks around 8 and 9 minutes, which correspond to dimeric or monomeric species that were too small (less than 100 kDa) to accurately determine their masses from standard curve due to the limitations of column. The column used for these experiments had a specified mass range of 2 MDa to 14.6 kDa for native proteins. It is obvious, however, that κ -casein both alone and in the presence of different concentrations of dextran, appeared to form aggregates, which were larger in the presence of greater amounts of dextran. The higher the concentration of dextran, the greater the loss of monomeric and dimeric κ -casein species with a concurrent increase in the amount of more

highly aggregated species. Thus, the presence of dextran caused greater aggregation of κ -casein.

4.8 Size exclusion HPLC time-course: change in the aggregate size of κ -casein in the presence and absence of 10% w/v dextran

Size exclusion HPLC was employed to investigate changes in the size of κ -casein molecules in the presence and absence of dextran over 24 hrs as the protein forms fibrils. The standard curve (Figure 4.20) was calculated as described in Section 4.6. except that in this experiment a different instrument with a dual wavelength detector and manual injection system was used. Manual injection resulted in slightly different retention times. As shown in Figure 4.21A, the majority of κ -casein samples loaded onto the column eluted as symmetrical peaks. However, in the presence of dextran, samples of κ -casein loaded onto the column did not elute as symmetrical peaks. Molecular masses of κ -casein aggregates, which eluted with retention times between 7.5-6.70 minutes were calculated using the standard curve for samples incubated in the presence and absence of 10% w/v dextran at 37°C over a 24 hour period. The resulting molecular masses vs incubation time are shown in Figure 4.22. According to this Figure with increasing time, the molecular mass of aggregated protein increased with a greater increase in the presence of dextran. Moreover, the first-order rate constant for the increase in aggregation of κ -casein changed from $(1.34 \pm 1.58) \times 10^{-1} \text{ min}^{-1}$ to $(14.7 \pm 6.02) \times 10^{-1} \text{ min}^{-1}$ with the addition of

10% w/v dextran (Figure 4.22). Thus, with time and in the presence of dextran, there was a shift to higher masses of aggregated κ -casein with concomitant loss of lower molecular weight species.

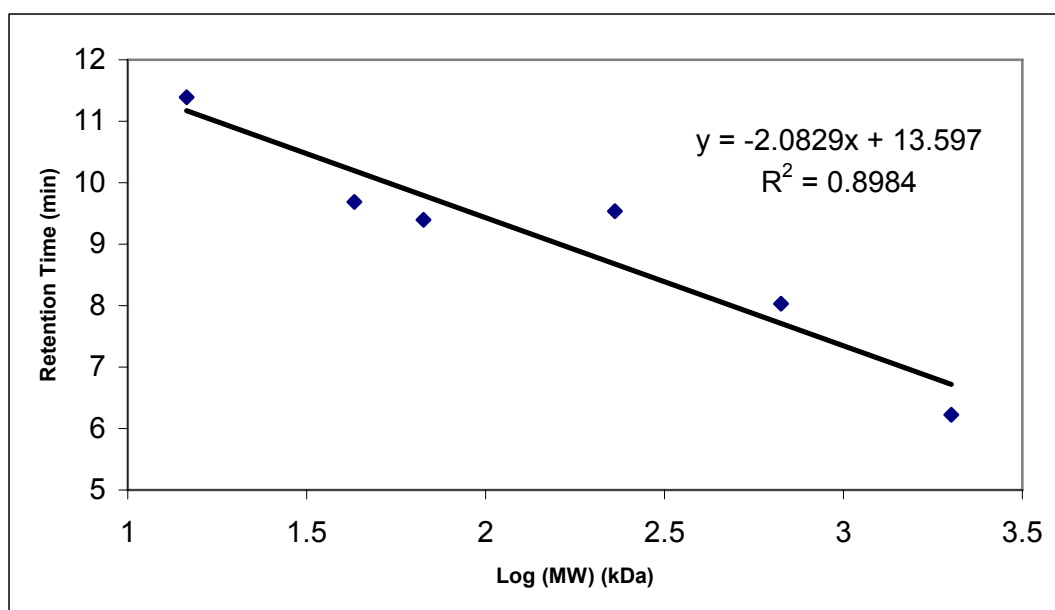


Figure 4.20: Size exclusion HPLC standard curve of retention time (min) versus log molecular mass (kDa) for ovotransferrin (78 kDa), bovine serum albumin (BSA) (67 kDa), catalase (230 kDa), bovine thyroglobulin (669 kDa) and blue dextran (2.0 MDa). The column used was the same as that for κ -casein in different concentrations of dextran (Figure 4.18) on a Shimadzu HPLC system with a SLC-10A vp pump system organizer, with a dual wavelength detector and manual injection (Tokyo, Japan).

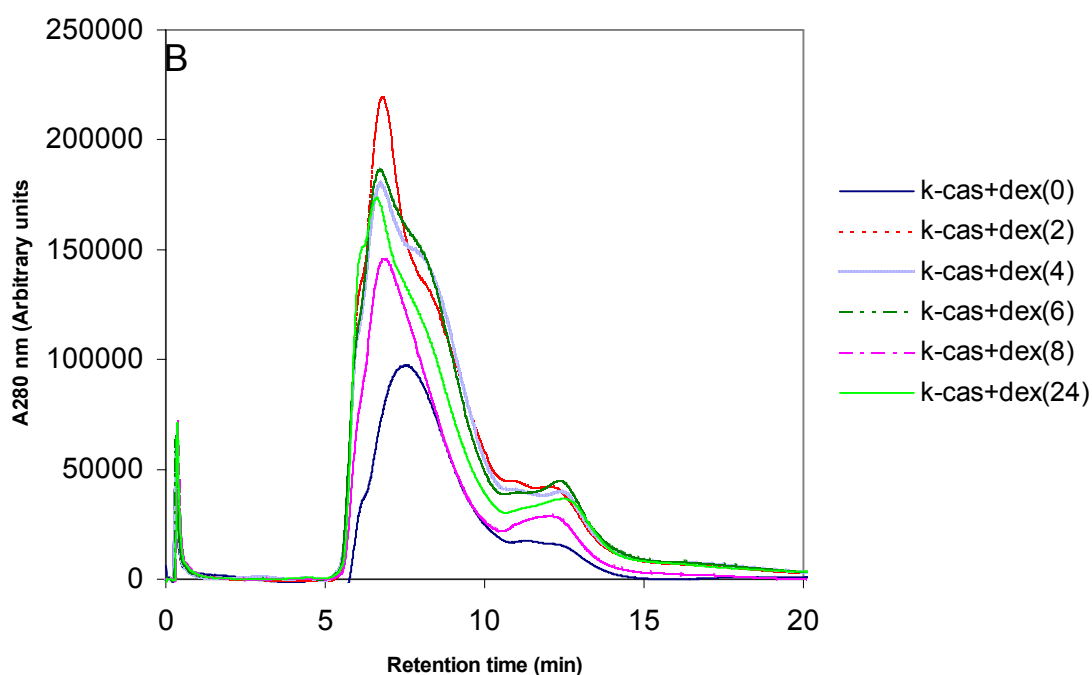
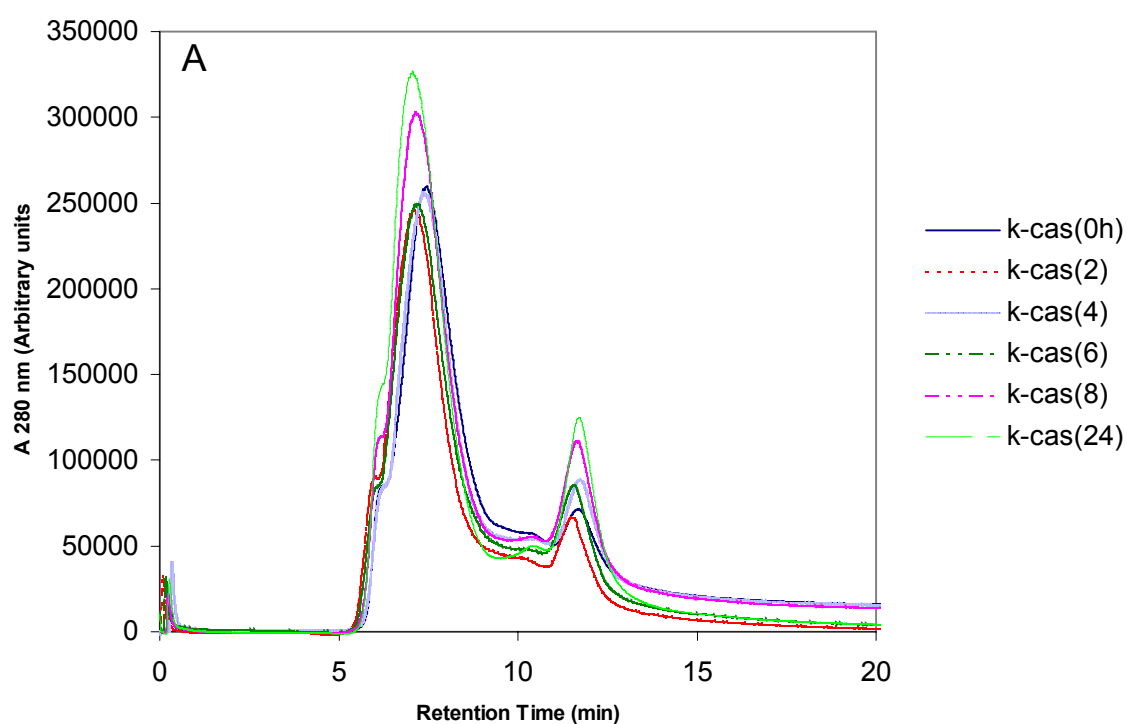


Figure 4.21: Size exclusion HPLC traces for κ -casein (10 mg/mL) aggregates, (A): in the absence of dextran, (B): in the presence of 10% w/v dextran at different incubation times. All samples were incubated at 37°C over the 24 hrs. 25 μ l of aliquots of samples in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 and pH 7.4 were injected onto the column.

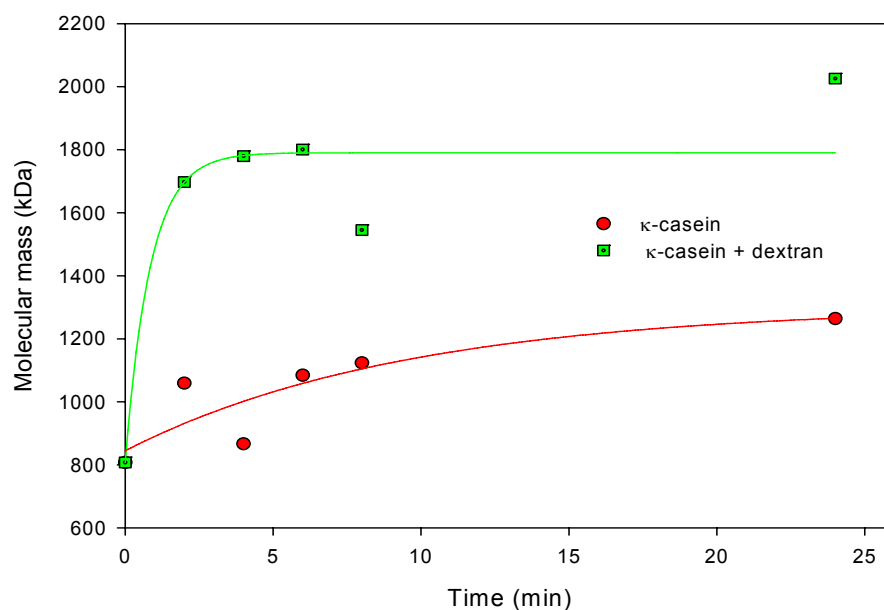
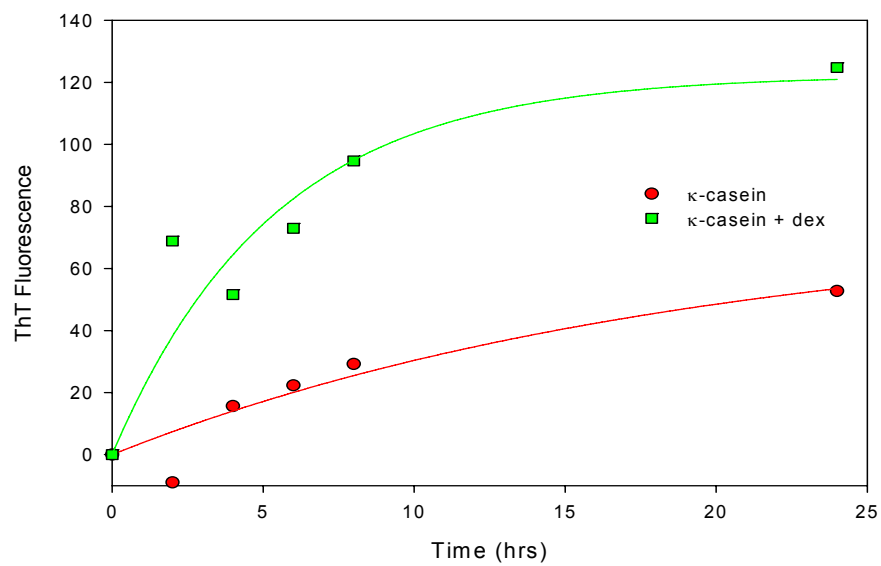


Figure 4.22. Average molecular masses (kDa) aggregates of κ -casein around the 7.5-6.70 minute retention time in the presence and absence of 10% w/v dextran with different incubation times. Average molecular masses were calculated using the standard curve (Figure 4.20).

In additional experiments, thioflavin T binding assays of the same samples as those employed in HPLC studies showed an increase in the fluorescence intensity of κ -casein samples with increasing time. Samples in the presence of dextran showed a greater increase than in the absence of dextran (Figure 4.23). This was also evident from rate constants of κ -casein which increased from $(0.82 \pm 0.54) \times 10^{-1} \text{ min}^{-1}$ in the absence of dextran to $(1.57 \pm 0.80) \times 10^{-1} \text{ min}^{-1}$ in the presence of dextran. This indicates that dextran increased the rate and extent of amyloid fibril formation by κ -casein.



4.23. Thioflavin T binding assay of κ -casein in the presence and absence of dextran at different times. All samples were incubated at 37°C for 3 hrs in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN₃ and pH 7.4.

4.9 Discussion

Amyloid fibril formation arises from the slow aggregation of intermediately folded protein states. During this process, the protein goes from its native soluble form to an insoluble fibril which is of great interest, as this process underlies a number of diseases e.g. Alzheimer's, Parkinson's, and Creutzfeldt-Jakob (Dobson et al. 2001, Treweek et al. 2003). *In vitro*, stresses that cause protein unfolding but not rapid aggregation (e.g. acidic pH or reduction) can trigger amyloid fibril formation (Hatters et al. 2002). In this study, the effects of a macromolecular crowding agent on amyloid fibril formation of α -lactalbumin, α_s - and κ -casein and the chaperone action of α -crystallin on amyloid fibril formation of α_s - and κ -casein was investigated in the presence and absence of dextran.

Macromolecular crowding agents increase the rate and extent of amyloid fibril formation for apolipoprotein C-II (Hatters et al. 2002a). Small heat shock proteins can prevent amyloid fibril formation of proteins by redirecting fibril forming species from a fibril-forming pathway to the amorphous aggregation (MacPhee et al. 2000, Hatters et al. 2001, Rekas et al. 2004).

Thioflavin T binding revealed that the increase in fluorescence intensity of α -lactalbumin at acidic pH and of reduced α_s - and κ -casein at neutral pH was due to amyloid fibril formation. Dextran increased the rate and extent of fibril formation by α -lactalbumin and κ -casein, which is in agreement with Hatters et al. (2002a) for fibril-forming of apolipoprotein-CII protein. This group showed the

same concentration-dependent effect of dextran on apolipoprotein-CII as found in the presence study for α -lactalbumin, and κ -casein. Electron micrographs supported the fibrillar structure of κ -casein under reducing conditions and an increase in the number and length of fibrils in the presence of dextran. However, α_s -casein did not undergo fibril formation. Instead, it aggregated into thioflavin T-binding spherical particles whose number increased in the presence of dextran the results of which were supported by TEM spectroscopy. This is agreement with Thorn et al. (2005) who reported the similar results of α_s -casein spherical particle formation under reducing conditions.

α -Crystallin effectively prevented amyloid fibril formation by reduced κ -casein. This is in agreement with Hatters et al (2001) and Rekas et al. (2004) who showed that α -crystallin prevented amyloid fibril formation in apolipoprotein C-II and α -synuclein, respectively. In this study, TEM data indicated that α -crystallin decreased the number of fibrils formed and significantly reduced the average fibril length of κ -casein as assessed by low levels of thioflavin T binding. In the presence of α -crystallin, the amorphous aggregate form of κ -casein were observed. This supported the proposal that chaperone proteins prevent amyloid formation by directing it to the amorphous aggregation (non toxic aggregates) pathway, which can be easily degraded (Muchowski et al. 2002). In the presence of dextran, amorphous aggregation promoted by α -crystallin significantly increased, indicating that under crowding conditions α -crystallin prevented amyloid fibril formation of the target protein by redirecting them to an amorphous state. This is in agreement with Rekas et al. (2004) who reported

the same result for α B-crystallin in preventing amyloid fibril formation of α -synuclein of under molecular crowding conditions.

α -Crystallin decreased the rate and the extent of amyloid fibril formation, however, in the presence of dextran, α -crystallin was less effective in preventing amyloid fibril formation. This is also consistent with the hypothesis that α -crystallin is a more efficient chaperone with slowly amorphously aggregating proteins (Lindner et al. 1998). The intrinsic fluorescence experiments on κ -casein in the presence and absence of α -crystallin and dextran also revealed that dextran caused an increase in the polarity of the environment of the tryptophan residue (position 76) in reduced κ -casein. This increase in polarity is most likely a result of a conformational change that either exposed Trp to the surface of the protein and therefore, to the solvent to a greater extent or brought Trp into closer proximity if charged groups on the protein. This may encourage the formation of undesirable contacts with other κ -casein molecules, which in turn increases the self assembly of κ -casein into a fibrillar structure under these conditions.

Previous studies (Lindner et al. 1998, 2001) showed that an increase in ANS binding in α -lactalbumin, insulin and ovotransferrin after addition of DTT occurred as a result of exposure of clustered hydrophobic residues in the molten globule state. Groves et al. (1992) also reported that reducing agents facilitate complete dissociation of disulfide-linked polymers of κ -casein. In this study, upon the addition of DTT to κ -casein, fluorescence intensity decreased.

κ -Casein is classified as an unfolded protein and in its native state exists as a molten globule-like structure with a significant amount of exposed hydrophobic area even in the disulphide-linked polymer (Dunker et al. 2002, Farrell et al., 2002, Thorn et al. 2005) as evidenced by the ANS fluorescence intensity of native κ -casein (Figure 4.12). Molecular modeling of κ -casein predicts that the secondary structure of κ -casein implies that it has a considerable amount of β -sheet, which is highly hydrophobic (Kumosinski et al. 1993). These hydrophobic areas in κ -casein are ideal sites for sheet-sheet interactions with other κ -casein molecules (Kumosinski et al. 1991, Kumosinski et al. 1993) as occurs during fibril formation. Pepper et al. (1982) reported that upon addition of a reducing agent, κ -casein becomes a high molecular mass polydisperse complex, in a concentration-dependent association with itself or with other proteins. Therefore, a decrease in ANS fluorescence intensity in reduced κ -casein implies that there is a conformational change in reduced κ -casein, which leads to a reduction in exposed clustered hydrophobicity, more likely as a result of being buried in the subsequent polymer formation (Kumosinski et al. 1993).

The addition of α -crystallin to reduced κ -casein increased the fluorescence intensity due to the interaction between the two proteins (Lindner et al. 1998). α -Crystallin prevented self assembly and eventual formation of fibrillar structure of reduced κ -casein possibly by binding to and shielding its hydrophobic region from solvent. However, in the presence of dextran, the fluorescence intensity of the reduced κ -casein (both in the presence and absence of α -crystallin), was

lower than the fluorescence intensity in the absence of dextran with a greater decrease observed for reduced κ -casein. We can conclude, therefore, that dextran decreased the degree of exposed hydrophobicity of κ -casein, possibly by promoting fibrillation. Under these conditions, α -crystallin was not able to prevent amyloid fibril formation as well as it was in the absence of dextran. This was also supported by thioflavin T binding and by size exclusion HPLC data, which showed greater aggregation of κ -casein in the presence of dextran. In addition, approximate molecular masses obtained from aggregated κ -casein in the presence and absence of 10% w/v dextran over 24 hrs from time course HPLC data and ThT binding of the same samples showed that dextran increased both the rate and extent of amyloid formation (Figures 4.22 and 4.23). In addition, sequential 1D ^1H NMR spectra of κ -casein upon reduction of the disulphide bonds with DTT in the presence and absence of 10% w/v dextran showed that dextran promoted the aggregation of reduced κ -casein. This was evident from a higher rate of signal decay of reduced κ -casein in the presence of the crowding agent consistent with the acceleration of the amyloid formation of κ -casein observed through ThT binding. In agreement with these data, Farrell et al. (2003) revealed that native κ -casein forms a self-associating polymeric species at 37°C. However, RCM- κ -casein (reduced and carboxymethylated) when heated up to 37°C exhibited an increase in average molecular mass due to self-association and the formation of fibrillar structure. The authors proposed that β -sheet-turn-sheet motifs might be responsible for fibril formation. Other studies (Vreeman et al. 1981, Rasmussen et al. 1992, Groves et al. 1998,

Farrell et al. 2003) reported that κ -casein exhibits four distinct types of self-association and the completely reduced form leads to fibril formation.

Near UV CD spectra of κ -casein also showed differences in the positions and intensity of bands in the spectra in the presence and/or absence of DTT and dextran. The effect of DTT and dextran on the tertiary structure of κ -casein and environment of aromatic and cysteine residues was therefore significant. With the results of tryptophan fluorescence, it appears that dextran caused alteration in the environment of tryptophan residues. Likewise NMR spectra (TOCSY) showed that dextran had little effect on the tertiary structure of κ -casein, which is a naturally unfolded protein.

4.10 Conclusion

A kinetic competition exists between amyloid formation of reduced proteins and their interaction with α -crystallin in the presence and absence of dextran. As shown by thioflavin T binding assays and NMR spectroscopy, the presence of dextran increased the rate of amyloid formation by susceptible proteins (α -lactalbumin, α_s -casein, and κ -casein). α -Crystallin was more efficient in preventing amyloid formation of κ -casein in the absence of dextran than in its presence. Possible causes of the effect of dextran on the rate of fibril formation of target proteins and on the chaperone action of α -crystallin were investigated by thioflavin T binding, intrinsic fluorescence spectroscopy, ANS-binding, NMR spectroscopy, TEM, and size exclusion HPLC.

Intrinsic fluorescence spectroscopy of κ -casein indicated that α -crystallin prevented amyloid fibril formation possibly by binding to the monomeric state of κ -casein and preventing its interaction with other κ -casein molecules and eventually forming fibrils. Dextran caused structural changes in the environment of the tryptophan residues of κ -casein on observation, which was supported by CD and NMR data. This may increase the dissociation of the κ -casein and increase its self-assembly.

ANS-binding experiments showed that dextran decreased the amount of exposed hydrophobicity in reduced κ -casein, consistent with amyloid formation of the protein and supported by ThT binding, TEM, NMR, and HPLC results. α -Crystallin decreased the amyloid formation of target protein, however the presence of dextran decreased the efficiency of α -crystallin to suppress fibrillation. Thus, α -crystallin is a less efficient chaperone with rapid amyloid fibril formation than with slow amyloid fibril formation, which was supported by thioflavin T binding, intrinsic fluorescence experiments and TEM. This is also in agreement with Lindner et al. (1998) and Carver et al. (2002) who reported that adding salts such as NaCl stimulated protein aggregation, and limited the efficiency of α -crystallin to suppress it.

In summary, dextran increases the rate of amyloid aggregation in target proteins. α -Crystallin suppresses the amyloid aggregation, but it is a poorer chaperone in the presence of dextran. Thus, kinetics factor may be

determinants in the reduced efficiency of α -crystallin as a molecular chaperone under crowding conditions.

Chapter 5

The effect of dextran on the rate of subunit exchange of α A-crystallin

α -Crystallin, the major lens protein, consists of two closely related subunits, α A- and α B-crystallin (Datta et al. 1999, Reddy et al. 2000, Horwitz et al. 2000, Horwitz et al. 2003, Meehan et al. 2004), as discussed in detail in Section 1.2. Both α A- and α B-crystallin, as well as α -crystallin, have been shown to display molecular chaperone activity (Horwitz et al. 1992a, Horwitz et al. 2000). Other studies have indicated that the chaperone action of α -crystallin increases with increasing temperature, which is accompanied, by an increase in exposed hydrophobicity (Maiti et al. 1988, Raman et al. 1995, Das et al. 1997, Reddy et al. 2000). Another contributor to the temperature dependence of α -crystallin chaperone action may be subunit exchange (van den Oetelaar et al. 1990, Sun et al. 1998). Subunit exchange between native and phosphorylated forms of α -crystallin and also between homogeneous α A/ α A-crystallin and heterogeneous α A/ α B-crystallin mixtures has been studied by isoelectric focusing and fluorescence resonance energy transfer (FRET) techniques (van den Oetelaar et al. 1990, Bova et al. 1997, Sun et al. 1998). Bova et al. (1997) showed that subunit exchange of α A-crystallin is strongly dependent on temperature and binding of target protein i.e. it increased with increasing temperature and decreased by interaction with reduced ovotransferrin.

Macromolecular crowding is ubiquitous and describes the normal conditions inside a living cell; it greatly promotes the self-assembly of proteins (van den Berg et al. 1999, Ellis et al. 2001) as discussed in detail in Section 1.4. In order to understand better the relationship between subunit exchange of α A-crystallin

and its chaperone activity, the macromolecular crowding agent, dextran, was used to mimic the crowded cellular interior.

5.1 Purification of α A- and α B-crystallin

To study the effect of dextran on the subunit exchange of α A-crystallin in the presence and absence of a target protein, the separation and purification of α A- and α B-crystallin from bovine α -crystallin was performed using the method of Steven et al. (1987) as described in the section 2.2.10. Figure 5.1 shows a typical elution profile for α A- and α B-crystallin at pH 2.5 on a size exclusion Sephadex G75 column. Elution of the protein was monitored at 280 nm using a UV detector. The elution time of α A-crystallin was approximately 6 hours, while α B-crystallin eluted after nearly 8 hours. α A-Crystallin is a tetramer while α B-crystallin is an unfolded monomer under acidic conditions in the presence of glycine, and thus, they can be separated by SEC (Size exclusion chromatography) (Steven et al. 1987).

The compositions of purified α A- and α B-crystallin were analyzed by SDS-PAGE as shown in Figure 5.2. Analysis of whole α -crystallin by SDS-PAGE revealed two distinct bands (lane 2), which corresponded to α A- and α B-crystallin. The lower molecular mass band in lane 3 corresponded to α A-crystallin (kDa) while the higher molecular mass band in lane 4 corresponded to α B-crystallin (kDa).

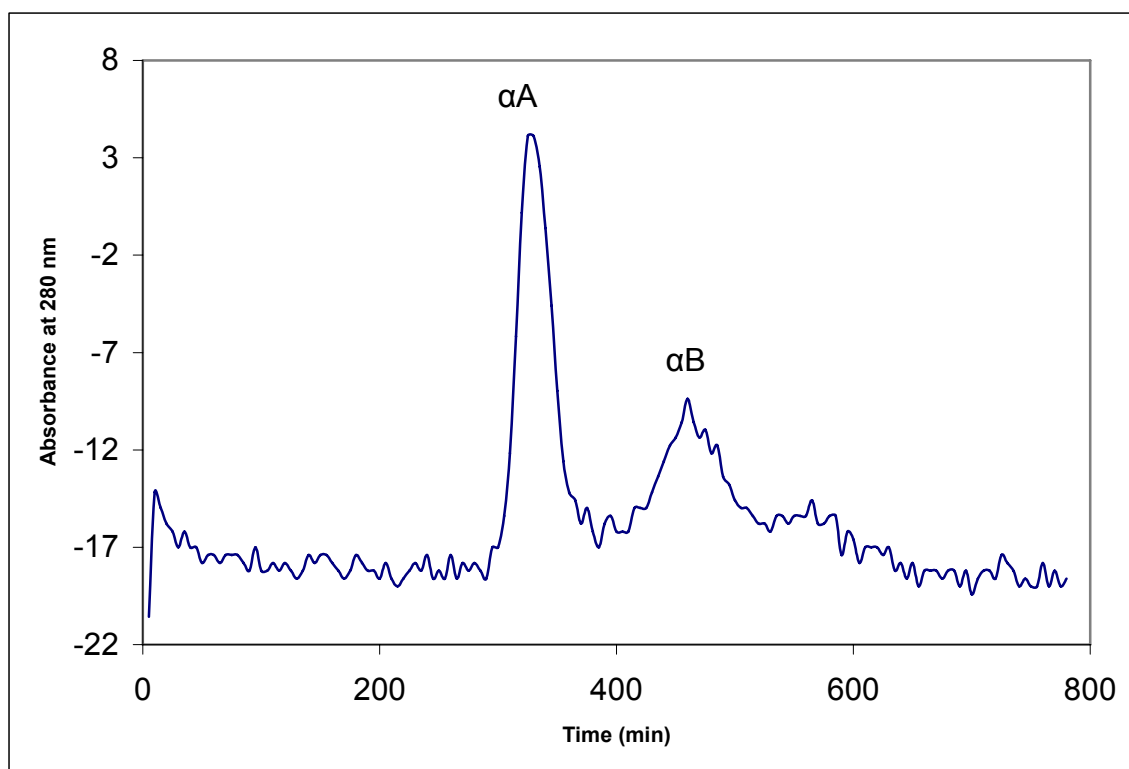


Figure 5.1: Elution profile of α -crystallin subunits on a G-75 Sephadex size-exclusion column. The protein was eluted with 0.1 M glycine, pH 2.5 at a flow rate of 9 mL/hr and fractions were collected at 4 minute intervals.

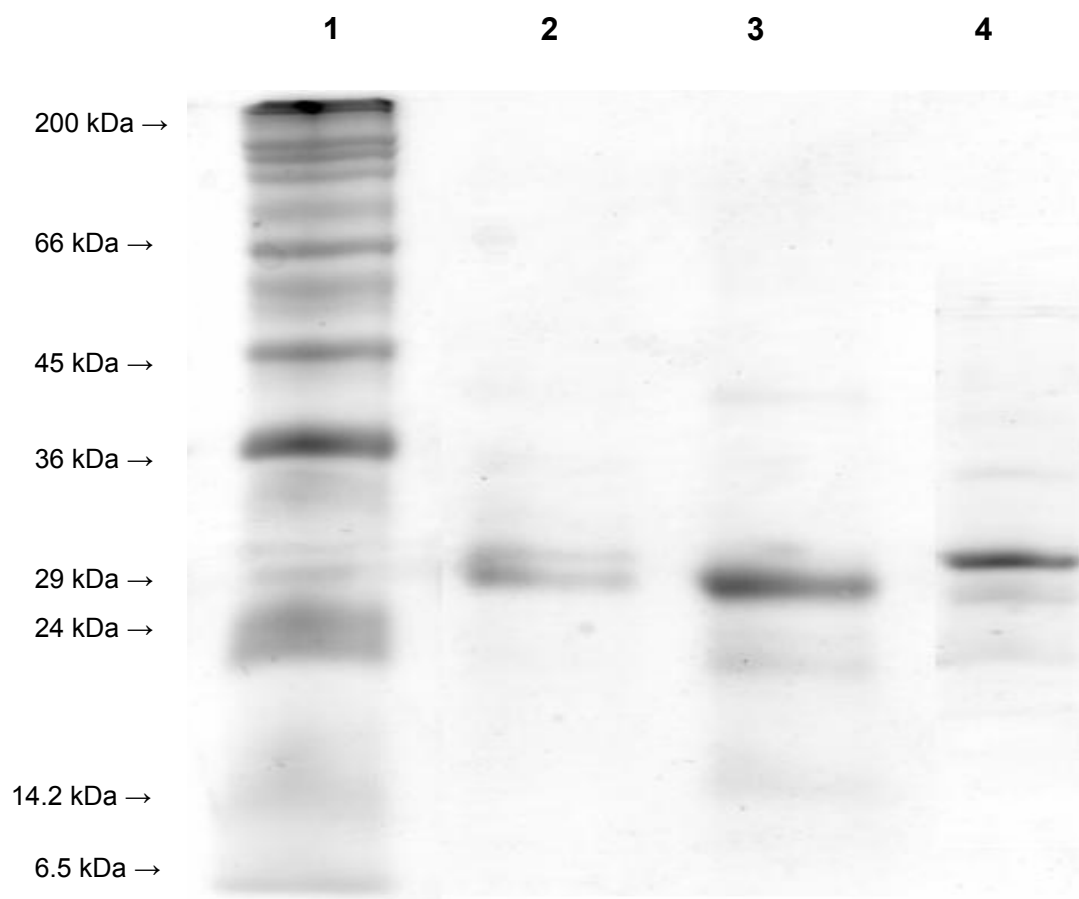


Figure 5.2: Purification of α A- and α B-crystallin from α -crystallin by SDS-PAGE. Lane 1: Wide range molecular weight markers Lane 2: α -crystallin, Lane 3: α A-crystallin, Lane 4: α B-crystallin

5.2 Chaperone activity of α A- and α B-crystallin subunits

5.2.1 Visible absorption spectroscopy of heated β_L -crystallin in the presence of α -, α A- and α B-crystallin

The ability of individual α -crystallin subunits, i.e. α A- and α B-crystallin, to prevent the aggregation of target proteins (e.g. β_L -crystallin, insulin, alcohol dehydrogenase (ADH)) has been reported previously (Horwitz et al. 1992, Datta et al. 1999, Santhoshkumar et al. 2001). Thus, to test the activity of separated α A- and α B-crystallin, their chaperone action in preventing the aggregation and

precipitation of β_L -crystallin was examined. Figure 5.3 shows the thermally induced aggregation of β_L -crystallin in the presence of α -, αA -, and αB -crystallin. All samples were incubated at 60°C. Under these conditions, a 0.25:1 molar ratio of αA -, αB and α -crystallin: β_L -crystallin provided nearly complete protection against aggregation and precipitation, consistent with the finding of Datta et al. (1999) who showed that 0.25:1 molar ratio of α -crystallin: β_L -crystallin (0.04 mg/mL of α -crystallin and 0.2 mg/mL of β_L -crystallin) prevented the aggregation of β_L -crystallin at 60°C. α -Crystallin provided comparable protection against the aggregation of β_L -crystallin (Figure 5.3).

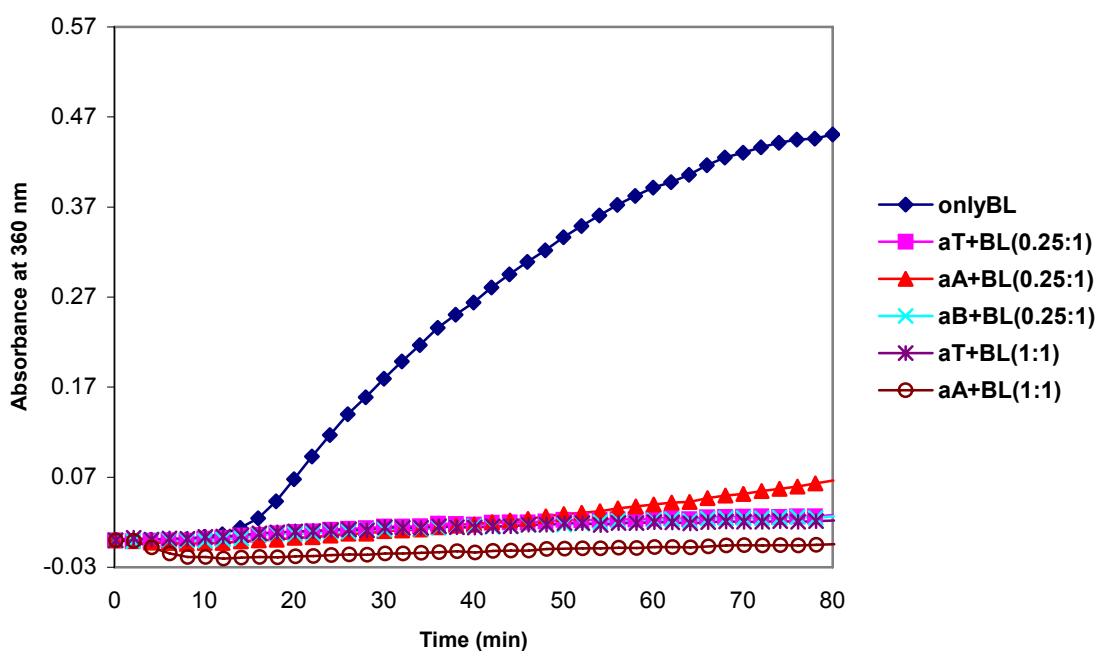


Figure 5.3: β_L -Crystallin (BL) (0.3 mg/mL) aggregation in the presence of α -, αA - and αB -crystallin. Protein was in total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , pH 7.4, and the incubation temperature was 60°C. The ratios indicated are w:w of α -, αA -, and αB -crystallin.

5.2.2 The chaperone activity of α - and α A-crystallin in the presence and absence of dextran at 37°C, 42°C and 45°C.

Figure 5.4 shows the effect of α - and α A-crystallin on the aggregation of reduced ovotransferrin in the presence and absence of 10% w/v dextran at different temperatures. It is apparent from these graphs that in the absence of dextran the rate of aggregation of reduced ovotransferrin increased with increasing temperature. In comparison, at 37°C, the presence of 10% w/v dextran slightly promoted ovotransferrin aggregation, at 42°C the rates were comparable while at 45°C the rate of aggregation in the presence of dextran slightly decreased. At all temperatures, α A-crystallin was more effective in preventing aggregation of ovotransferrin than α -crystallin both in the presence and absence of dextran (Figure 5.4). As expected, both α A- and α -crystallin were more effective chaperones at higher temperatures in the absence of dextran. However, in the presence of dextran both α - and α A-crystallin were less effective chaperones with aggregating ovotransferrin, which became more obvious at higher temperature.

The rate constants of aggregation of reduced ovotransferrin in the presence and absence of α -, α A-crystallin and 10% w/v dextran at different temperatures are summarized in Table 5.1 by fitting the exponential function, $F(t) = A_1 + A_2 (1 - e^{-kt})$ to data using Sigmaplot software (version 8.0) where $F(t)$

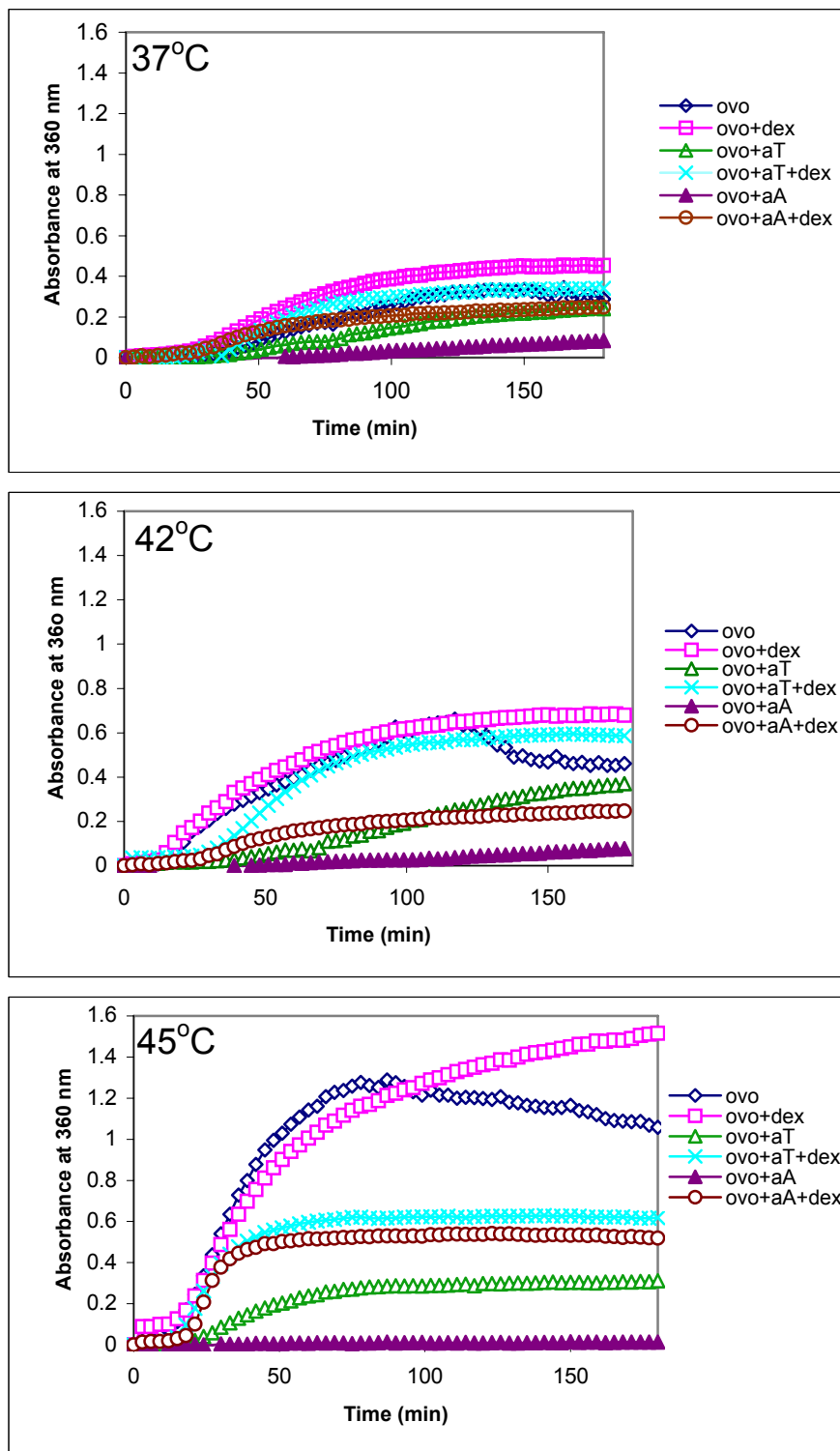


Figure 5.4: Ovotransferrin (0.66 mg/mL) aggregation in the presence of α - or α A-crystallin (1.0:0.5) molar ratio and 10% w/v dextran at 37°C, 42°C, and 45°C. Protein solutions were incubated in 50 mM phosphate buffer, 0.1 M NaCl, 0.05% (w/v) NaN_3 and pH 7.4. Aggregation of ovotransferrin was initiated by the addition of 20 mM DTT. The rate constants of ovotransferrin aggregation were obtained by fitting an exponential function to the light scattering data as described in Section 2.2.12.1.

is a exponential function of t , A_1 and A_2 are constants, at time 0, $F(0) = A_1 + A_2$, k is rate constant and t is time (in minutes). Figure 5.5 shows the effect of temperature on the chaperone action of αA - and α -crystallin in the presence and absence of dextran.

Table 5.1: Summary of rate constants for the aggregation of reduced ovotransferrin in the presence of αA - and α -crystallin at 37°C, 42°C, and 45°C. Rate constants were calculated by fitting exponential functions to light scattering data using Sigmaplot software (version 8.0).

Sample	Rate constant at 37°C $\times 10^{-2}$ (min⁻¹)	Rate constant at 42°C $\times 10^{-2}$ (min⁻¹)	Rate constant at 45°C $\times 10^{-2}$ (min⁻¹)
Ovotransferrin	6.61 \pm 0.50	7.43 \pm 0.15	7.84 \pm 0.69
Ovotransferrin + dextran	7.14 \pm 0.43	7.47 \pm 0.01	7.56 \pm 0.52
αA-crystallin + ovotransferrin	3.41 \pm 0.66	3.31 \pm 0.13	1.48 \pm 0.81
αA-crystallin + ovotransferrin + dextran	4.67 \pm 0.37	5.80 \pm 0.85	7.38 \pm 0.82
α-crystallin + ovotransferrin	5.12 \pm 0.36	3.80 \pm 0.44	3.63 \pm 1.20
α-crystallin + ovotransferrin + dextran	6.40 \pm 0.51	7.05 \pm 0.35	7.36 \pm 0.59

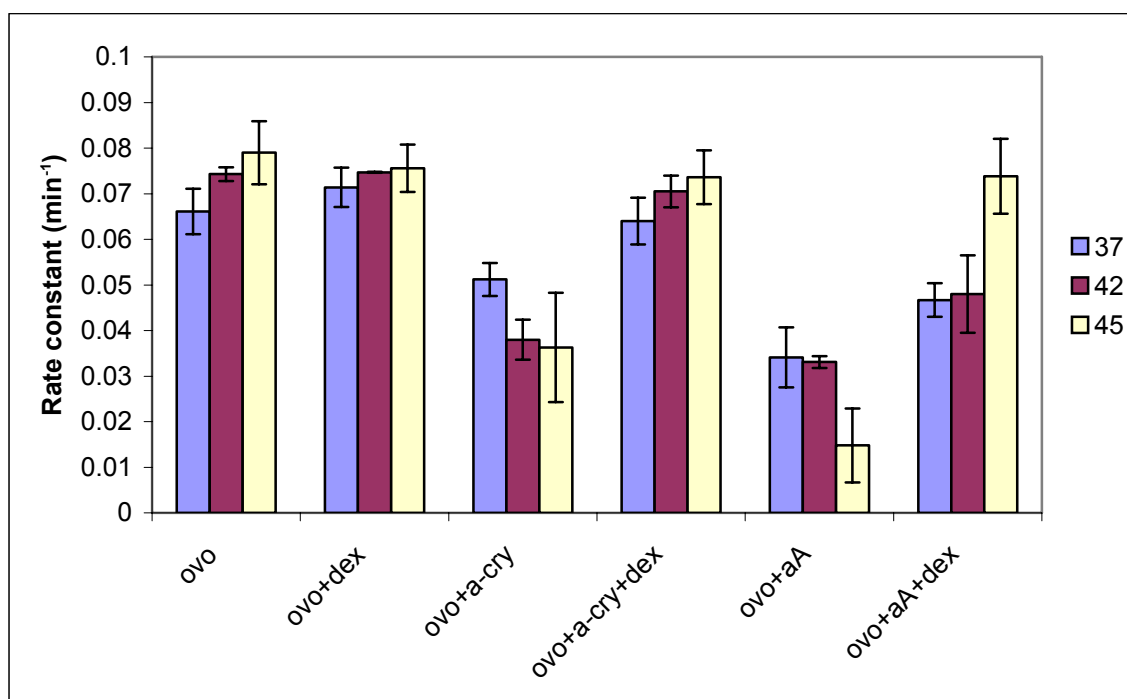


Figure 5.5: The effect of increasing temperature on the chaperone action of α A- and α -crystallin from light scattering profiles of reduced ovotransferrin in the presence of 0.5:1 molar ratio of α A- and α -crystallin:ovotransferrin at 37°C, 42°C and 45°C. Rate constants were obtained by fitting the exponential function $F(t) = A_1 + A_2 (1 - e^{-kx})$ to data, which was obtained from light scattering, using Sigma Plot software (version 8.0).

5.3 Subunit exchange rates of α A-crystallin

5.3.1 Labeling of α A-crystallin with lucifer yellow iodoacetamide (LYI) and 4-acetamido-4'-((iodoacetyl)amino)-stilbene-2,2'-disulfonic acid (AIAS)

AIAS and LYI are dyes that bind to proteins at their free cysteine residues (Heim et al. 1996, Bova et al. 2000). In α A-crystallin, Cys 131 is in the C-terminal region, and being the only cysteine residue in the protein, is not involved in disulphide bond formation (Bova et al. 2000). FRET (Fluorescence resonance energy transfer) is a technique, which is based on the transfer of energy between a donor and acceptor (Cheung et al. 2003) and can be used to

measure the degree of association of molecules. During a FRET experiment, AIAS acts as an energy donor and LYI acts as an energy acceptor (Bova et al. 1997, Bova et al. 2000). Excitation of the donor molecule leads to emission from the acceptor molecule when both molecules are close enough for energy transfer to occur. Therefore, FRET can be used to monitor direct protein-protein interaction in an AIAS and LYI-labeled protein blend (Pollok et al. 1999). Labeling of α A-crystallin was performed using the method of Bova et al. (1997), as described in Section 2.2.12.1. Figure 5.6 shows typical elution profiles for fluorescently labeled α A-crystallin on a Sephadex G-25 size exclusion column used to separate unreacted AIAS and LYI from labeled α A-crystallin. Elution of the protein was monitored at 280 nm using a UV detector. The elution time of both AIAS and LYI labeled α A-crystallin was approximately 2 hrs.

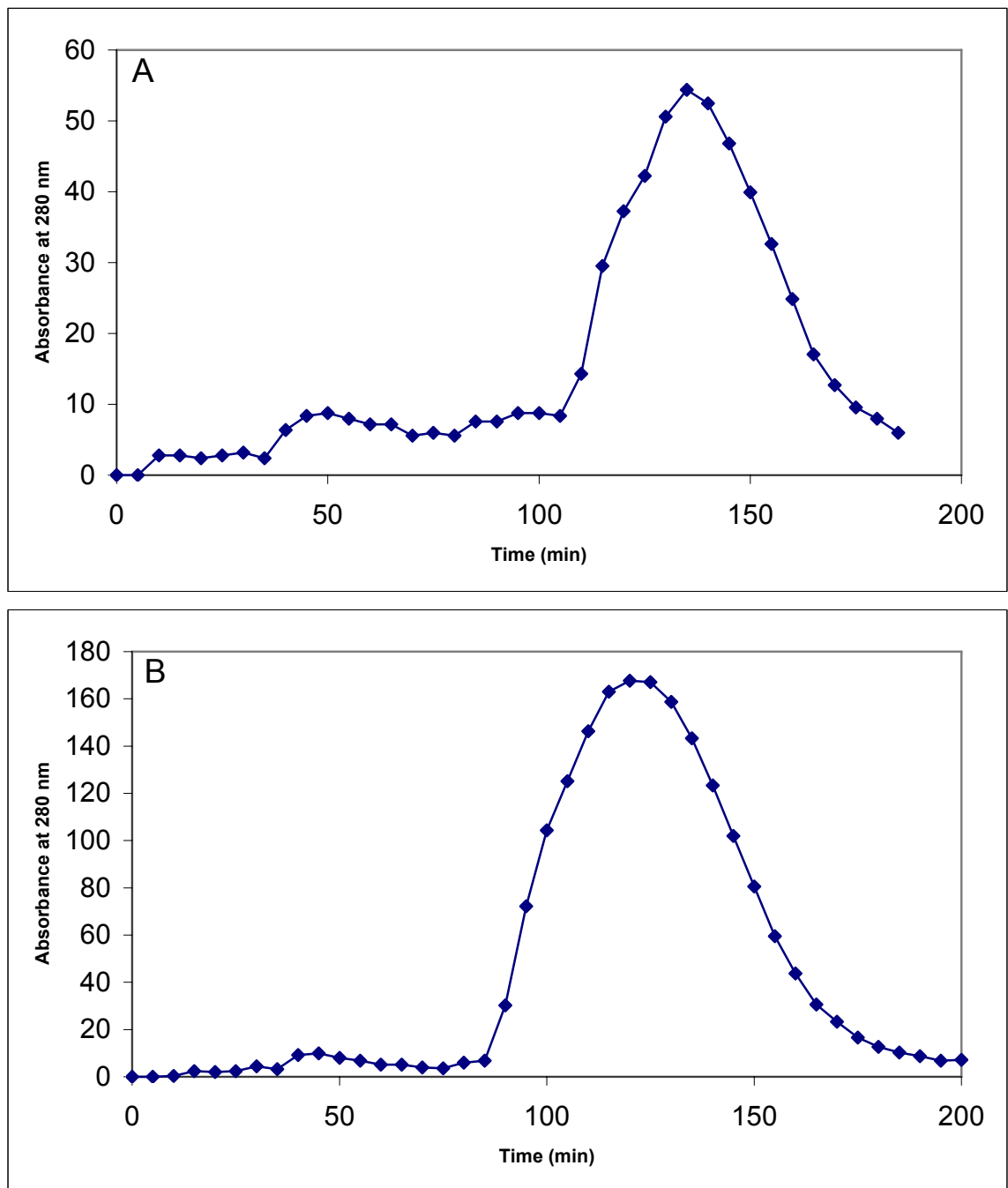


Figure 5.6 Elution profiles for (A): AIAS-labeled α A-crystallin and (B): LYI-labeled α A-crystallin on a Sephadex G-25 column. The protein was eluted with 50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 2 mM DTT at a flow rate of 20 mL/hr and fractions were collected at 4 minute intervals.

5.3.2 Fluorescence resonance energy transfer (FRET) measurements during subunit exchange in α A-crystallin

5.3.2.1 Determination of the rate constant of subunit exchange in labeled α A-crystallin by FRET

In order to measure the rate of subunit exchange in α A-crystallin, 0.4 mg/mL of AIAS-labeled α A-crystallin and 0.4 mg/mL of LYI-labeled α A-crystallin were mixed. Upon mixing, the fluorescence intensity of both AIAS- and LYI-labeled α A-crystallin was altered. Emission spectra of sample excited at 335 nm and 435 nm for AIAS and LYI respectively were recorded from 350-600 nm and time dependent changes in emission were observed. Figure 5.7 shows a time-dependent increase in LYI emission intensity at 525 nm and a time-dependent decrease in AIAS emission intensity at 415 nm, due to the progression of subunit exchange with time (0, 12, 21, 95, 120, 180, 210, 240 min). No change in fluorescence was observed after 4 hrs at 37°C indicating that the exchange was completed.

By measuring the decrease in donor fluorescence and the increase in acceptor fluorescence with time at the respective wavelengths, and fitting exponential function to these data the rate of subunit exchange in the subsequent sections were estimated.

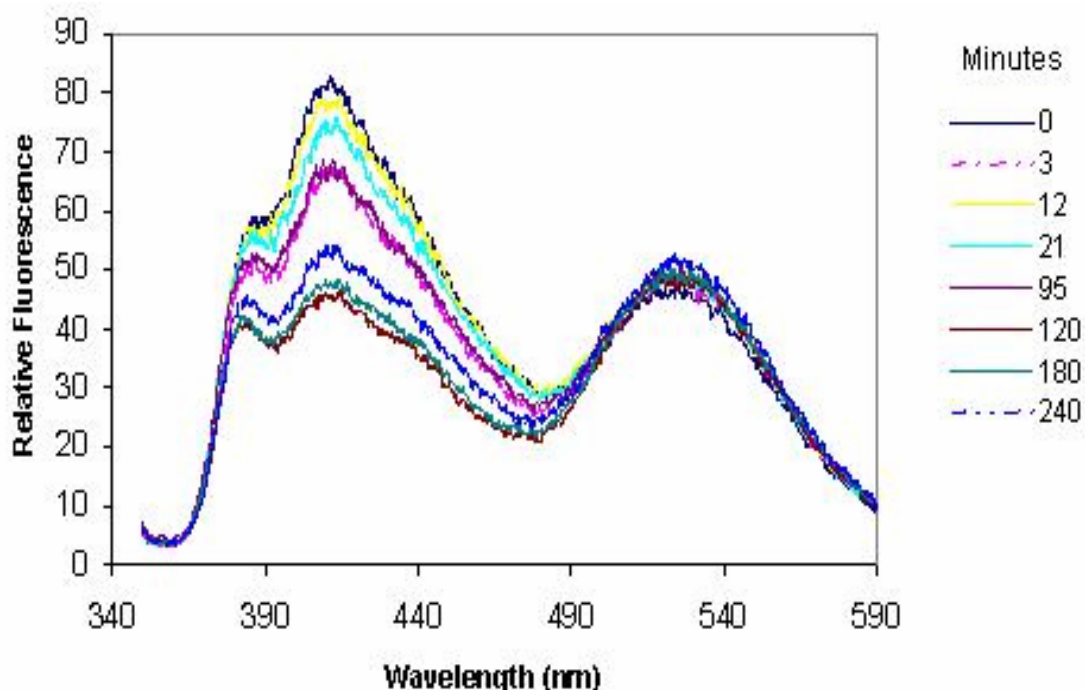


Figure 5.7: Time dependent changes in the emission spectra of AIAS-labeled α A-crystallin due to subunit exchange. Emission spectra at 335 nm were recorded at different times after mixing equal amounts of AIAS- and LYI-labeled α A-crystallin.

5.3.2.2 The effect of 10% w/v dextran on the rate of subunit exchange of α A-crystallin at 37°C.

Figure 5.8 shows a plot of emission intensity for the two populations of labeled α A-crystallin in the absence of dextran. The upper panel shows a decrease in AIAS emission intensity at 415 nm as a function of time and the lower panel shows an increase in LYI emission intensity at 545 nm as a function of time after mixing of the two labeled proteins in an equimolar ratio. The rate constant for subunit exchange (k) was obtained using the first-order rate equation, i.e. $F(t) = C_1 + C_2 e^{-kt}$, (Bova et al. 1997) where $F(t)$ is the exponential function of the fluorescence intensity at 415 nm for AIAS and 545 nm for LYI for each given time point, C_1 and C_2 are fluorescence intensities determined using the

condition $C_1+C_2=1$ at time 0 and C_1 is the fluorescence intensity at time. The rate constants determined for subunit exchange, were $(7.72 \pm 1.36) \times 10^{-2} \text{ min}^{-1}$ for AIAS (A) and $(6.23 \pm 1.20) \times 10^{-2} \text{ min}^{-1}$ for LYI (B) and time for complete exchange was about 60 min in both cases.

The effect of dextran on the subunit exchange rate of α A-crystallin was then investigated. Time-dependent changes in FRET due to subunit exchange of labeled α A-crystallin in the presence of 10% w/v dextran are shown in Figure 5.9. Dextran led to 30% and 20% decrease in the rate of subunit exchange of AIAS- and LYI-labeled α A-crystallin respectively. The exchange rate constants of $(5.42 \pm 1.86) \times 10^{-2} \text{ min}^{-1}$ and $(4.64 \pm 1.10) \times 10^{-2} \text{ min}^{-1}$ were obtained from fitting exponential functions to time courses of AIAS fluorescence intensity at 415 nm and the LYI fluorescence intensity at 545 nm. In addition, the time for complete exchange increased to 70 min compared to 60 min in the absence of dextran for both case. A time course quenching control experiment showed that using samples of LYI in the presence and absence of dextran gave very similar results (i.e. rate constant $(11 \pm 0.02) \times 10^{-2} \text{ min}^{-1}$ vs $(9.9 \pm 0.03) \times 10^{-2} \text{ min}^{-1}$ in the absence of dextran). This shows that the effect of dextran in subunit exchange experiment is independent of LYI quenching by dextran. Thus, dextran led to a small but significant decrease in the rate of subunit exchange of α A-crystallin.

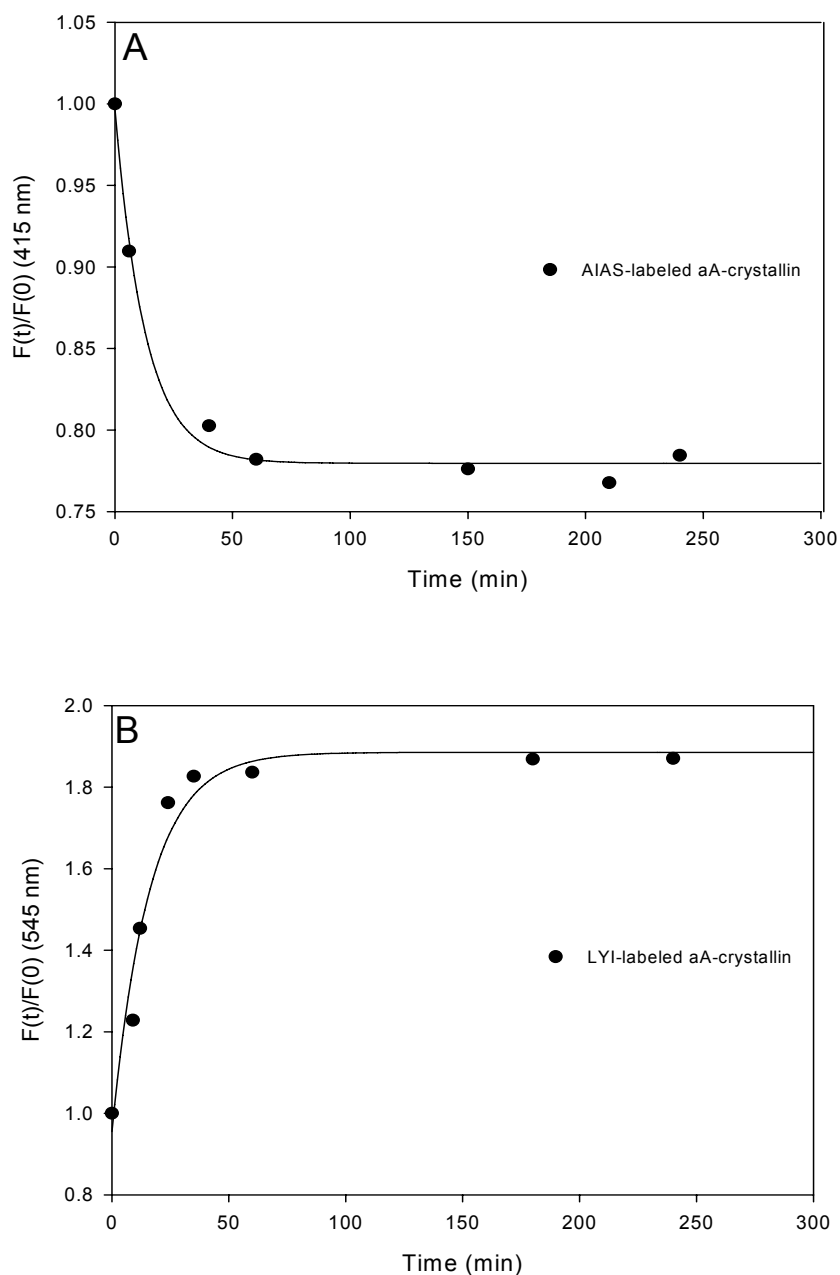


Figure 5.8: Time-dependent changes in (A) AIAS and (B) LYI fluorescence during subunit exchange of labeled α A-crystallin in the absence of 10% w/v dextran. (A): decrease in relative fluorescence intensity of AIAS at 415 nm and (B): increase in relative fluorescence intensity of LYI at 545 nm after mixing an equal amount of AIAS- and LYI-labeled α A-crystallin. Experiments were conducted for 4 hrs at 37°C in 50 mM sodium phosphate, 100 mM NaCl, 2 mM DTT and pH 7.5. The rate constants were calculated by fitting rate constants were calculated by fitting exponential decay and rise to max functions $F(t) = C_1 + C_2 e^{-kt}$ and $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the data.

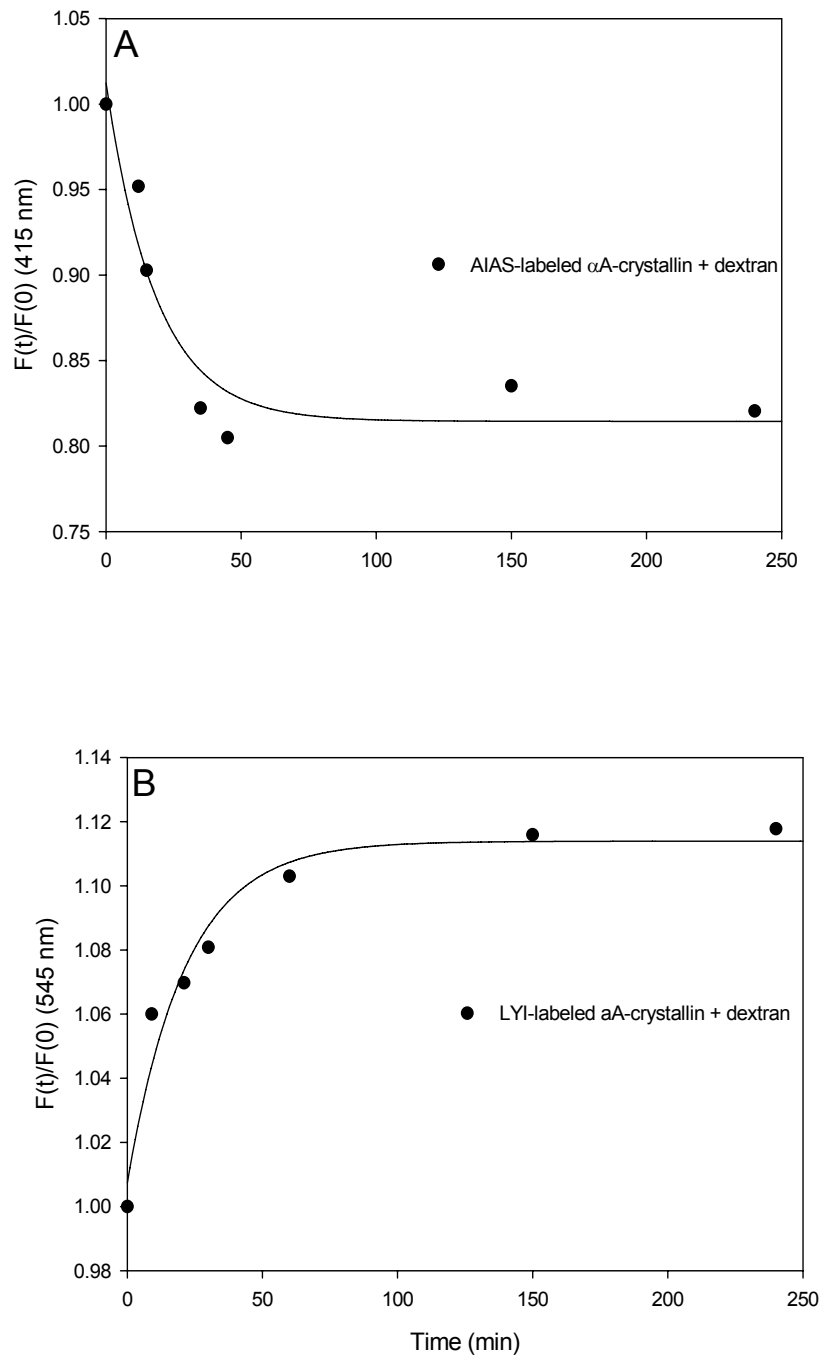


Figure 5.9: Time-dependent changes in (A) AIAS and (B) LYI fluorescence during subunit exchange of labeled α A-crystallin in the presence of 10% w/v dextran. (A): decrease in relative fluorescence intensity of AIAS at 415 nm and (B): increase in relative fluorescence intensity of LYI at 545 nm after mixing an equal amount of AIAS- and LYI-labeled α A-crystallin. Experiments were conducted for 4 hrs at 37°C in 50 mM sodium phosphate, 100 mM NaCl, 2 mM DTT and pH 7.5. The rate constants were calculated by fitting exponential decay and rise to max functions $F(t) = C_1 + C_2 e^{-kt}$ and $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the data.

5.3.2.3 The effect of reduced ovotransferrin on the rate of subunit exchange of labeled α A-crystallin in the presence and absence of 10% w/v dextran

In order to investigate the effect of bound target protein on the rate of the α A-crystallin subunit exchange reaction, ovotransferrin and DTT were added to labeled α A-crystallin in a 1.0:0.5 molar subunit ratio of ovotransferrin and α A-crystallin and incubated for 3 hours at 37°C. Figure 5.10 shows the time-dependent changes in the subunit exchange of labeled α A-crystallin in the presence of reduced ovotransferrin. Exchange rates constants of $(2.90 \pm 0.63) \times 10^{-2} \text{ min}^{-1}$ and $(3.15 \pm 0.13) \times 10^{-2} \text{ min}^{-1}$ were obtained by measuring the decrease in AIAS fluorescence intensity at 415 nm and the increase in LYI fluorescence intensity at 545 nm, respectively. These rate constants are lower than those observed for free α A-crystallin, i.e. 59% lower for AIAS-labeled α A-crystallin and 50% lower for LYI-labeled α A-crystallin (Figure 5.9). The addition of reduced ovotransferrin increased the time taken to complete exchange 3-fold from 60 minutes to ~180 minutes in both AIAS and LYI labeled α A-crystallin. The decrease in α A-crystallin subunit exchange rate caused by binding of reduced ovotransferrin is consistent with Bova et al. (1997) who reported that a 2:1 molar ratio of α A-crystallin:ovotransferrin decreased the rate of subunit exchange by 35%.

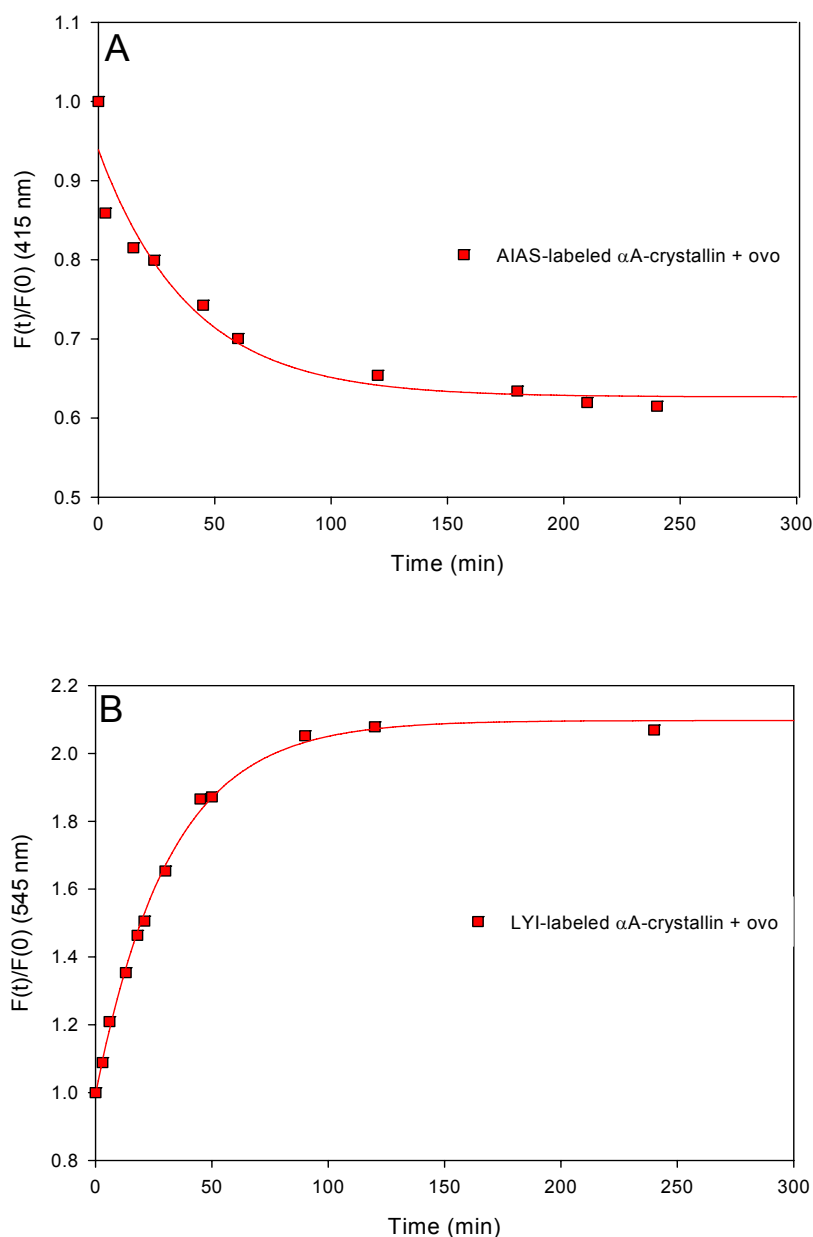


Figure 5.10: Time-dependent changes in (A) AIAS and (B) LYI fluorescence during subunit exchange of labeled α A-crystallin and ovotransferrin. (A): decrease in relative fluorescence intensity of AIAS at 415 nm and (B): increase in relative fluorescence intensity of LYI at 545 nm after mixing an equal amount of AIAS- and LYI-labeled α A-crystallin. Experiments were conducted for 4 hrs at 37°C in 50 mM sodium phosphate, 100 mM NaCl, 2 mM DTT and pH 7.5. The rate constants were calculated by fitting exponential decay and rise to max functions $F(t) = C_1 + C_2 e^{-kt}$ and $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the data.

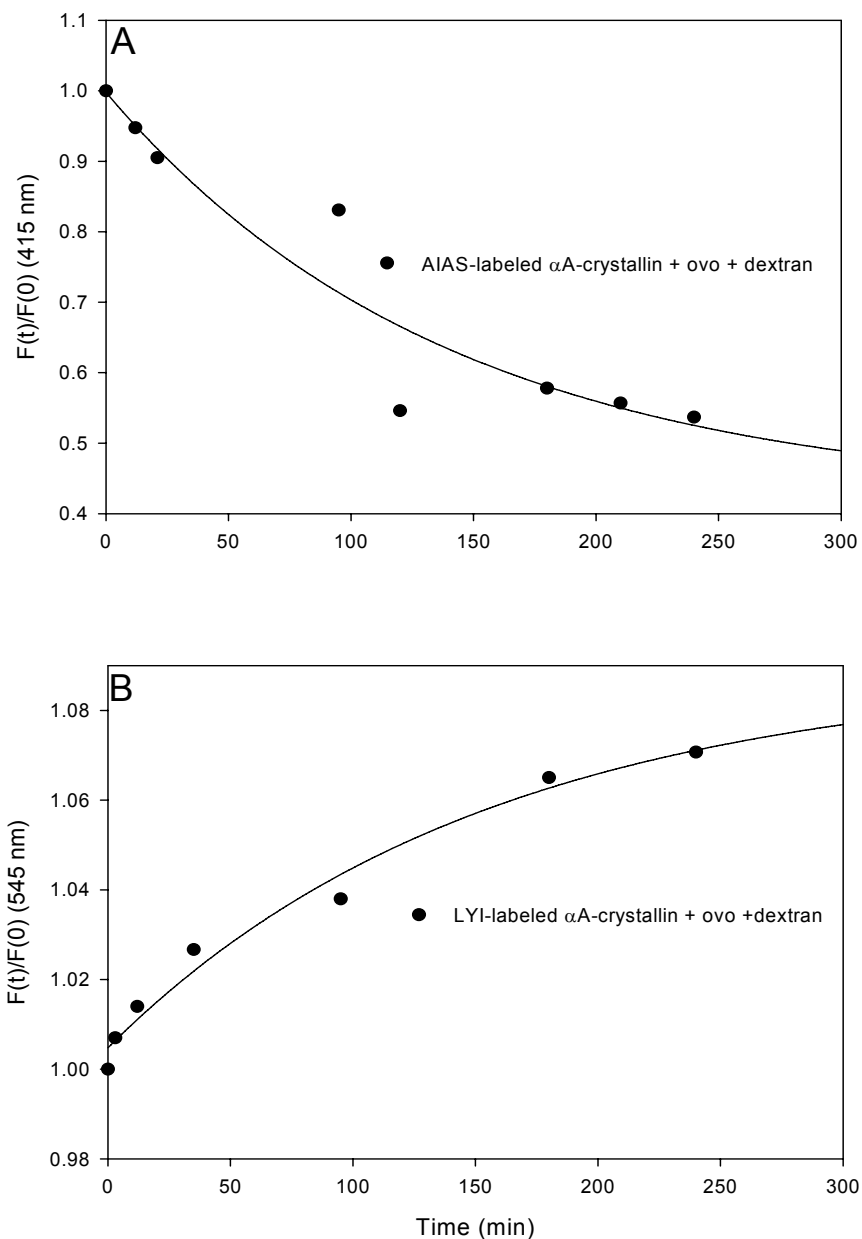


Figure 5.11: Time-dependent changes in (A) AIAS and (B) LYI fluorescence during subunit exchange of labeled α A-crystallin in the presence of ovotransferrin and 10% w/v dextran. (A): decrease in relative fluorescence intensity of AIAS at 415 nm and (B): increase in relative fluorescence intensity of LYI at 545 nm after mixing an equal amount of AIAS- and LYI-labeled α A-crystallin. Experiments were conducted for 4 hrs at 37°C in 50 mM sodium phosphate, 100 mM NaCl, 2 mM DTT and pH 7.5.m. The rate constants were calculated by fitting exponential decay and rise to max functions $F(t) = C_1 + C_2 e^{-kt}$ and $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the data.

The presence of 10% w/v dextran in the mixture of α A-crystallin and reduced ovotransferrin (Figure 5.11) further decreased the α A-crystallin subunit exchange rate to $(7.16 \pm 5.96) \times 10^{-3} \text{ min}^{-1}$ and $(6.47 \pm 2.82) \times 10^{-3} \text{ min}^{-1}$, for AIAS- and LYI-labeled α A-crystallin respectively. These Figures are 76% and 79%, lower for AIAS and LYI labeled α A-crystallin respectively, compared to those recorded in the absence of dextran. Moreover, the time for completion of the exchange increased from 180 min to more than 240 min in both AIAS- and LYI-labeled α A-crystallin.

Rate constants for α A-crystallin subunit exchange measured under different conditions are summarised in Table 5.2.

Table 5.2: Summary of apparent rate constants for subunit exchange experiments of labeled α A-crystallin under different conditions at 37°C.

Sample	AIAS-labeled αA-crystallin rate constant $\times 10^{-2} \text{ (min}^{-1}\text{)}$	LYI-labeled αA-crystallin rate constant $\times 10^{-2} \text{ (min}^{-1}\text{)}$
αA-crystallin	7.22 ± 1.36	6.23 ± 1.20
αA-crystallin + dextran	5.42 ± 1.86	4.64 ± 1.10
αA-crystallin + ovotransferrin	2.90 ± 0.63	3.16 ± 0.13
αA-crystallin + dextran + ovotransferrin	0.716 ± 0.60	0.647 ± 0.28

5.3.2.4 The effect of molecular crowding on subunit exchange rate of α A-crystallin at different temperatures

In order to investigate the effect of temperature on the rate of the subunit exchange of α A-crystallin in the presence and absence of dextran, experiments were performed at 42°C and 45°C. Figure 5.12 shows the time-dependent changes in the subunit exchange of labeled α A-crystallin at 42°C. Exchange rate constants of $(1.55 \pm 0.62) \times 10^{-1} \text{ min}^{-1}$ and $(1.07 \pm 0.16) \times 10^{-1} \text{ min}^{-1}$ were obtained for AIAS and LYI fluorescence signals, respectively, which are approximately 2.15 and 1.70 fold higher than those obtained at 37°C (Table 5.3). In addition, the time for complete exchange decreased to 20 and 30 minutes compared to 60 minutes at 37°C for AIAS- and LYI-labeled α A-crystallin, respectively, illustrating the effect of temperature on enhancing the rate of subunit exchange (Table 5.4). In contrast, the presence of dextran decreased the exchange rate constants to $(3.39 \pm 1.09) \times 10^{-2} \text{ min}^{-1}$ and $(3.04 \pm 0.62) \times 10^{-2} \text{ min}^{-1}$ for AIAS fluorescence intensity at 415 nm and the LYI fluorescence intensity at 545 nm, respectively. These values were 4.6 and 3.5 fold lower than the corresponding rates in the absence of dextran for AIAS- and LYI-labeled α A-crystallin, respectively. This is comparable with the data at 37°C, in that the exchange rate in the presence of dextran also decreased but less significantly. In addition, the times taken for complete exchange at 42°C in the presence of dextran decreased to 50 and 40 minutes compared to 70 minutes at 37°C in AIAS- and LYI-labeled α A-crystallin respectively implying that at

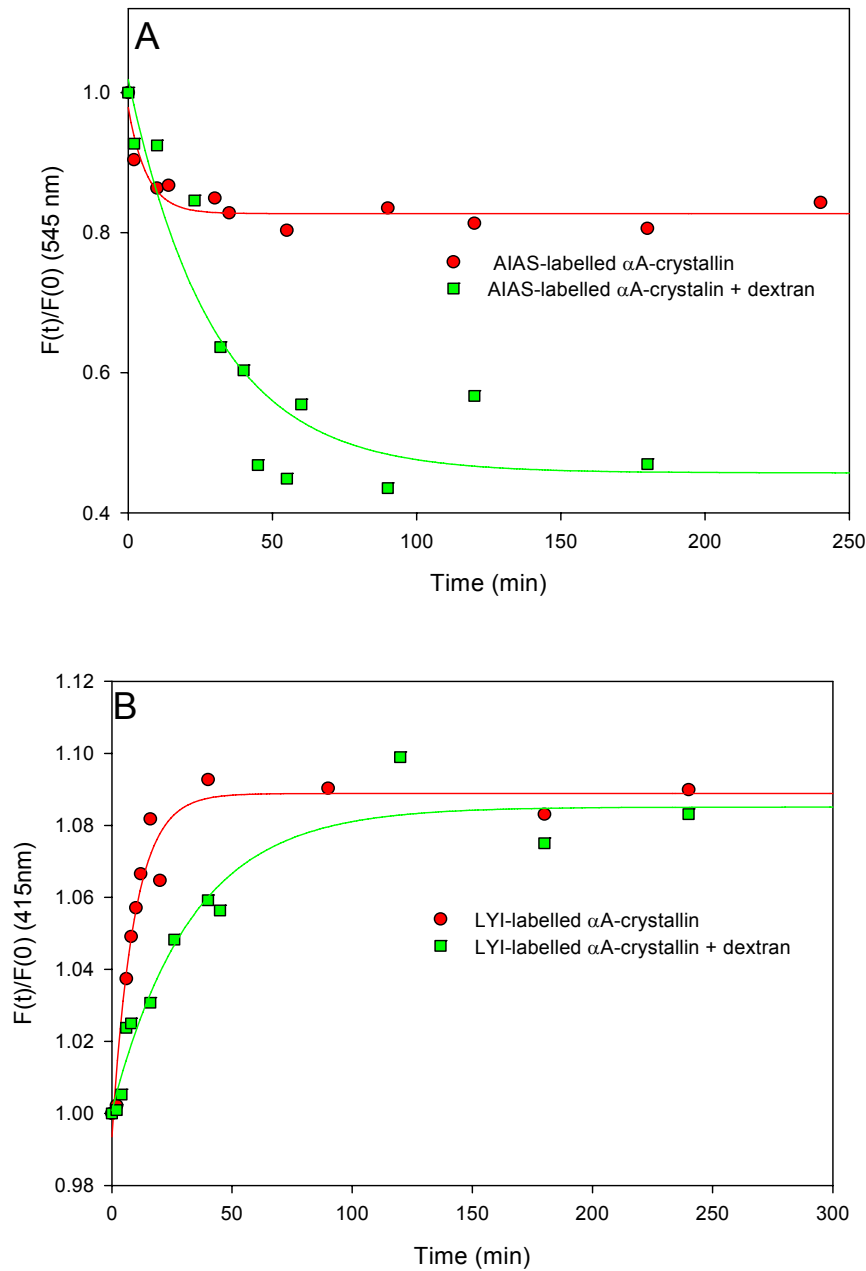


Figure 5.12: Time-dependent changes in (A) AIAS and (B) LYI fluorescence during subunit exchange of labeled α A-crystallin in the presence and absence of 10% w/v dextran at 42°C. (A): decrease in relative fluorescence intensity of AIAS at 415 nm and (B): increase in relative fluorescence intensity of LYI at 545 nm after mixing an equal amount of AIAS- and LYI-labelled α A-crystallin. Experiments were conducted in 4 hrs at 42°C in 100 mM NaCl, 2 mM DTT, 50 mM sodium phosphate, and pH 7.5. The rate constants were calculated by fitting exponential decay and rise to max functions $F(t)=C_1 + C_2 e^{-kt}$ and $F(t)=A_1 + A_2 (1-e^{-kt})$ to the data.

higher temperature, dextran has a greater effect on the rate of subunit exchange.

Increasing the temperature to 45°C increased the rate constants significantly to $(4.05 \pm 3.08) \times 10^{-1} \text{ min}^{-1}$ and $(3.55 \pm 4.58) \times 10^{-1} \text{ min}^{-1}$ for AIAS- and LYI-labeled α A-crystallin, respectively, (Figure 5.13). These rate constants are 5.6 and 5.7 fold higher than rate constants calculated at 37°C and 2.6 and 3.2 fold higher than rate constants calculated at 42°C for AIAS- and LYI-labeled α A-crystallin respectively. Moreover, the time for complete exchange at 45°C was 14 and 10 minutes for AIAS and LYI-labeled α A-crystallin respectively, while at 37°C and 42°C, the times taken for complete exchange were 60 and 20 minutes for both case.

At 45°C and in the presence of dextran, the exchange rate constants of $(7.66 \pm 1.88) \times 10^{-2} \text{ min}^{-1}$ and $(4.13 \pm 1.43) \times 10^{-2} \text{ min}^{-1}$ were obtained for AIAS and LYI signal, respectively. The exchange rate was 5.3 times lower for AIAS and 7.3 times for LYI than in the absence of dextran at 45°C. In addition, the time to complete exchange was 30 and 40 minutes, compared to 14 and 10 minutes in the absence of dextran for AIAS- and LYI labeled α A-crystallin respectively. This means that at higher temperature, dextran has a considerable effect on decreasing the rate of subunit exchange. Considering the data obtained at 37°C, where dextran decreased the rate of subunit exchange only 1.4 times and the time to complete exchange was 70 minutes compared to 60 minutes in the absence of dextran in both case, these results revealed that at

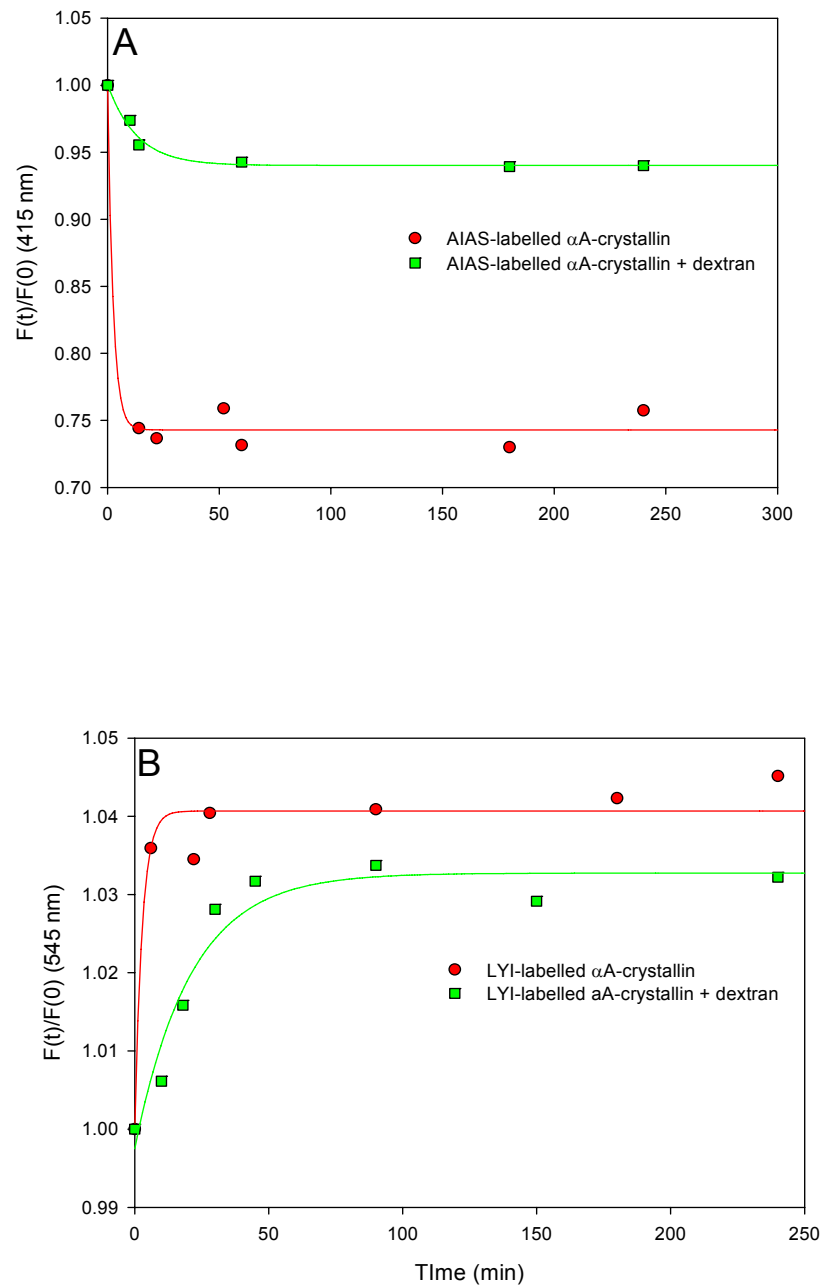


Figure 5.13: Time-dependent changes in (A) AIAS and (B) LYI fluorescence during subunit exchange of labeled α A-crystallin in the presence and absence of 10% w/v dextran at 45°C. (A): decrease in relative fluorescence intensity of AIAS at 415 nm and (B): increase in relative fluorescence intensity of LYI at 545 nm after mixing an equal amount of AIAS- and LYI-labeled α A-crystallin. Experiments were conducted in 4 hrs at 45°C in 50 mM sodium phosphate, 100 mM NaCl, 2 mM DTT, and pH 7.5. The rate constants were calculated by fitting exponential decay and rise to max functions $F(t) = C_1 + C_2 e^{-kt}$ and $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the data.

higher temperature dextran was more effective in slowing down α A-crystallin subunit exchange. Furthermore, a comparison of rate constants for α A-crystallin and dextran mixtures at 37°C, 42°C, and 45°C indicated that in the presence of dextran, subunit exchange of α A-crystallin was not affected significantly by increasing temperature (Table 5.3).

The rate constants for α A-crystallin in the presence and absence of dextran at 37°C, 42°C, and 45°C are shown in Figure 5.13.

The effects of temperature on rate of subunit exchange and time for complete exchange are summarized in Tables 5.3 and 5.4.

Table 5.3: Summary of rate constants for subunit exchange experiments of α A-crystallin (A): AIAS fluorescence and (B): LYI fluorescence at 37°C, 42°C, and 45°C.

A	Sample	AIAS fluorescence rate constant $\times 10^{-2}$ (min⁻¹)		
		37°C	42°C	45°C
	αA-crystallin	7.22 \pm 1.36	15.5 \pm 1.68	40.48 \pm 3.08
	αA-crystallin + dextran	5.42 \pm 1.86	3.39 \pm 1.09	7.64 \pm 1.88

B	Sample	LYI fluorescence rate constant $\times 10^{-2}$ (min⁻¹)		
		37°C	42°C	45°C
	αA-crystallin	6.23 \pm 1.20	10.73 \pm 1.56	35.54 \pm 1.46
	αA-crystallin + dextran	4.64 \pm 1.10	3.04 \pm 0.62	4.78 \pm 1.43

Table 5.4: Summary of time for complete exchange in various temperatures. N.D. refers to those experiments that were not undertaken in this study. Note that the calculated times were based on reaching the plateau region.

Sample	Time for complete exchange (min) at different temperatures		
	37°C	42°C	45°C
AIAS-/LYI-labeled αA- crystallin	60/60	20/30	14/10
AIAS-/LYI-labeled αA crystallin + ovotransferrin	180/180	N.D.	N.D.
AIAS-/ LYI-labeled αA- crystallin +dextran	70/70	50/40	30/40
AIAS-/ LYI-labeled αA-crystallin + ovotransferrin +	240/240	N.D.	N.D.

The effect of temperature on the rate of subunit exchange in the presence and absence of dextran is shown in Figure 5.14.

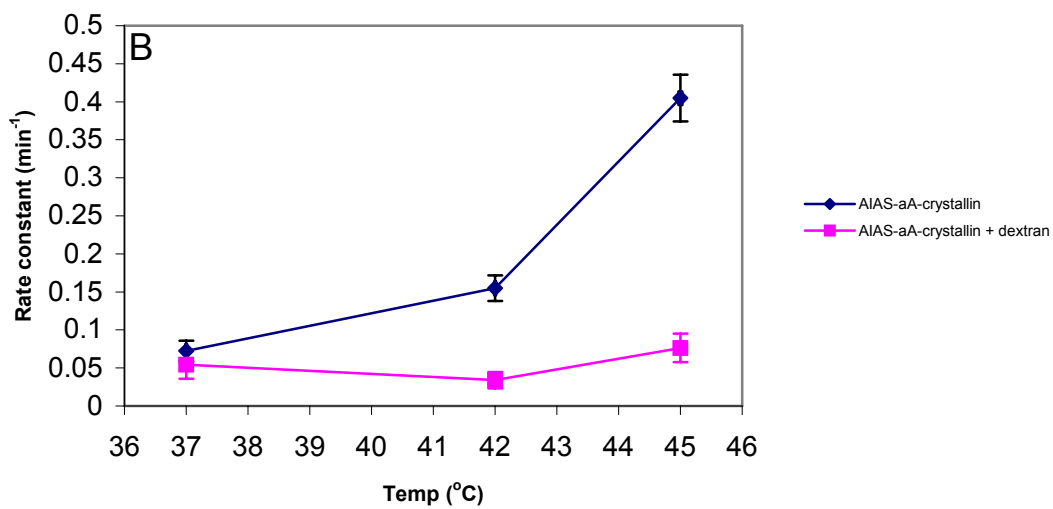
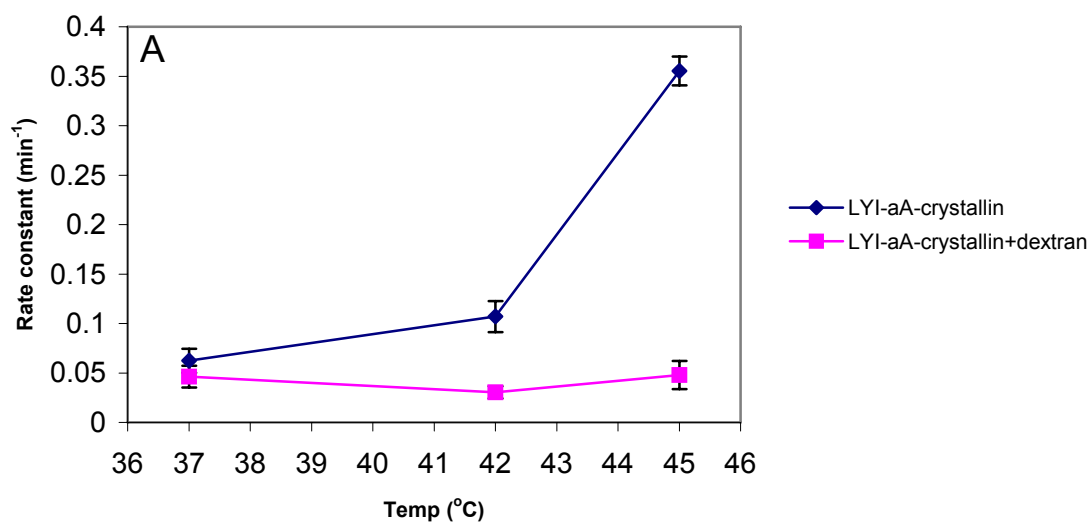


Figure 5.14: Temperature-dependent changes in the rate of α A-crystallin subunit exchange measured by (A): AIAS and (B): LYI fluorescence in the presence and absence of 10% w/v dextran.

5.4 Circular Dichroism (CD) spectroscopy of α -crystallin in the presence and absence of dextran at different temperatures

In order to investigate the effect of dextran on temperature-induced changes in the tertiary structure of α -crystallin, near-UV CD spectra of α -crystallin in the presence and absence of 10% w/v dextran were acquired at different temperatures. Due to the large absorbance of dextran in the far-UV, it was not possible to acquire CD spectra of α -crystallin in this wavelength region and hence monitor the effect of dextran on the secondary structure of the protein. However, dextran has little absorbance in the near-UV region of the spectrum. The near-UV CD spectrum of α -crystallin provides information on the environment of aromatic residues in the protein. As shown in Figure 5.15A, α -crystallin exhibits a negative ellipticity at around 291-300 nm from tryptophan residues and a larger positive peak at 262-266 nm from its phenylalanine residues, respectively (Technical report., E.C.S. 2001). The variation in ellipticity at 264 nm with temperature is shown in Figure 5.16 in the presence and absence of dextran. Spectra of α -crystallin showed a significant decrease in ellipticity at this wavelength with increasing temperature. Decreased ellipticity indicates that the environment of the phenylalanine residues has changed, consistent with the protein becoming partially unfolded at elevated temperature.

In the presence of dextran, the change in ellipticity at 264 nm was not significant (Figure 5.15B). Similar effects were observed at all wavelengths across the near-UV region. Thus, with increasing temperature the environment of the

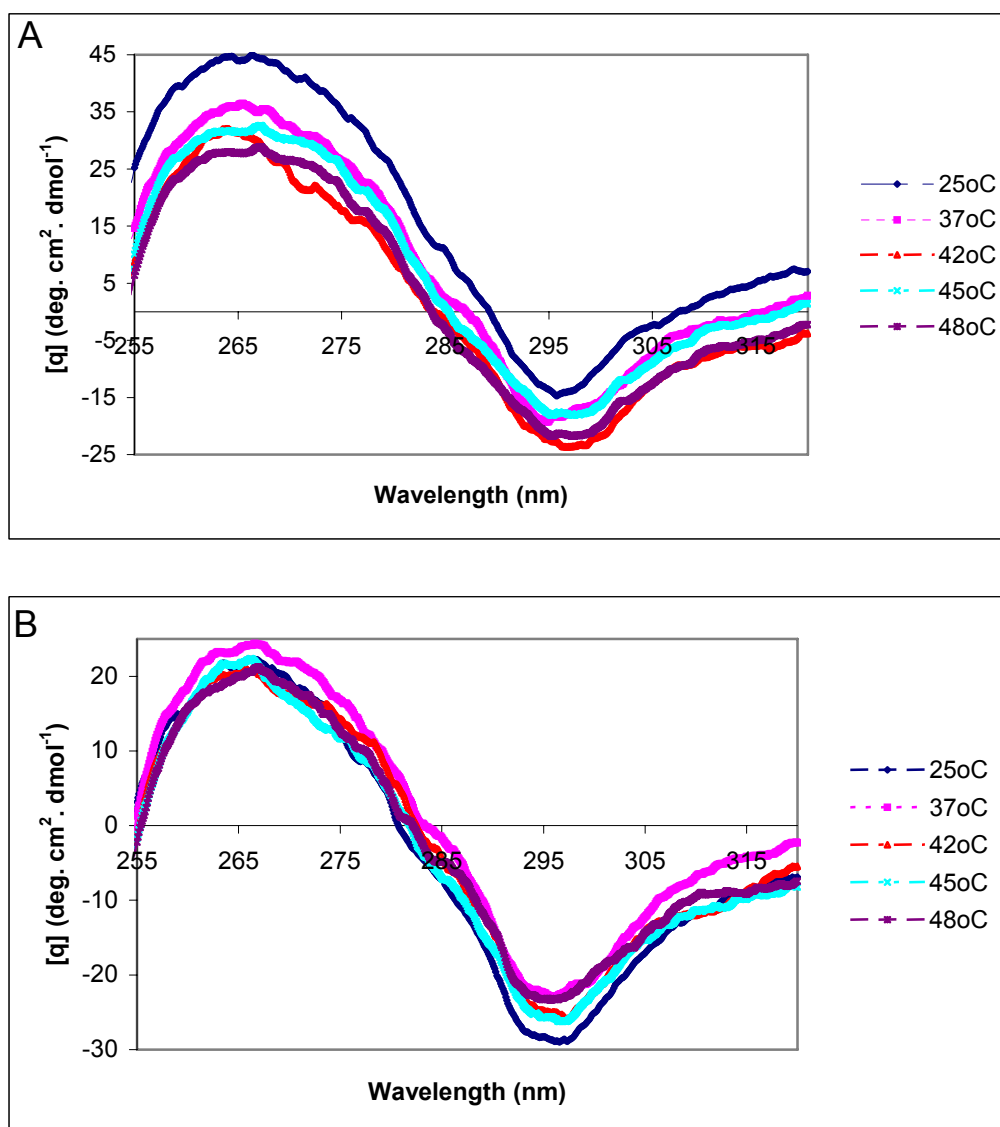


Figure 5.15: Near-UV CD spectra of α -crystallin at temperatures between 25°C and 48°C (A) in the absence and (B) presence of 10% w/v dextran. α -Crystallin was at a (3 mg/mL) in 10 mM phosphate buffer, pH 7.0. Spectra were acquired on a Jasco 810 spectropolarimeter with a 1 mm pathlength cell.

aromatic amino acids in α -crystallin was altered which correlated with α -crystallin undergoing tertiary structural alterations. Other studies have shown

that α -crystallin undergoes significant structural change in this temperature range (Raman et al. 1995, Raman et al. 1997). In the presence of dextran, however, little structural change with temperature occurred for α -crystallin implying that dextran protected the protein from temperature-induced structural changes.

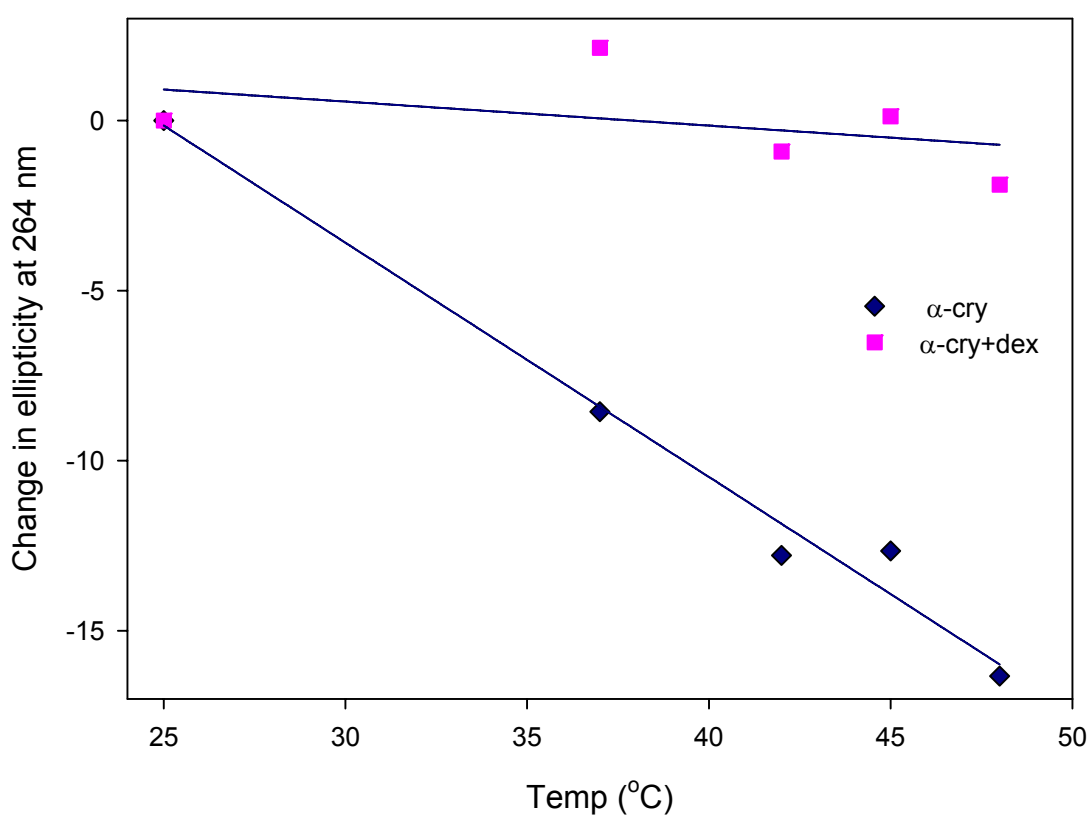


Figure 5.16: Decrease in the ellipticity of the CD signal at 264 nm of α -crystallin in the presence and absence of 10% w/v dextran at different temperatures.

5.5 Intrinsic fluorescence of α -crystallin in the presence and absence of dextran at different temperatures

To investigate the effect of dextran on the environment of the tryptophan residues in α -crystallin with increasing temperature the intrinsic fluorescence spectra of α -crystallin in the presence and absence of 10% w/v dextran were recorded. A comparison of maximum fluorescence wavelength (λ_{\max}) and fluorescence intensity at λ_{\max} of α -crystallin, in the presence and absence of 10% w/v dextran at different temperatures is shown in Figures 5.17A and B, respectively. There was a progressive decrease in the fluorescence intensity with temperature and a progressive increase in the maximum fluorescence wavelength over the range 25°C-48°C indicating that with increasing temperature, the tryptophan residues of α -crystallin become more exposed to solvent. In the presence of dextran, the change in the fluorescence intensity and wavelength were small, implying that dextran stabilised α -crystallin and prevented conformational changes that would have exposed its tryptophan residues to solvent. In additional control experiments, the intrinsic fluorescence of L-tryptophan in the presence and absence of dextran gave exactly the same results (Figure 5.18).

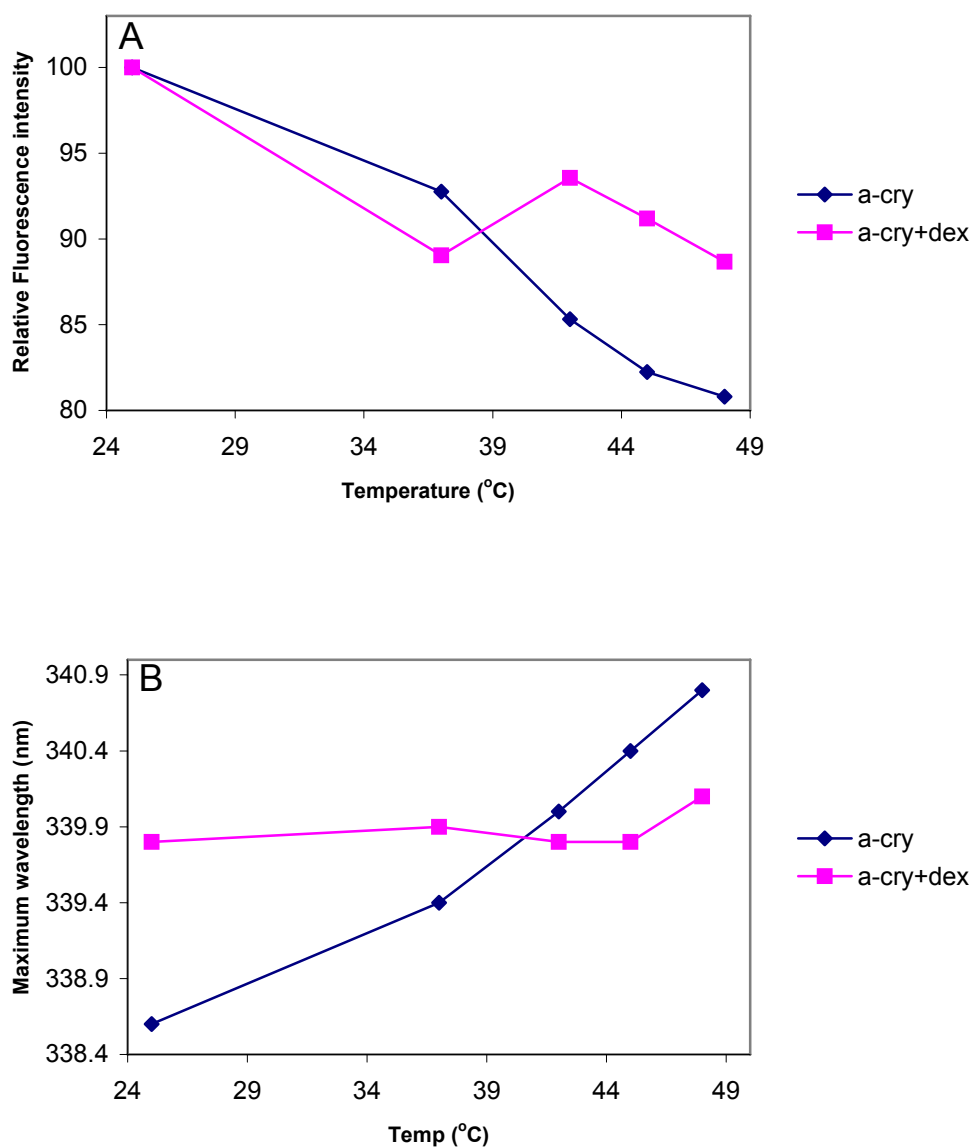


Figure 5.17: Intrinsic fluorescence of α -crystallin at temperatures between 25°C and 48°C in the presence and absence of dextran (A) fluorescence intensity and (B) wavelength. Protein concentration was 15 μ M in 10 mM phosphate buffer, pH 7.0. Fluorescence values are relative to 100% fluorescence at 25°C.

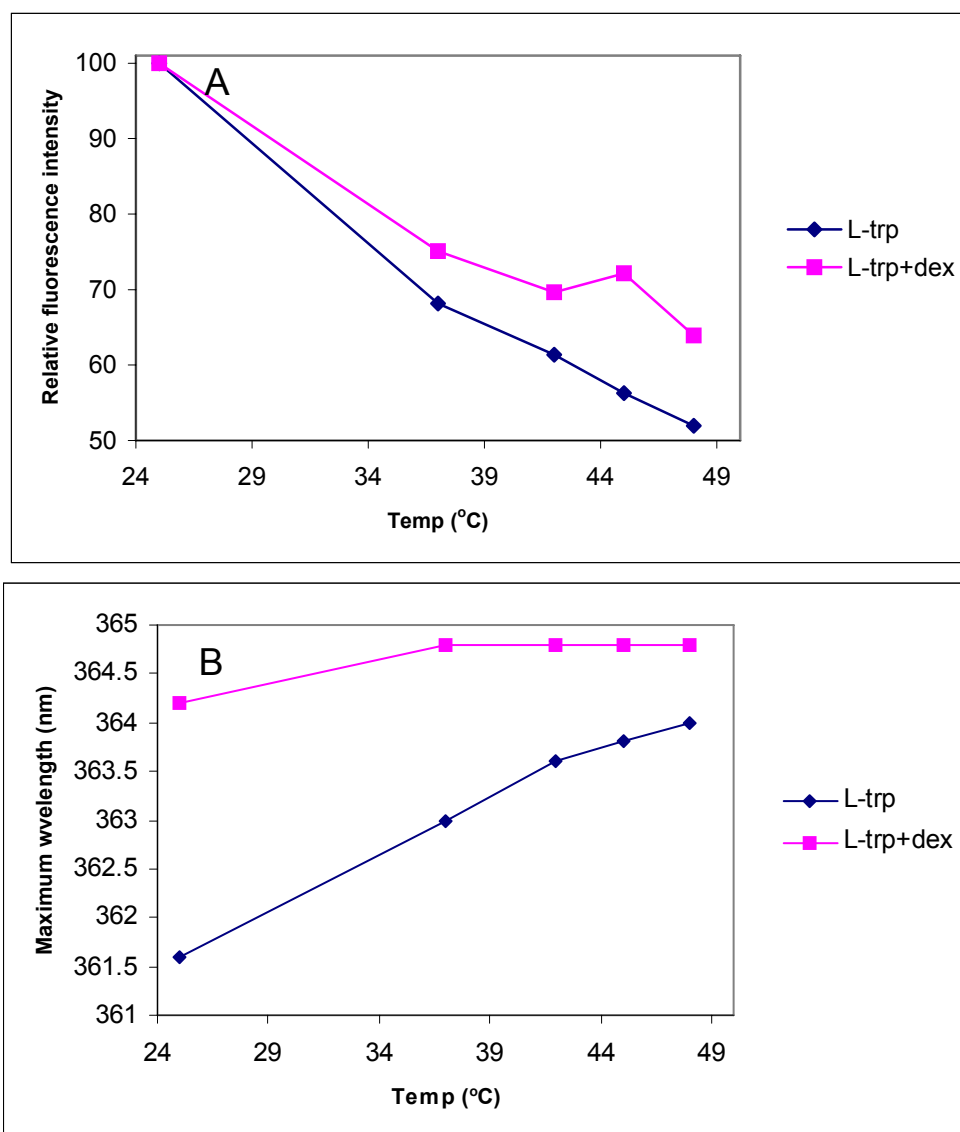


Figure 5.18: Intrinsic fluorescence of L-tryptophan at temperatures between 25°C and 48°C in the presence and absence of dextran (A) fluorescence intensity and (B) maximum wavelength. Amino acid concentration was 18.75 μ M in 10 mM phosphate buffer, pH 7.0. Fluorescence values are relative to 100% fluorescence at 25°C.

5.6 Discussion

Relative values of the rate constants of α A-crystallin subunit exchange obtained for AIAS and LYI fluorescence changes were consistent at all temperatures studied. At 37°C, these data (0.072 min^{-1} for AIAS- and 0.062 min^{-1} for LYI-labeled α A-crystallin) were very similar to those of Bova et al. (1997), who reported rate constants of 0.075 min^{-1} and 0.069 min^{-1} at 37°C for fluorescence changes of AIAS- and LYI-labeled α A-crystallin, respectively. In the presence of 10% w/v dextran at 37°C, the rate of subunit exchange of α A-crystallin decreased by approximately 26% and the time for complete exchange increased by approximately 14% for both case (Tables 5.3 and 5.4).

Binding of reduced ovotransferrin to α A-crystallin decreased the rate of subunit exchange by 59% and 50% for AIAS and LYI-labeled α A-crystallin, respectively, and significantly increased the time required for the completion of the exchange of both labeled subunits. Since ovotransferrin is a large protein (subunit mass $\sim 78 \text{ kDa}$), it is likely that a molecule of ovotransferrin binds to several subunits of α A-crystallin, effectively cross-linking them together, and thus markedly slowing down their exchange (Bova et al. 1997, Bova et al. 2000). In the presence of reduced ovotransferrin, 10% w/v dextran further slowed down the subunit exchange of α A-crystallin in both case (by $\sim 90\%$). In addition, in the presence of dextran, the time for the completion of subunit exchange was significantly longer than in the absence of dextran. The cumulative effect of reduced ovotransferrin and dextran on the rate of subunit exchange was larger than the sum of the individual effects of these components (by about 15%), suggesting

an enhancement of the effect of ovotransferrin on the rate of subunit exchange of α A-crystallin in the presence of dextran. In summary, these data show that dextran slows down subunit exchange of α A-crystallin and this effect is more evident in the presence of a bound target protein.

Between 37°C and 45°C, the chaperone action of α A-, and α -crystallin in preventing reduced ovotransferrin from aggregating was enhanced. The increase in chaperone activity of α -crystallin at higher temperature has been reported in a variety of studies (Raman et al. 1995, Reddy et al. 2000, Srinivas et al. 2001). Raman et al. (1995) showed that at temperatures above 30°C, a structural change occurred in α -crystallin, which correlated with its enhanced chaperone activity. It was suggested that this increased chaperone activity of α -crystallin occurred as a result of an increase in exposed hydrophobic surface area. Reddy et al (2000) also reported the increase in chaperone activity of α A-crystallin upon increasing temperature above 30°C.

This study found that at higher temperature, α A-crystallin was a better chaperone than α -crystallin in preventing aggregation of reduced ovotransferrin, which is consistent with the effect observed by Datta. et al. (1999) in preventing insulin aggregation. The chaperone ability of α A- and α -crystallin when interacting with reduced ovotransferrin increased with temperature (Figure 5.4). Dextran, however, interfered with α - and α A-crystallin chaperone action and eliminated the effect of temperature on their chaperone activity (in fact both proteins were slightly worse chaperones at higher temperature in the presence of dextran (Figure 5.4)). This correlates with the finding that increasing

temperature did not alter significantly the subunit exchange rate of α A-crystallin in the presence of dextran. Possible reasons for this observation could encompass one or more of the following: (a) a decreased efficiency of interaction between reduced ovotransferrin and α A-crystallin in the presence of dextran, (b) an increased rate of aggregation of ovotransferrin at higher temperature affecting α -crystallin's chaperone efficiency and (c) dextran stabilizing α -crystallin and thereby diminishing the structural changes that occur to α -crystallin at higher temperature (Raman et al. 1995, Raman et al. 1997). In relation to point (a), the temperature-dependent decrease in the effect of dextran on the rate of ovotransferrin aggregation may be a consequence of dextran slowing down diffusion and limiting chance encounters of reduced ovotransferrin molecules with α -crystallin (Ellis et al. 1999a and b). With regard to point (b), the rate of ovotransferrin aggregation in the presence of dextran increases only slightly at higher temperatures (Figure 5.4) in contrast to the much larger effect in the absence of dextran. Thus, this is unlikely to be a major factor in the observation of an absence of temperature dependence α -crystallin chaperone ability in the presence of dextran.

The third point discussed above (c) was examined by monitoring structural changes in α -crystallin with temperature in the presence and absence of dextran (Figures 5.15 and 5.16). Near-UV CD spectra of α -crystallin showed, for example, differences in phenylalanine emission intensity bands at different temperatures indicating that the effect of temperature on the tertiary structure of α -crystallin was significant in the absence of dextran, as has been observed by

others (Raman et al. 1997, Das et al. 1997) Santhoshkumar et al. (2001) reported that a phenylalanine mutant of α A-crystallin (F71G) had decreased chaperone activity compared to the wild type protein and that by increasing temperature to 45°C, a complete loss of chaperone activity in the mutant was observed. It was concluded that the N-terminal phenylalanine-rich region (residues 71-80) contributes to the chaperone-like function of α -crystallin (Santhoshkumar et al. 2001, Plater et al. 1996). In this study, increasing temperature changed the structure of α -crystallin in the region of the phenylalanine residues. Concomitant with this temperature-dependence, the chaperone activity of protein increased with temperature. In the presence of dextran, there was little change in ellipticity with temperature in the near-UV region of the CD spectrum arising from the phenylalanine residues and in other regions of the spectrum. Intrinsic fluorescence spectroscopy of α -crystallin in the presence and absence of dextran indicated that the environment of the tryptophan residues (the N-terminal domain of the protein at positions 9 in α A and 9 and 60 in α B) was not altered significantly with increasing temperature in contrast to the situation in the absence of dextran. Thus, all these data are consistent with the notion that, in contrast to the condition in the absence of dextran, α -crystallin did not undergo significant structural alteration with temperature in the presence of dextran.

The lack of significant temperature dependence in α A-crystallin subunit exchange rate in the presence of dextran is in marked contrast to the situation in the absence of dextran where subunit exchange rate increased significantly

with temperature (Table 5.3). In the crowded environment of the cell this observation has relevance, i.e. from our results, variation in subunit exchange with temperature in a cellular environment may not be an important factor in regulating chaperone action.

Upon increasing the temperature from 37°C to 42°C, the rate constant for subunit exchange of α A-crystallin increased 2.1- and 1.7-fold for AIAS and LYI signal, respectively and the time for the completion of exchange decreased by approximately 67%. These results differ from those of Bova et al. (1997), who reported a 4.2-fold increase in the rate constant of subunit exchange and a 76% reduction in the time for complete exchange at 42°C relative to 37°C. This difference may be due to the use of recombinant α A-crystallin by Bova et al. (1997) while in the present study a native protein, which was partially phosphorylated and had been separated from α B-crystallin at acidic conditions, was used. An increase in temperature to 45°C further increased the subunit exchange rate of α A-crystallin and decreased the completion time. Our data confirm that subunit exchange rate increases with temperature and show that faster subunit exchange correlates with enhanced chaperone ability of α A-crystallin. One explanation for this is that faster subunit exchange leads to greater availability of the chaperone-binding sites due to a greater abundance of dissociated species (Raman et al. 1995, Haslbeck et al. 1999, Reddy et al. 2000). Coupled with this, the structural changes in α -crystallin, which occur at higher temperature (Haslbeck et al 1999; Das et al. 1997) may expose more of its chaperone-binding sites to solution.

The increased chaperone activity of α -crystallin at higher temperatures, coinciding with the faster subunit exchange supports the hypothesis that the dissociated form of α -crystallin has enhanced chaperone activity compared to the aggregated species, possibly because the dissociated species has its chaperone binding site more exposed to solution (Raman et al.1995 Reddy et al. 2000). During chaperone action, these dissociated forms interact with their stressed target protein and their association rate is altered, leading to a decrease in the rate of α -crystallin subunit exchange, as shown by our data with reduced ovotransferrin. These results are consistent with the reports that the quaternary structure of Hsp26 changes dramatically at heat shock temperature (37°C), i.e. an oligomeric form of Hsp26 was observed at room temperature, while a dimeric species was observed at 43°C (Haslbeck, et al.1999). At this higher temperature, the chaperone ability of Hsp26 was also enhanced.

Our data showed that dextran decreased the rate of subunit exchange both in the presence and absence of reduced ovotransferrin. While the chaperone ability of α -crystallin increased with temperature, dextran eliminated this effect, or even made α -crystallin a less efficient chaperone at higher temperatures. As molecular crowding did not cause enhancement with temperature of ovotransferrin aggregation, a possible reason for this is the decreased interaction between α A-crystallin subunits and/or α A-crystallin subunits and reduced ovotransferrin in the presence of dextran implying decreased diffusion of the molecules. Both of these processes involve complex formation, i.e. aggregation of ovotransferrin subunits and subunit exchange of α A-crystallin,

were slower in the presence of dextran. Crowding has both thermodynamic and kinetic effects on biochemical reaction rates (Ellis et al. 1999a and b; van den Berg et al. 1999). If the overall rate of reaction between two defined species is limited by the encounter rate of the reacting molecules, then crowding will reduce the overall rate by reducing diffusion (Ellis et al. 1999 a and b) To validate this hypothesis, it would be worth considering studies on temperature dependence of diffusion rates in the presence of molecular crowding.

5.7 Conclusions

In this chapter, the hypothesis that subunit exchange may be relevant to the chaperone activity of α -crystallin was tested. It has been shown that at higher temperatures, the rate of subunit exchange of α A-crystallin increased and this correlated with its enhanced chaperone activity, possibly by facilitating dissociation of α -crystallin into its subunits (Raman et al. 1995, Reddy et al. 2000, Srinivas et al 2001). However, with more relevance to the *in vivo* situation, the presence of a bound target protein and/or a crowding agent decreased the rate of subunit exchange. In particular, the decrease in the rate of α -crystallin subunit exchange is possibly due to the interaction of the target protein with α A-crystallin subunits. Molecular crowding further decreased the rate of subunit exchange of mixture of α A-crystallin and target protein. Although the α A-crystallin subunit exchange rate increased with temperature between 37°C and 45°C, in the presence of dextran it did not, however, show significant temperature-dependency. This was accompanied by reduced chaperone

activity of α -crystallin with temperature in the presence of dextran and little overall structural change in the protein as monitored by near-UV CD and fluorescence spectroscopy. Thus, from this work, it is apparent that although subunit exchange in α -crystallin plays an integral role in regulating its chaperone action *in vitro*, this aspect may be less crucial in the crowded intracellular environment.

Chapter 6

The effect of α -crystallin on the heat-induced aggregation of β -lactoglobulin

β -Lactoglobulin is a major whey protein of milk (Puyol et al. 1994, Manderson et al. 1998, Verheul et al. 1998), which at physiological pH values exists as a dimer (Croguennec et al. 2003, Livney et al. 2003a). Each monomer has a molecular weight of 18.3 kDa, contains 162 amino acid residues, two disulphide bonds (C106-C119 and C66-C160) and one free cysteine residue (C121) (Manderson et al. 1999, Creamer et al. 2004, Livney et al. 2004). Upon heating, β -lactoglobulin dissociates into monomers, leading to exposure of hydrophobic groups and the free sulfhydryl group (Hoffman et al. 1997, Fox. 1998). This then gives rise to aggregation and/or polymerization (Iammeti et al. 1995, Hoffmann et al. 1997, Galani et al. 1999, Croguennec et al. 2003, Livney et al. 2003a). β -Lactoglobulin is sensitive to heating at 60-100°C. During heating, the protein undergoes an initial dissociation of dimers followed by conformational changes which increase the exposure of previously buried hydrophobic groups and the thiol group (Cairoli et al. 1994, Roefs et al. 1994, Hoffmann et al. 1997a, Galani et al. 1999, Croguennec et al. 2003).

Aggregation of β -lactoglobulin occurs mainly via intermolecular disulphide bond exchange, i.e. the aggregation is not (in the main) due to nucleation-dependent processes (Sebastianus et al. 1994, Hoffmann et al. 1997, Creamer et al. 2004) but arises from polymerization. In a previous study (Devlin et al. 2003) indicated that α -crystallin did not inhibit the aggregation of a serpin (AT (antitrypsin)) which aggregated via a polymerization reaction but did inhibit a serpin aggregation (ACT(antichymotrypsin)) when occurring via a nucleation-dependent mechanism. Therefore, in order to explore the effect of α -crystallin

on β -lactoglobulin aggregation, a heating assay of β -lactoglobulin at different pH values in the presence and absence of α -crystallin was performed.

6.1 The effect of pH on the aggregation of β -lactoglobulin

It has been reported previously that increasing pH increases the rate of aggregation and precipitation of heated β -lactoglobulin (Hoffmann et al. 1999, Law et al. 2000). Accordingly, the effect of pH on the rate of aggregation and precipitation of β -lactoglobulin was studied by heating the sample at 70°C and at different pH values between 6.5 and 8.0. As pH increased, the rate of aggregation of β -lactoglobulin also increased (Figure 6.1, Table 1).

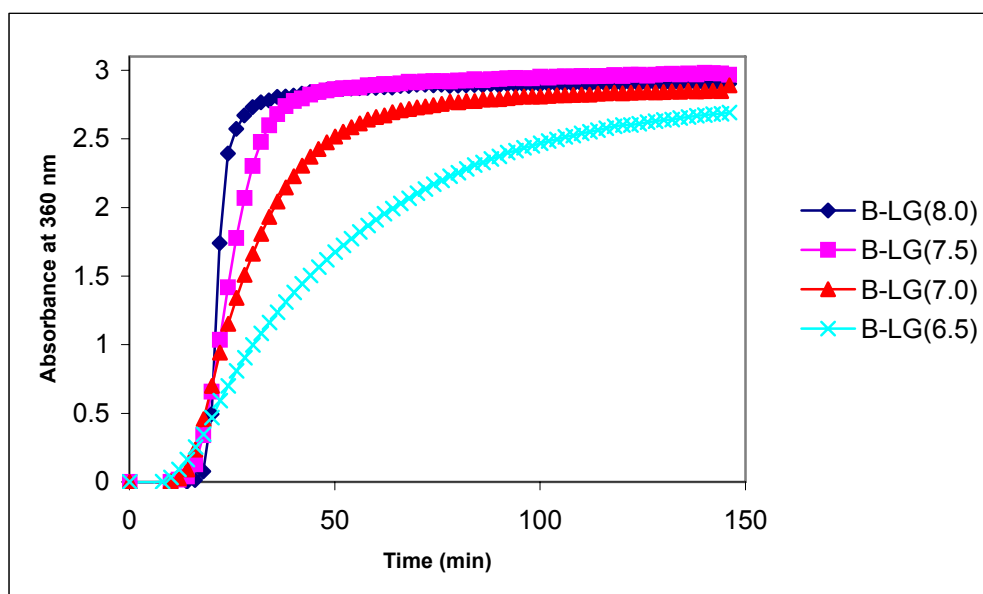


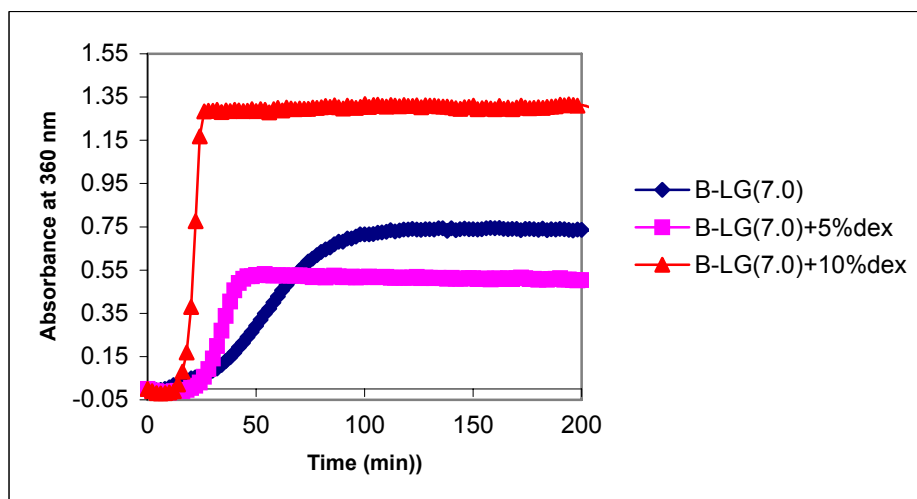
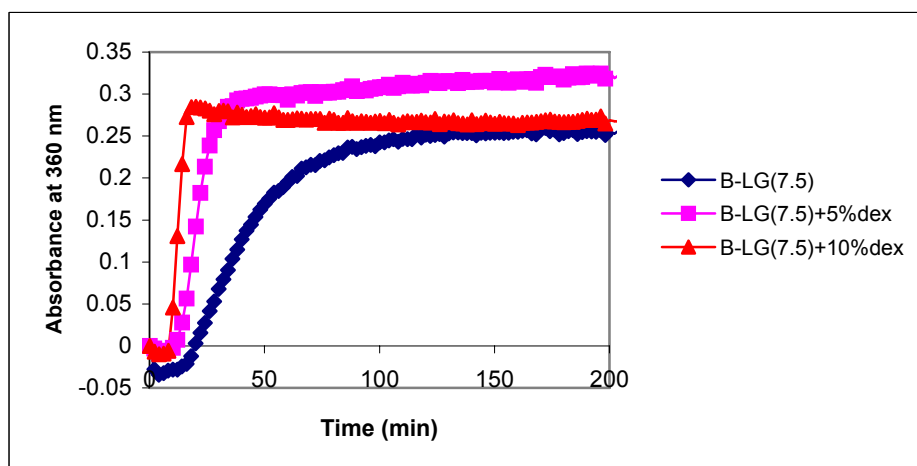
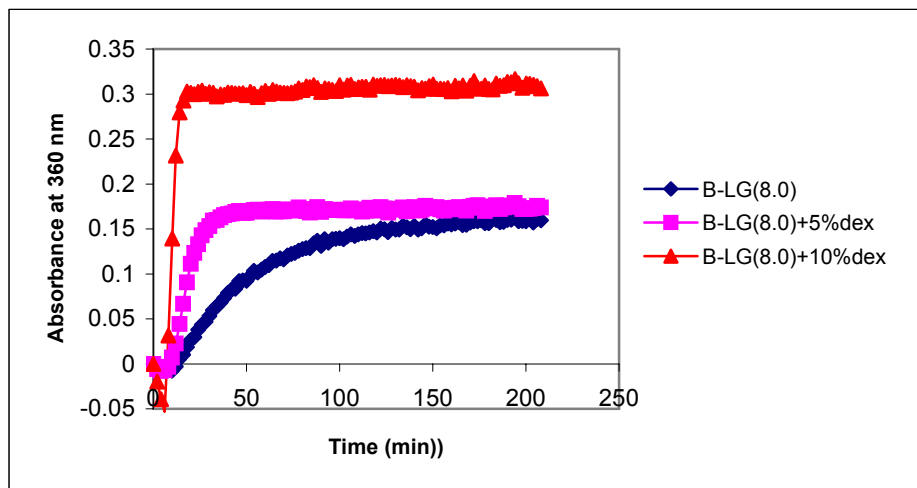
Figure 6.1: Heat-induced aggregation of β -lactoglobulin at 5 mg/mL and different pH values. Protein was in a total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , 100 mM NaCl and 2.5 mM EDTA and the incubation temperature was 70°C.

Table 6.1: Summary of rate constants for the aggregation of heat-stressed β -lactoglobulin at different pH values. The standard deviation obtained from Sigmaplot upon regeneration calculation.

β -lactoglobulin	Rate constants $\times 10^{-1}$ (min^{-1})
pH (8.0)	4.37 ± 0.45
pH (7.5)	1.20 ± 0.17
pH (7.0)	0.99 ± 0.34
pH (6.5)	0.47 ± 0.09

6.2 The effect of dextran on heated β -lactoglobulin at different pH values

In order to study the effect of macromolecular crowding on the aggregation and precipitation of β -lactoglobulin at different pH values, dextran was added to give concentrations of 5% and 10% w/v. As shown in Figure 6.2, the rate of aggregation and precipitation of β -lactoglobulin increased at all pH values in the presence of dextran. This implies that the presence of a macromolecular crowding agent enhances the aggregation and precipitation of β -lactoglobulin.



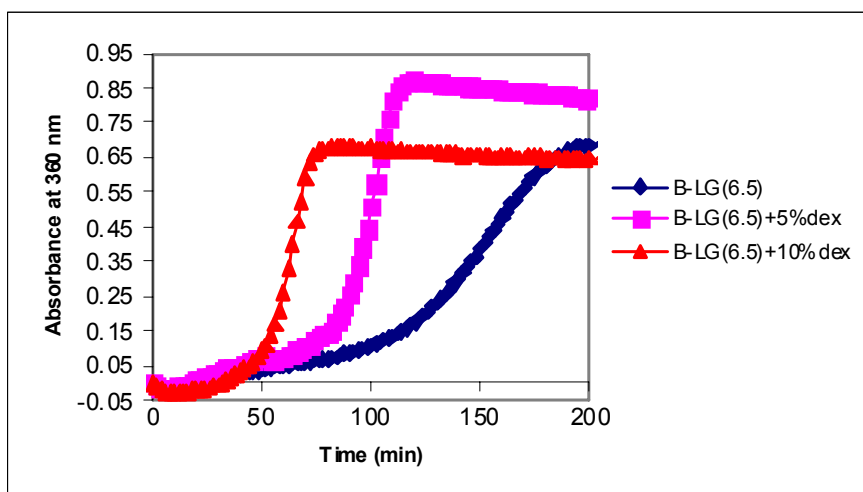
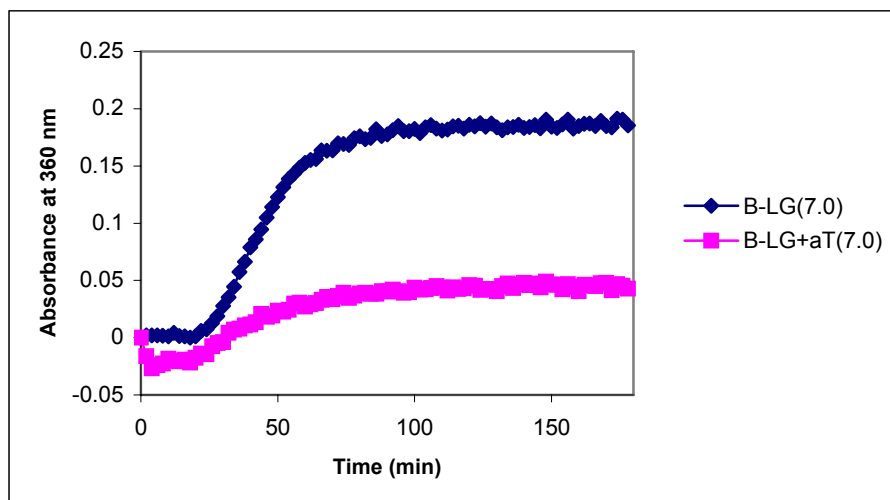
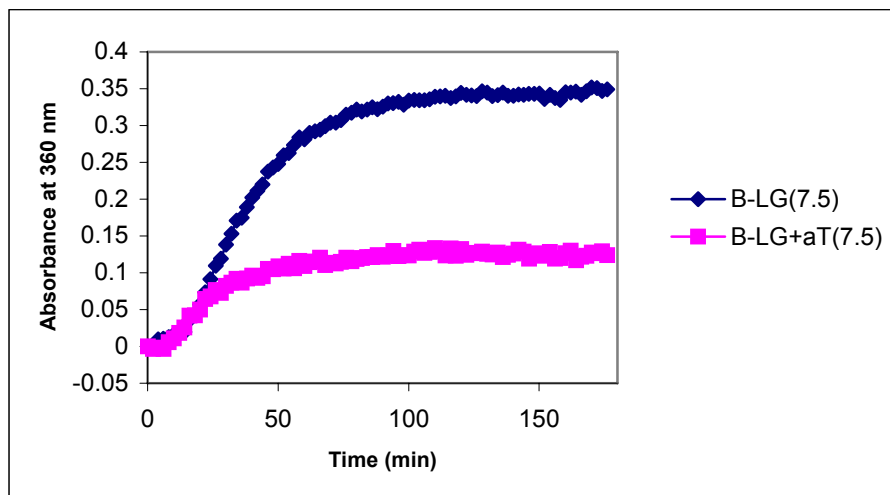
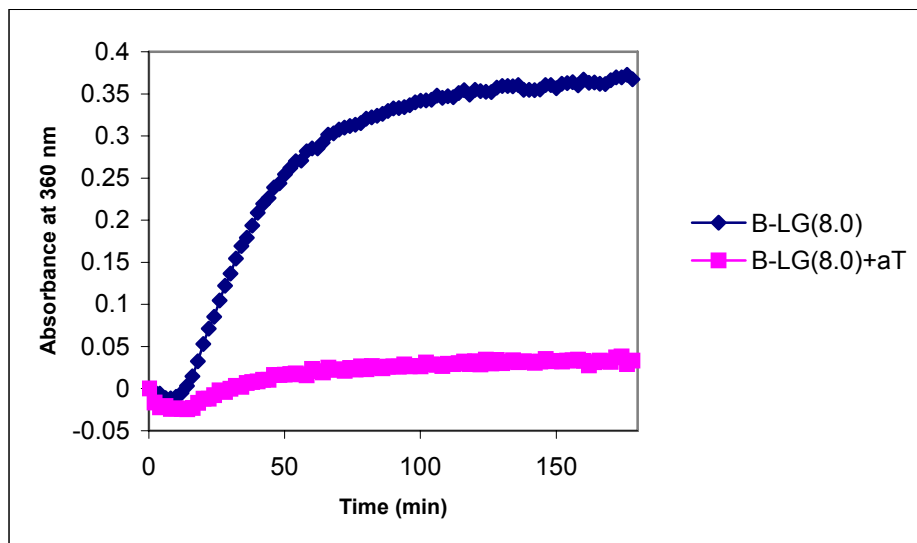


Figure 6.2: Heat-induced aggregation of β -lactoglobulin at 5 mg/mL in the presence of 5% and 10% w/v concentrations of dextran and different pH values. Protein was in a total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , 100 mM NaCl and 2.5 mM EDTA and the incubation temperature was 70°C.

6.3 The chaperone action of α -crystallin in preventing aggregation of heated β -lactoglobulin at different pH values

The chaperone-like action of α -crystallin in preventing the aggregation of β -lactoglobulin was examined at different pH values. As shown in Figure 6.3, α -crystallin is able to prevent the aggregation of β -lactoglobulin at all pH values tested (i.e. 6.5, 7.0, 7.5 and 8.0). The efficiency of α -crystallin as a molecular chaperone increased with increasing pH as demonstrated by its greater protection of β -lactoglobulin against aggregation. At pH 8.0 and at the time point of 178 minutes, the aggregation of β -lactoglobulin was suppressed ~92.2% in the presence of a 1:1 (w:w) ratio of α -crystallin: β -lactoglobulin. At pH values of 7.5, 7.0, and 6.5 at the same time point, α -crystallin prevented the aggregation of β -lactoglobulin by ~76%, ~72%, and ~43% respectively.



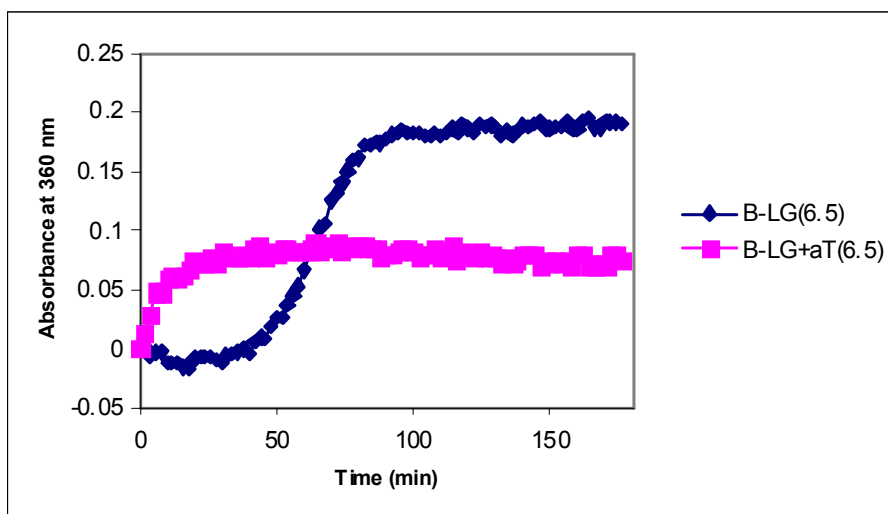


Figure 6.3: Heat-induced aggregation of β -lactoglobulin at 5 mg/mL in the presence and absence of α -crystallin at different pH values (8.0, 7.5, 7.0, and 6.5). Protein was in a total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , 100 mM NaCl and 2.5 mM EDTA and the incubation temperature was 70°C.

Pellets and supernatants of the protein solutions (after heat induced aggregation experiments) at pH values of 8.0 and 7.0 were examined by SDS-PAGE for the distribution protein (Figure 6.4). Bands corresponding to ~18 kDa in mass were observed at pH 7.0 and pH 8.0 both in the pellet and supernatant. Two bands corresponding to ~18 and 20 kDa in mass were also observed for β -lactoglobulin and α -crystallin at pH 7.0 and pH 8.0 both in the pellet and supernatant. The strong bands seen in Lanes 3 and 5 in Figure 6.4 A show the precipitation of β -lactoglobulin at pH 7.0 and 8.0 values. In the supernatant the bands are very weak (Figure 6.4B) indicating that β -lactoglobulin mostly

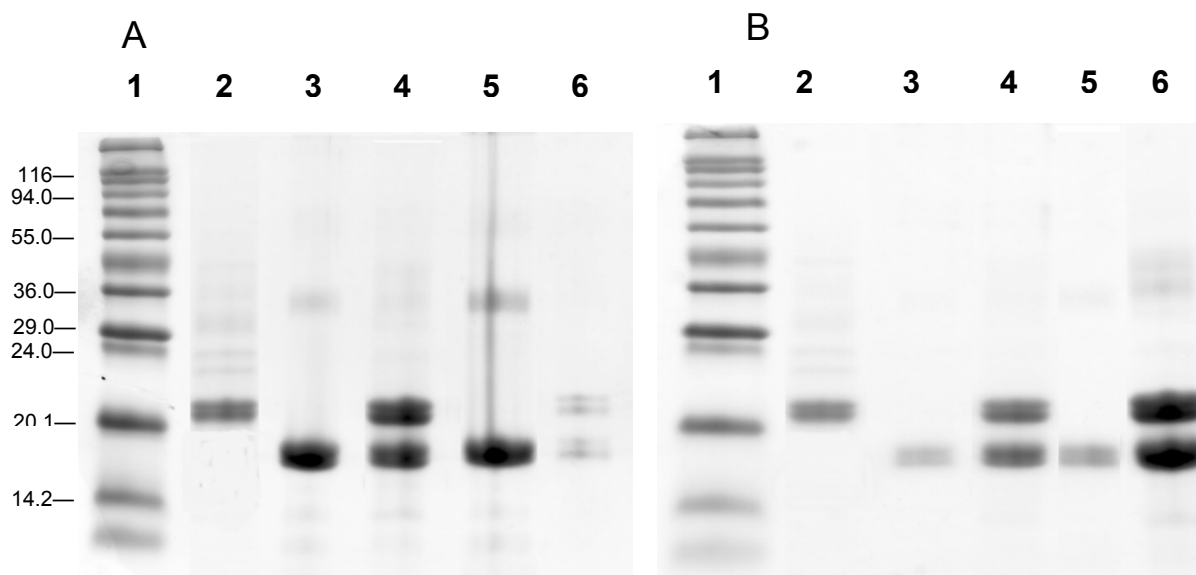
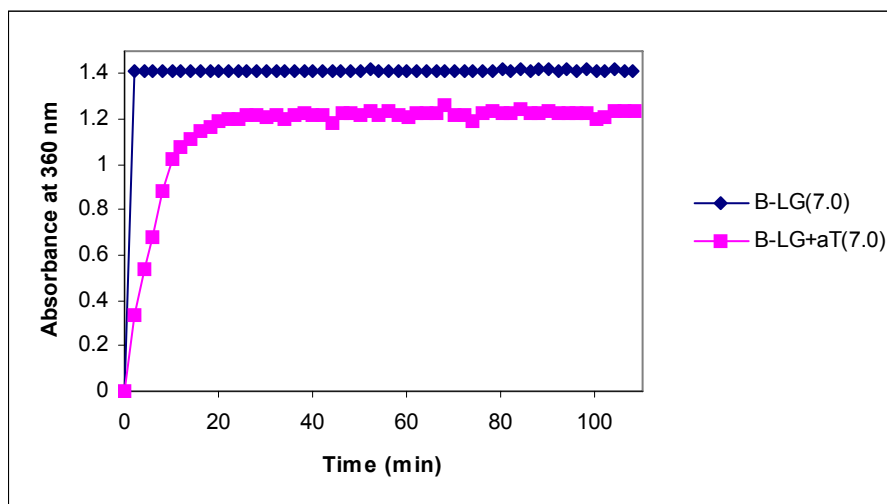
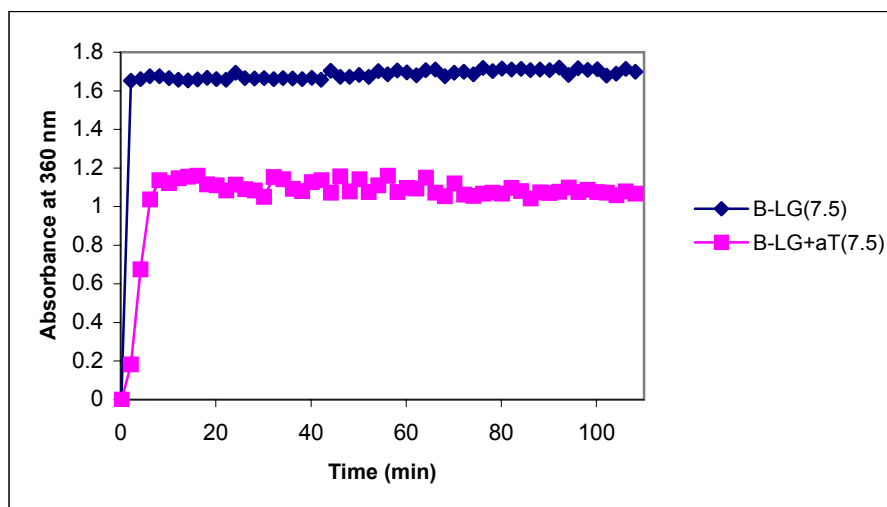
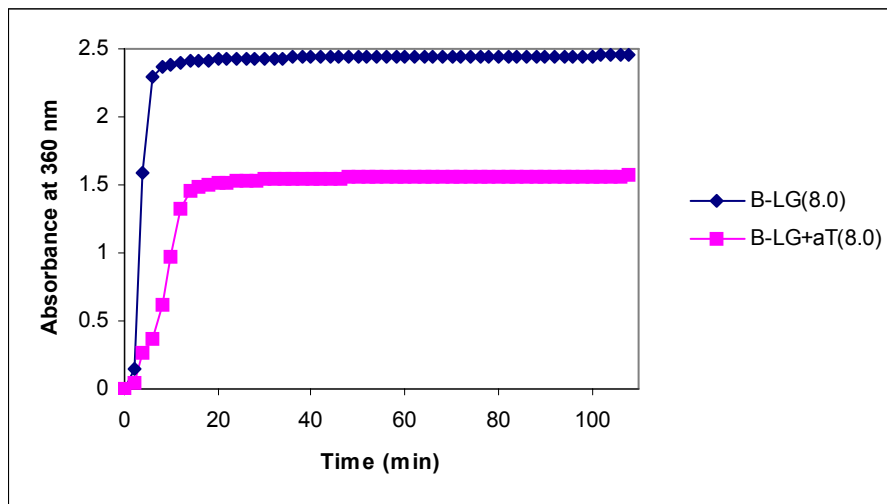


Figure 6.4: SDS-PAGE gels showing the expression of (A) pellet and (B) supernatant of β -lactoglobulin heat-induced aggregation samples in the presence and absence of α -crystallin at pH 7.0 and 8.0 values. For both gels Lane 1: Wide range molecular weight markers, Lane (2) α -crystallin, Lane (3): β -lactoglobulin at pH 7.0, Lane (4): β -lactoglobulin and α -crystallin at pH 7.0 , Lane (5): β -lactoglobulin at pH 8.0, Lane (6): β -lactoglobulin and α -crystallin at pH 8.0.

precipitated under these conditions when heated. Lanes 4 and 6 show the bands of β -lactoglobulin and α -crystallin at pH 7.0 and pH 8.0, respectively. At pH 7.0, the intensity of the bands are stronger in the pellet compared to the supernatant. This means that most of protein precipitated and α -crystallin was not effective in preventing the precipitation of β -lactoglobulin. In fact, α -crystallin and β -lactoglobulin co precipitated indicating that α -crystallin itself was destabilized under these conditions. At pH 8.0, very faint bands were present in the pellet suggesting little precipitation while very strong bands in the supernatant are consistent with both proteins being present. This means that at pH 8.0, α -crystallin was successful in forming a soluble complex with β -lactoglobulin to prevent its aggregation and eventual precipitation. Thus, these data are consistent with α -crystallin being a more efficient chaperone at higher pH values as observed in the chaperone assay (Figure 6.2).

6.4 Chaperone action of α -crystallin in preventing the aggregation of reduced and heated β -lactoglobulin at different pH values

The chaperone-like action of α -crystallin in preventing aggregation of reduced and heated β -lactoglobulin was examined at different pH values to compare the chaperone activity of α -crystallin with the non-reduced form of β -lactoglobulin (Figure 6.5). With increasing pH, the aggregation of reduced β -lactoglobulin increased even more than in the absence of DTT (compare Table 6.2 with Table 6.1). The efficiency of α -crystallin as a molecular chaperone decreased in the presence of DTT as demonstrated by its poorer protection of β -lactoglobulin against aggregation. At pH 8.0 and the time point of 100 minutes, the aggregation of β -lactoglobulin was suppressed by approximately 36% with a 1:1 ratio (w:w) of α -crystallin to β -lactoglobulin. At pH values of 7.5, 7.0, and 6.5 at the same time point, α -crystallin prevented the aggregation of β -lactoglobulin by approximately 34%, 15%, and 14% respectively. These results show that DTT increased the rate of aggregation of heated β -lactoglobulin and in this situation α -crystallin is a less efficient chaperone in preventing the aggregation of β -lactoglobulin. Furthermore, the chaperone ability of α -crystallin increased with increasing pH. This is consistent with other data showing that the chaperone ability of α -crystallin is less efficient for rapidly aggregating target protein (Lindner et al. 1998, Chapter 3).



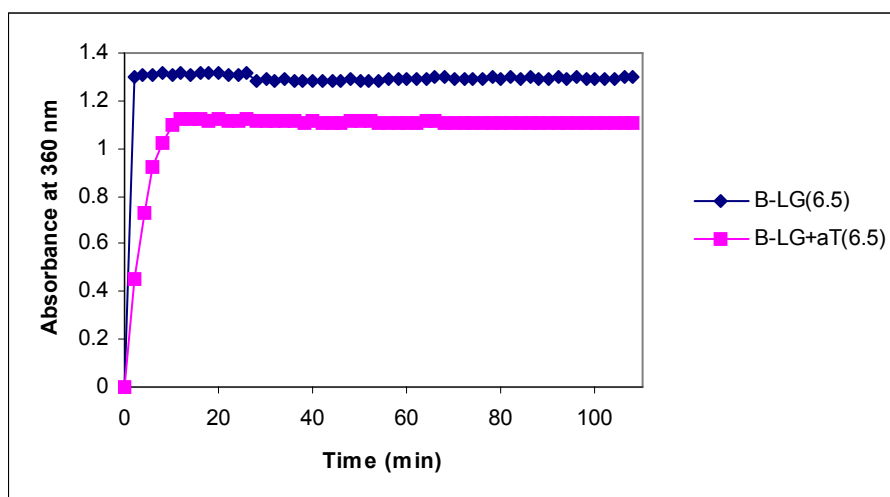


Figure 6.5: Heat-induced aggregation of reduced β -lactoglobulin at 5 mg/mL in the presence and absence of α -crystallin and DTT at different pH values. Protein was in a total volume of 1 mL of 50 mM phosphate buffer, 0.05% (w/v) NaN_3 , 100 mM NaCl, 2.5 mM EDTA with 20 mM DTT and the incubation temperature was 70°C.

Table 6.2: Summary of rate constants for the aggregation of reduced and heated β -lactoglobulin at different pH values. The standard deviation obtained from Sigmaplot upon regeneration calculation

β -lactoglobulin	Rate constants $\times 10^{-1}$ (min^{-1})
pH (8.0)	5.71 ± 0.30
pH (7.5)	1.50 ± 1.5
pH (7.0)	1.01 ± 0.54
pH (6.5)	1.47 ± 1.1

6.5 Discussion

β -Lactoglobulin aggregates via a combination of disulphide bond exchange (which leads to polymerization) and a nucleation-dependent mechanism due to hydrophobic association (Hoffmann et al. 1997b, Croguennec et al. 2004). Mainly heat-induced aggregation of β -lactoglobulin occurs through the formation of a dimer-monomer transition state of the protein. This transition state is characterized by a decrease in charge on the histidine residues of β -lactoglobulin and an increased number of free thiol groups which in turn increases intermolecular thiol/disulfide linkage (Law et al. 2000).

At pH values from 6.5 to 8.0, the aggregation and eventual precipitation of β -lactoglobulin was investigated. The rate of aggregation of heat-stressed β -lactoglobulin increased with increasing pH, which is consistent with the pH dependence of disulphide bond cleavage (Hoffmann et al. 1999, Law et al. 2000). Law et al. (2000) found that the rate of denaturation of β -lactoglobulin was increased at higher pH values. A possible reason for this is that by increasing pH, the net negative charge on the protein also increases, which in turn encourages intramolecular electrostatic repulsion. In order to counteract this increased charge, dissociation from the dimer to the monomer form of β -lactoglobulin is favoured. (Qi et al. 1995, Law et al. 2000). This also increases the activity of thiol groups and hence their tendency to form covalent bonds, which eventually leads to unfolding and aggregation of, heated β -lactoglobulin (Qi et al. 1995, Law et al. 2000).

The effect of the molecular chaperone, α_s -casein, on preventing the aggregation of one of its natural targets, heat-stressed β -lactoglobulin, has been previously demonstrated (Morgan et al. 2005), whereby α_s -casein effectively suppressed thermally induced aggregation of β -lactoglobulin at 70°C and pH 7.0 and this chaperone ability of α_s -casein decreased with increasing pH which is the opposite in fact seen for α -crystallin. Devlin et al. (2003) observed that α -crystallin was not able to prevent the aggregation of serpin (AT), which aggregates via a polymerization mechanism rather than a nucleation-dependent mechanism. However, α -crystallin inhibited the aggregation of a serpin (ACT), which aggregated via a nucleation-dependent mechanism. Thus, would α -crystallin inhibit β -lactoglobulin aggregation and if so, would this decrease as the pH increased and via aggregation polymerization predominated? To answer these questions. the chaperone action of α -crystallin was investigated in preventing the aggregation of heat stress β -lactoglobulin at different pH values to determine its efficiency. It was found that at all pH values tested, α -crystallin prevented the aggregation of heat-stressed β -lactoglobulin and this effect increased with increasing pH. Incomplete suppression of protein aggregation and precipitation by α -crystallin implies that the aggregation of β -lactoglobulin occurs as a result of intermolecular disulphide bond polymerization. It has also been demonstrated that the chaperone action of α -crystallin increased with increasing pH (Brockwell. 2004). At lower pH values, the aggregation of β -lactoglobulin occurs more by hydrophobic (nucleation-dependent) mechanisms since disulphide bond cleavage is highly pH dependent (i.e. it is reduced at lower pH values) (Prabakaran et al. 1997, Otte et al. 2000). Thus, it was

proposed that even though α -crystallin's chaperone action is reduced at lower pH values, it should be able to prevent β -lactoglobulin aggregation better at lower rather than at higher pH values. In fact, in this study it was found that despite increasing hydrophobic aggregation of β -lactoglobulin at lower pH, α -crystallin was a better chaperone at higher pH. In agreement with this, SDS-PAGE results confirmed the better chaperone action of α -crystallin at higher pH. These results imply that the increase in polymerization of β -lactoglobulin at higher pH and the inability of α -crystallin to suppress this is offset by the enhanced chaperone ability of α -crystallin to suppress nucleation-dependent aggregation at higher pH.

Aggregation of β -lactoglobulin occurs via both hydrophobic interactions and thiol/disulphide exchange reactions (Hoffmann et al. 1997b, Croguennec et al. 2004). Increased aggregation of heated β -lactoglobulin in the presence of DTT showed that aggregation of β -lactoglobulin occurs both via intramolecular and intermolecular disulphide bond formation (hydrophobic interactions and a nucleation-dependent). Under these conditions β -lactoglobulin aggregates much faster and α -crystallin is not a good chaperone (as previously reported, Chapters 3 and 4) to prevent its aggregation.

At room temperature and physiological pH, β -lactoglobulin exists as a dimer in which the monomers are noncovalently linked but dissociates into monomers upon heating (Verheul et al. 1999). β -Lactoglobulin undergoes heat-induced structural changes which involve the exposure of buried hydrophobic groups and thiol groups (Ptitsyn et al. 1995, Croguennec et al. 2004). By contrast, Otte

et al. (2000) provided evidence that polymerization of β -lactoglobulin into a gel network does not occur via disulphide linkages. They explain that disulphide linkages occur initially during heating and the association of the soluble aggregates into a network occurs by non-covalent bonding which is a hydrophobic and/or hydrogen bond interaction. The aggregation of β -lactoglobulin can be described using a kinetic model (Roefs et al. 1994). In this kinetic model, the aggregation of β -lactoglobulin is described by analogy with a radical-addition polymerization and consists of three steps. In the initiation step, the β -lactoglobulin dimer splits into monomers, a process in which reversible aggregation is followed by an irreversible step, which is the real initiation reaction. This real initiation is a first-order reaction, and the conformation of β -lactoglobulin changes in such way that the free thiol group becomes reactive. This reactive thiol group reacts via thiol/disulphide exchange. The polymerization process stops when two reactive intermediates react with each other, forming a polymer without a reactive thiol group (Roefs et al. 1994). According to these data, at all pH values tested, aggregation of β -lactoglobulin occurs via both intermolecular exchange and hydrophobic interaction as showed in this study by the increasing aggregation of β -lactoglobulin in the presence of DTT. It appears that increasing chaperone action of α -crystallin at higher pH rather than the hydrophobic aggregation by β -lactoglobulin at lower pH is the predominant force for the chaperone action of α -crystallin. One explanation is that by increasing pH, the chaperone action of α -crystallin increases, which prevents both hydrophobic aggregation and non-covalent association of β -lactoglobulin into a gel network. Another possibility could be

that α -crystallin reverses the dimer-dissociation of heat-induced aggregation of β -lactoglobulin, which is a necessary step in the aggregation process of β -lactoglobulin (Cairolì et al. 1994).

6.6 Conclusion

This study reveals that upon heating, β -lactoglobulin aggregation increased with increasing pH. As shown by light scattering profiles, the presence of dextran or DTT led to more rapid aggregation and precipitation of β -lactoglobulin. Increasing aggregation of β -lactoglobulin occurred in the presence of DTT implying that the aggregation of β -lactoglobulin occurs via both intra and intermolecular disulphide bond formation. α -Crystallin prevented the aggregation of heat-stressed β -lactoglobulin and was a more efficient chaperone at higher pH values. In the presence of DTT, however, α -crystallin was a less efficient chaperone due to faster aggregation of heated and reduced β -lactoglobulin.

Chapter 7

Nuclear Magnetic Resonance (NMR) spectroscopic and chaperone studies of dogfish α -crystallin

Professor Augustan from university of Melbourne kindly donate a sample of dogfish α -crystallin therefore, this provided a good opportunity to investigate the structure of its C-terminal extension and compare it to bovine α -crystallin

Dogfish α -crystallin with a molecular mass of around 400,000-710,000 Da comprises approximately 17% of the lens cortex in the adult dogfish (de Jong et al. 1975, Bindels et al. 1983). Using gel filtration, one can separate α -crystallin from other water-soluble proteins of the dogfish lens (Leenen et al. 1981), which can be further separated to α A- and α B-crystallins by ion exchange chromatography in the presence of urea (de Jong et al. 1988). Dogfish α -crystallin consists of α A-, and α B-crystallins (in an approximately 1:3 ratio) which have three and one cysteine residues respectively (de Jong et al. 1988). This is the opposite of bovine α -crystallin in which the ratio of α A- and α B-crystallin is 3:1 with one and no cysteine residues respectively (Horwitz et al. 2000, Derham and Harding 2002, Lentze et al. 2003).

Total lens of dogfish α -crystallin contains three types of crystallins (α , β and γ), which is the same as the mammalian lens. The β -crystallin fraction has two fractions (β_H and β_L) with a subunit mass range of approximately 24,000-29,500 Da. The γ -crystallins are monomers and have a molecular mass under 20,000 Da. Analysis of adult and embryo dogfish nuclei by gel filtration and electrophoresis showed that they contain none and hardly any α -crystallin, respectively (Leenen et al. 1981). They have a high molecular weight β -crystallin subunit and some γ -crystallin subunits (Leenenn et al. 1981). In older embryos and in adult lens nuclei, γ -crystallins are the major component. In the

200

adult cortex, α - and β -crystallin (mostly β_H -crystallin) are the predominant proteins. γ -Crystallin is also present but to a lesser degree than in embryo and adult nuclei.

Spiny dogfish (*Squalus acanthias*), evolutionarily, is a very old animal (de Jong et al. 1993, Ringholm et al. 2003). Amino acid analysis of dogfish α -crystallin has confirmed the homology of its primary sequence to bovine α -crystallin (de Jong et al. 1976, de Jong et al. 1988). It is therefore probable that the homologous structural domains of bovine and dogfish α -crystallin have comparable biological properties and functions (de Jong et al. 1988). ^1H NMR spectroscopy of bovine α -crystallin (Carver et al. 1992, Carver et al. 1998) revealed polar, highly exposed and flexible short C-terminal extensions in both the αA - and αB -subunits. In this study, the structural similarity of dogfish and bovine α -crystallin in the C-terminal region were compared by acquisition of ^1H NMR spectra of dogfish α -crystallin and by comparing its chaperone ability with that of bovine α -crystallin. ^1H 2D TOCSY and NOESY NMR spectra were acquired and reduction and heating chaperone assays were performed.

7.1 ^1H 2D TOCSY and NOESY NMR spectroscopy of dogfish α -crystallin

Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were conducted on dogfish α -crystallin as described in Section 2.2.7. ^1H 1D NMR spectra of dogfish α -crystallin before and after 2D acquisition are shown in Figure 7.1 to monitor if any structural

changes occurred during acquisition of NMR spectra. It is apparent from the spectra that the conformation of the protein was stable for 24 hrs at 25°C in the NMR spectrometer the fact that there was no difference in them. Resonance intensities were measured at discrete frequencies from a baseline connecting the region from 0.0 ppm to 9.0 pm after phasing of the spectra. Spectral plots were scaled to the height of the resonance at 5.8 ppm.

Cross-peaks in 2D spectra arising from residues in the C-terminal extension of dogfish α -crystallin are shown in Figures 7.2A and B. The sequential assignment (Wuthrich. 1986) of the residues in the C-terminal extensions was made using through bond correlations via ^1H - ^1H WATERGATE TOCSY, and through space correlations via WET NOESY (States et al. 1982). Residues were assigned from the chemical shifts of NH, H α , H β cross peaks and then correlated with random coil α -CH chemical shift values for amino acids when followed by either alanine or proline residues accordingly (Wishart et al. 1995). The ^1H NMR chemical shifts for dogfish α -crystallin are listed in Table 7.1.

The proline residues only give cross peaks in the aliphatic region of the spectrum since they have no NH proton. The assignment of proline residues is shown in Figure 7.3 and listed in Table 7.1.

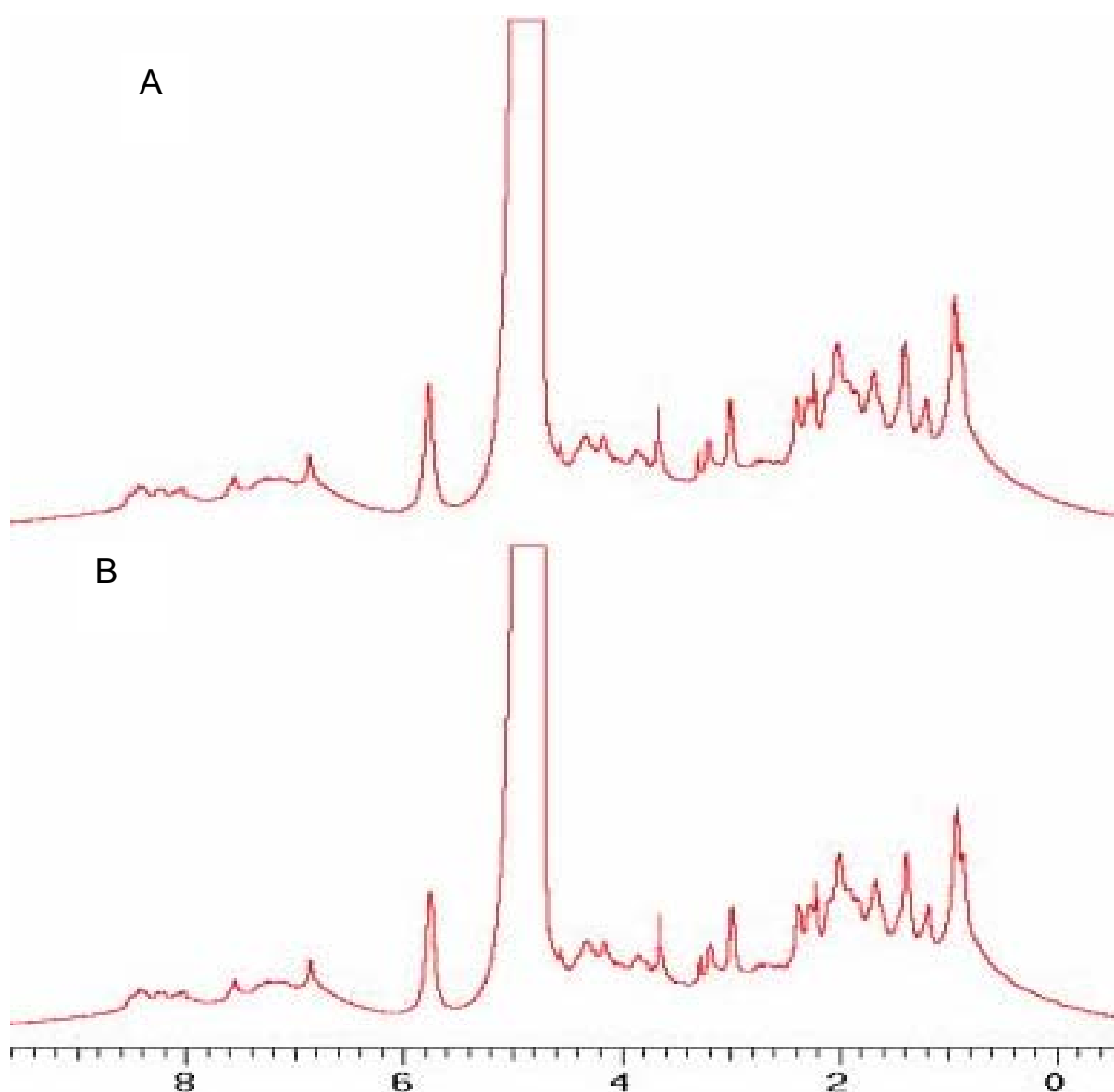
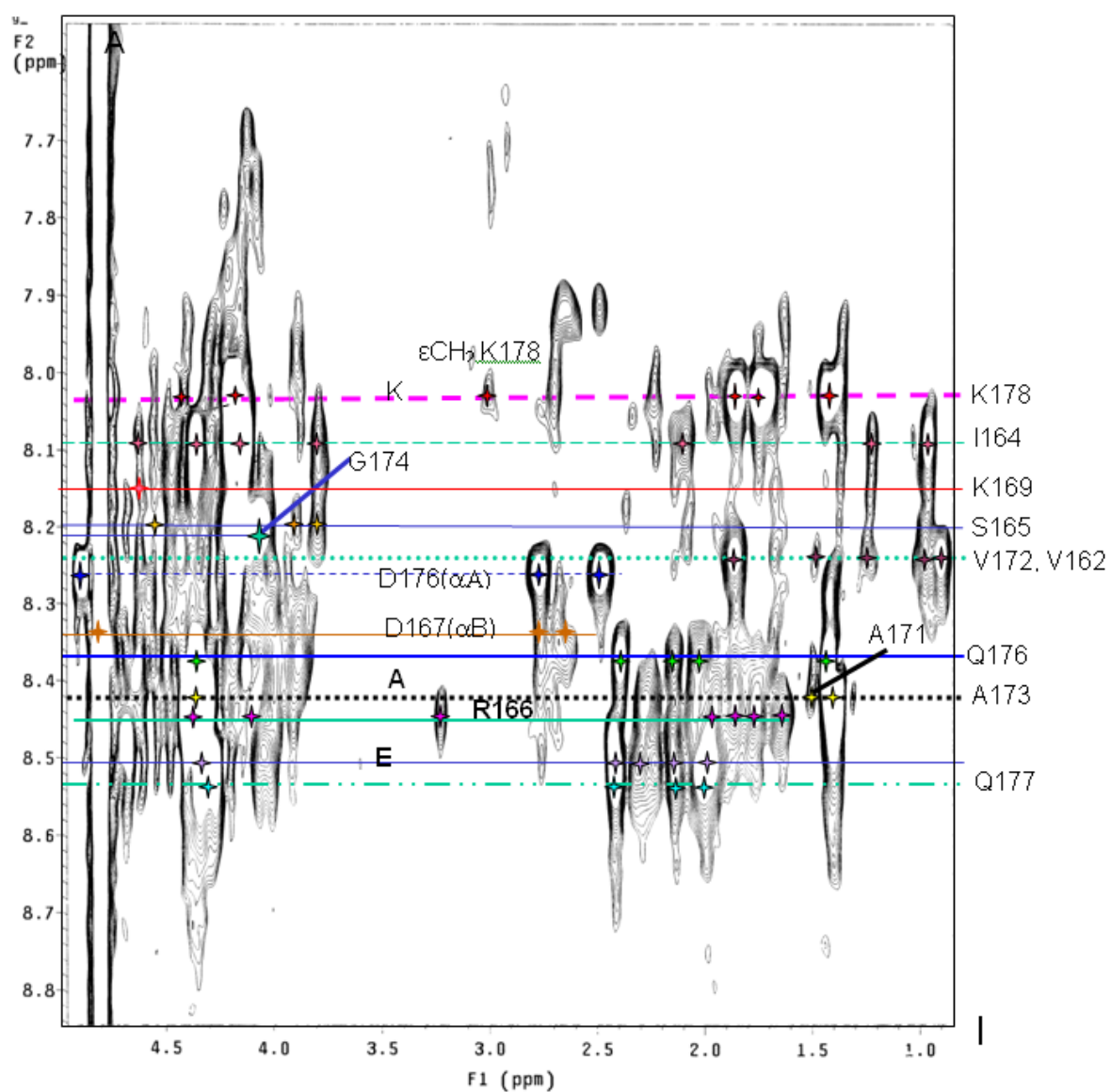


Figure 7.1: ^1H 1D NMR spectra of dogfish α -crystallin (A): zero time (B): after 24 hrs. The experiments were conducted in 100 mM phosphate buffer, 90% H_2O , 10% D_2O , and pH 6.5, at 25°C . Peak intensities were referenced to the resonance at 5.8 ppm.



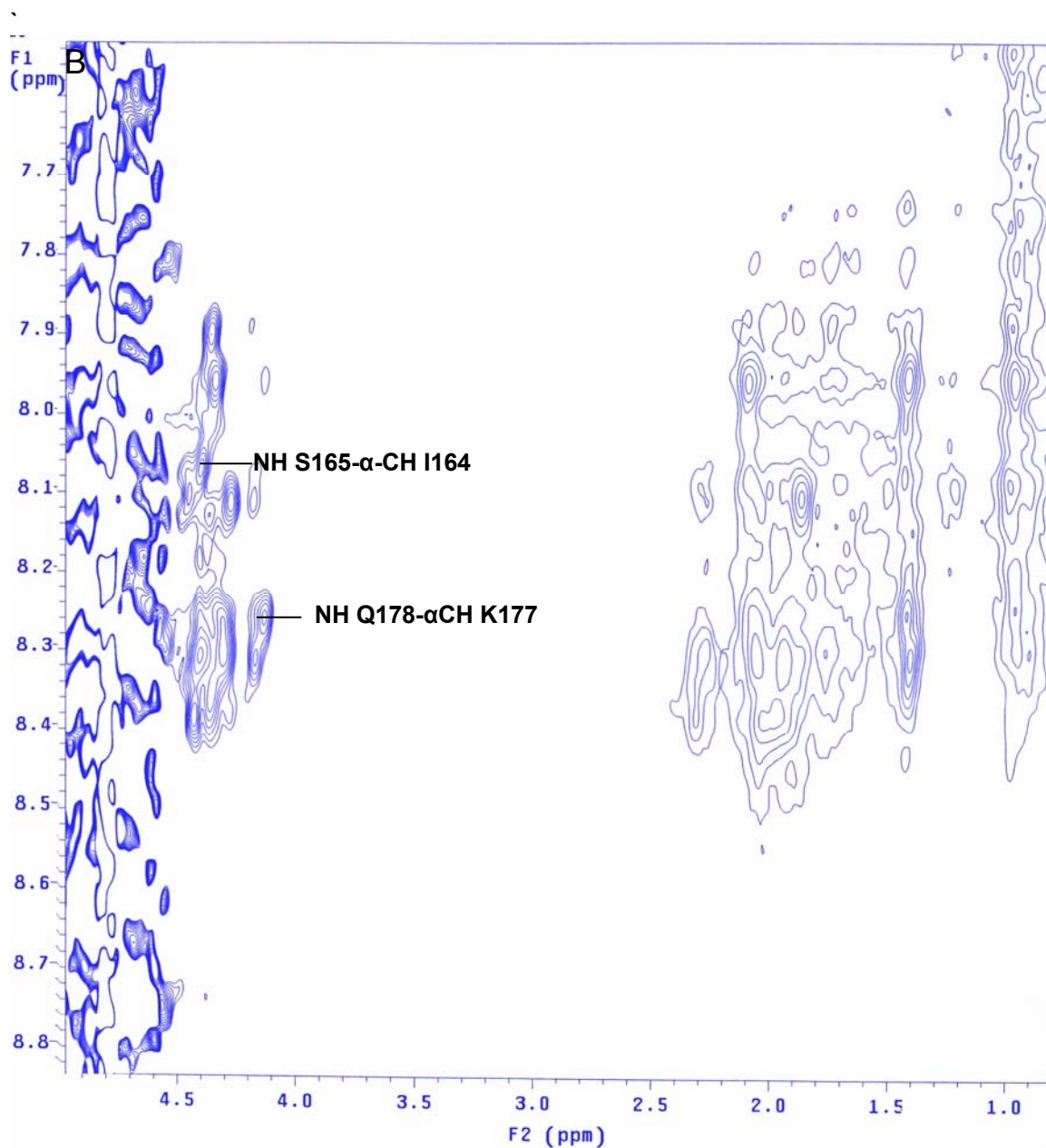


Figure 7.2: (A) TOCSY and (B) WET NOESY spectra of dogfish α -crystallin. Residues were assigned from the NH to α , β and γ cross peaks in TOCSY and NH to α -CH region in NOESY spectra.

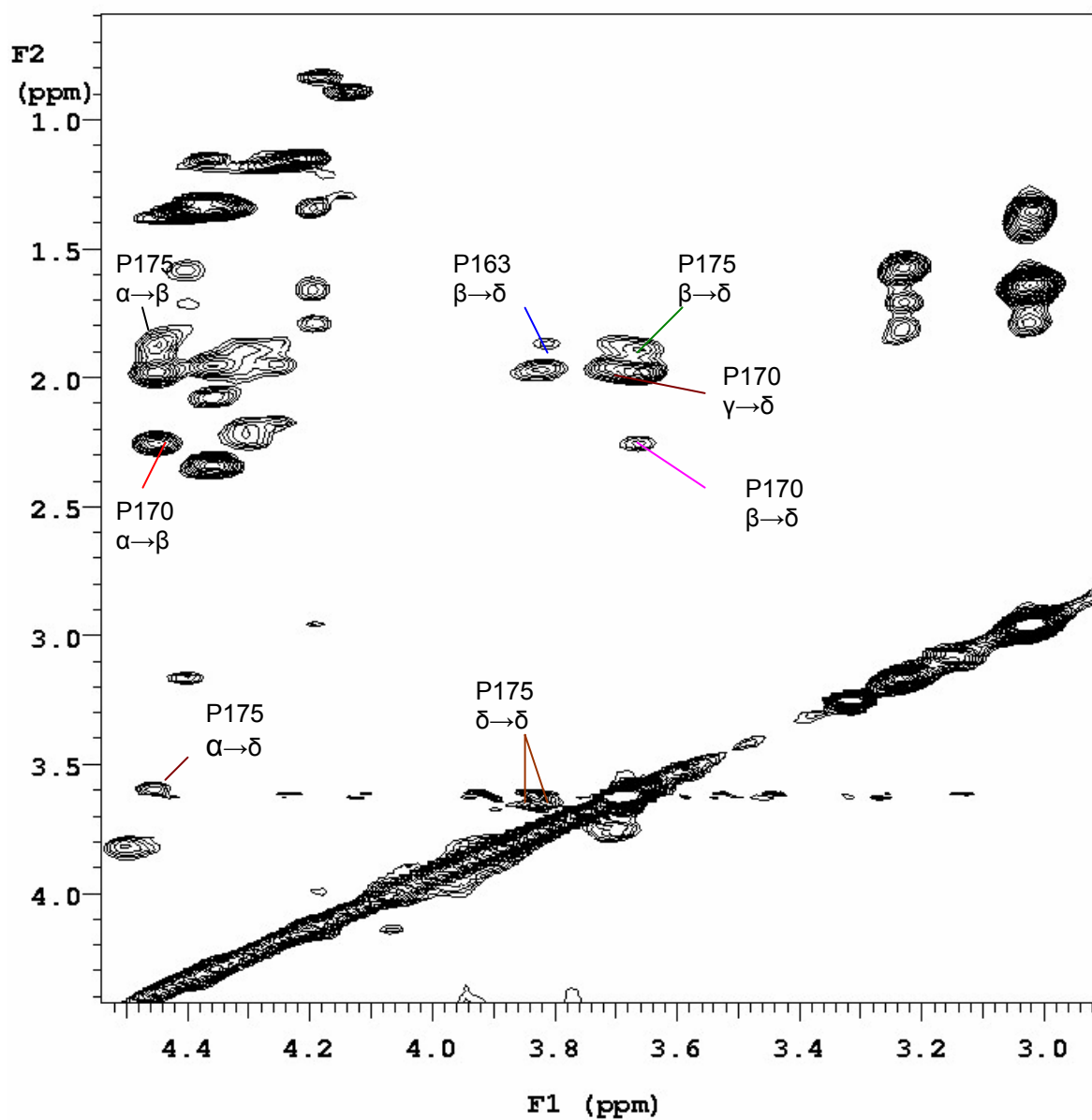


Figure 7.3: A portion of the aliphatic region of a TOCSY spectrum of dogfish α -crystallin showing the proline residue assignments.

Table 7.1: ^1H chemical shifts of C-terminal residues of dogfish αB -crystallin in 100 mM phosphate buffer, pH 6.5 at 25°C. Residues marked with an asterisk are those followed by proline.

Residue	NH	H$^\alpha$	H$^\beta$	Others
V162*	8.22	4.45	1.86	γCH_3 0.91
P163	-	4.40	1.88	
I164	8.08	4.20	2.10	γCH_2 1.04, 1.30, γCH_3 0.96
S165	8.19	4.53	3.80, 3.90	
R166	8.46	4.40	1.96, 2.04	γCH_2 3.23, 3.23, ϵNH 7.3
D167	8.34	4.64	2.68, 2.76	
E168	8.5	4.34	2.02, 1.97	γCH_2 2.30, 2.30
K169*	8.15	4.54	1.70	
P170	-	4.45	2.28	
A171	8.41	4.30	1.51	
V172	8.23	4.18	1.86	γCH_3 0.96
A173	8.42	4.32	1.41	
G174*	8.19	4.05		
P175	-	4.46	1.92	γCH_2 2.20, 1.98, δCH_3 3.64, 3.66
Q176	8.34	4.39	2.14, 2.03	γCH_2 2.43
Q177	8.52	4.32	1.99, 2.02	γCH_2 2.43
K178	8.02	4.30	1.75, 1.87	γCH_2 1.44, 1.44, ϵCH_2 3.02

A comparison was made between the α -CH chemical shifts determined for resonances in the spectrum and those given by Wishart et al. (1991) in order to determine the relative flexibility of the C-terminal extension (Table 2, Figure 7.4).

Table 7.2: Chemical shifts for α -CH resonances of dogfish α B-crystallin in 100 mM phosphate buffer, pH 6.5, at 25°C compared to random coil values of Wishart et al. (1995).

Residue	H$^{\alpha}$ shift determined (ppm)	H$^{\alpha}$ shift given by Wishart et al. (ppm)
V162*	4.45	4.44
P163	4.40	4.42
I164	4.20	4.17
S165	4.53	4.47
R166	4.40	4.34
D167	4.64	4.64
E168	4.34	4.35
K169*	4.54	4.60
P170	4.45	4.42
A171	4.30	4.32
V172	4.18	4.12
A173	4.32	4.32
G174*	4.05	4.13
P175	4.46	4.42
Q176	4.39	4.34
Q177	4.32	4.34
K178	4.30	4.32

The referenced random coil values have ^1H chemical shifts within ± 0.1 of those for dogfish αB -crystallin, which indicates that they originate from a flexible and unstructured region in the protein (Wishart et al. 1991) (Figure 7.4).

Figure 7.4: Deviation from random coil chemical shifts (Wishart et al. 1995) for the ^1H α -CH resonances of the C-terminal domain of dogfish α -crystallin.

From Figures 7.2, 7.3, and 7.4 and Tables 7.1 and 7.2, it is apparent that the last 17 amino acids of the B subunit (V162-K178) have great conformational flexibility and are highly exposed to solvent and adopt little ordered conformation. Resonances from the majority of the amino acids of the A subunit were not observed due to its low concentration relative to the B subunit (Leenen et al. 1981)

7.2 Chaperone assay of dogfish α -crystallin

The chaperone action of bovine α -crystallin in preventing the aggregation of a variety of target proteins has been demonstrated by many workers (e.g. Horwitz et al. 1992, Lindner et al. 1998, Carver et al. 2002). The chaperone activity of dogfish α -crystallin was compared with bovine α -crystallin as described in Sections 3.2.2., 3.2.4, 3.2.5. and 3.2.7. In order to compare chaperone action of dogfish and bovine α -crystallin in preventing aggregation of proteins, reduction and heating assays were undertaken. The unfolding, aggregation and eventual precipitation of insulin and β_L -crystallin were induced by the reduction of the disulfide bonds using DTT and heating, respectively, as described in Methods (Sections 2.2.2.1 and 2.2.2.2).

7.2.1 Reduction assay of dogfish α -crystallin

To study the chaperone action of dogfish α -crystallin to prevent DTT induced aggregation of a target protein, insulin was used as previously described (Section 2.2.2.1). A 0.25:1.0 molar ratio of dogfish α -crystallin:insulin, at the time point of 180 min, completely suppressed aggregation of insulin, as shown in Figure 7.5. By comparison bovine α -crystallin under the same experimental conditions (Section 3.2.4, Figure 3.5A) only suppressed 41% of the aggregation of reduced insulin (at a 1.0:0.2 molar ratio of insulin: α -crystallin). Thus in this assay, dogfish α -crystallin is a much more efficient chaperone in preventing the aggregation of reduced insulin.

In addition, an experiment showing the concentration-dependence of the chaperone action of dogfish α -crystallin was performed with lower concentrations of dogfish α -crystallin (1:0.075 and 1:0.125 molar ratios of insulin: α -crystallin) (Figure 7.5). At 1:0.075 and 1:0.125 molar ratios of insulin:dogfish α -crystallin and at time point of 150 min dogfish α -crystallin suppressed aggregation of reduced insulin by 79% and 89%, respectively, compared to the control in the absence of dogfish α -crystallin.

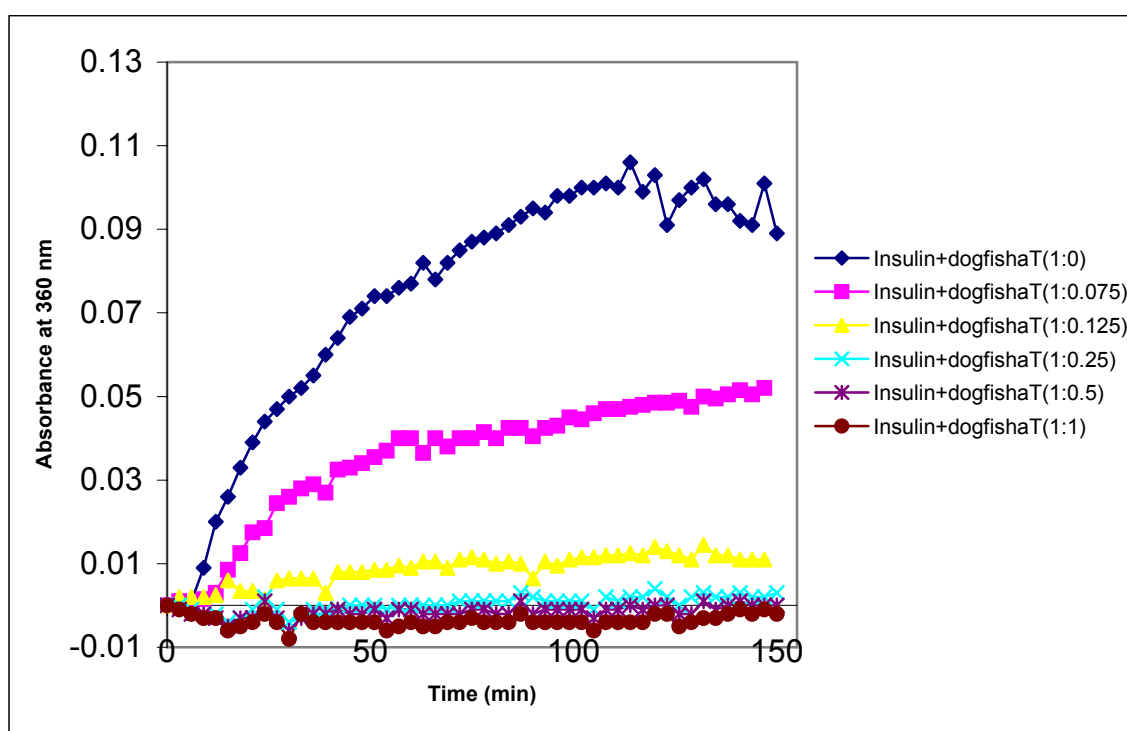


Figure 7.5: Insulin (0.42 mg/mL) aggregation in the presence and absence of dogfish α -crystallin (α T). The protein was incubated in 50 mM sodium phosphate buffer, 0.03% (w/v) NaN_3 , pH 7.2, at 37°C. Aggregation of insulin was initiated by the addition of 20 mM DTT. The molar ratios of insulin:dogfish α -crystallin are indicated. The experiment was done in duplicate.

7.2.2 Heat stress assay of β_L -crystallin in the presence and absence of dogfish α -crystallin

The chaperone action of bovine α -crystallin to suppress heat-induced aggregation of proteins has been well described (Horwitz et al. 1997, Section 3.2.7). To determine chaperone activity of dogfish α -crystallin to prevent heat induced aggregation of the target protein, β_L -crystallin was used as previously described (Section 2.2.2.2.). A 1.0:0.25 w:w ratio of dogfish α -crystallin: β_L -crystallin suppressed aggregation of heated β_L -crystallin by 97% as shown in Figure 7.6. This is similar to bovine α -crystallin where, under the same experimental conditions (Section 3.2.7, Figure 3.9A), a 1.0:0.25 w:w ratio of β_L -crystallin to α -crystallin completely suppressed aggregation of β_L -crystallin. This indicates that at high temperatures (60°C), the chaperone efficiency of bovine α -crystallin and dogfish α -crystallin against the heat-induced aggregation of β_L -crystallin is comparable.

In addition, an experiment showing concentration-dependence of the chaperone action of dogfish α -crystallin was performed with lower concentrations of α -crystallin (1.0:0.05 and 1.0:1.0 w:w ratio of β_L -crystallin: α -crystallin). At a 1.0:0.1 w:w ratio of β_L -crystallin:dogfish α -crystallin and at a time point of 80 minutes, dogfish α -crystallin suppressed the aggregation of heated β_L -crystallin by 77.5% compared to a control of β_L -crystallin in the absence of α -crystallin.

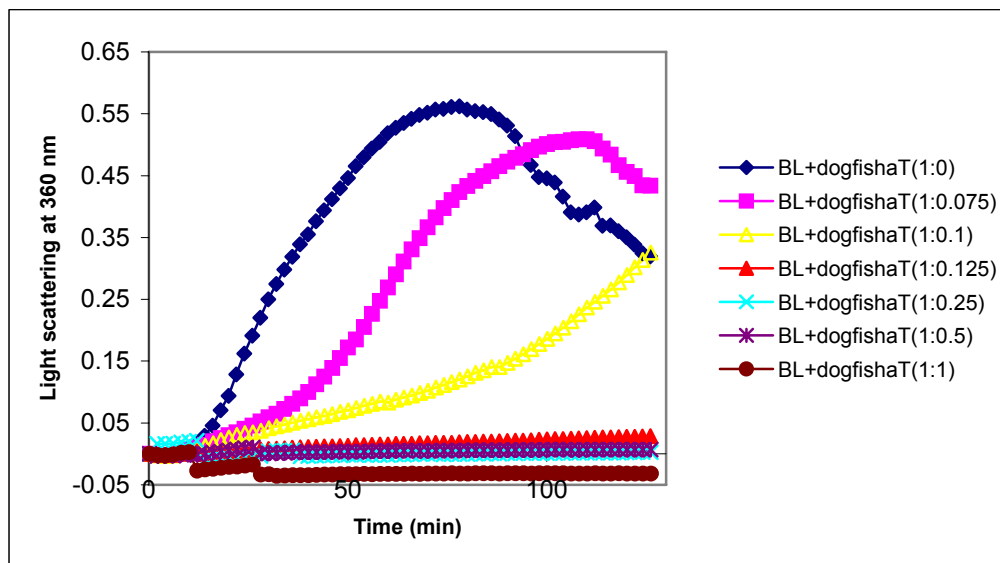


Figure 7.6: β_L -crystallin (0.3 mg/mL) aggregation in the presence and absence of different concentrations of dogfish α -crystallin (αT). The protein was incubated in 50 mM sodium phosphate buffer, 0.03% (w/v) NaN_3 , pH 7.2 at 60°C. Heating induced the aggregation of β_L -crystallin. The w:w ratios of β_L -crystallin:dogfish α -crystallin are indicated. The experiment was done in duplicate.

7.3 Discussion

The spiny dogfish is a very old fish in evolutionary terms which arose approximately 420-430 million years ago (Ringholm et al. 2003). In the phylogenetic tree of small heat-shock proteins, dogfish α A- and α B-crystallin diverged much earlier than the mammalian proteins, e.g. human and cow (de Jong et al. 1993). Thus, would α -crystallins impose additional evolutionary conservation in their structure and function? Puri et al. (1983) observed that α -crystallins from many vertebrates, e.g. cow, horse, rabbit, rat and spiny dogfish are recognized by antibodies raised against bovine α -crystallin. They concluded that the regions of the α -crystallin protein responsible for antibody binding have been conserved and did not change during evolution. Leunissen et al. (1990) also proposed that eye lens crystallin avoided extreme changes in their charge during evolution. This could be useful in the lens environment, where distribution of charged amino acid residues could affect the correct packing of the crystallins (de Jong et al. 1993). In this study, further investigation using ^1H NMR spectroscopy and chaperone assays were done to explore further the effect of evolution on the structure and function of α -crystallin.

A ^1H NMR study of bovine α -crystallin (Carver et al. 1998) showed that the last nine amino acids of α A- and the last 11 amino acid of the α B subunits have significant flexibility and lack of structure and thereby constitutes a C-terminal extension. Figure 7.6 shows the aligned amino acid sequences of the C-terminal region of the two dogfish and bovine dogfish α -crystallin subunits (de Jong et al. 1988, Carver et al. 1998). As with bovine α A- and α B-crystallin,

there is little sequence similarity between the flexible C-terminal regions of α A and α B in dogfish α -crystallin. However, comparing dogfish α A and α B sequences with their respective bovine α A and α B sequences, there is 67% sequence similarity in both cases (de Jong et al. 1988). This implies that the C-terminal extension of bovine and dogfish α -crystallin have a similar structure and function.

Figure 7.6: Amino acid sequences in the C-terminal region of the A and B subunits of dogfish and bovine α -crystallin (Carver et al. 1998, de Jong et al. 1998). The NMR-visible flexible C-terminal extensions are underlined. Resonances from the flexible C-terminal extension of dogfish α A-crystallin were difficult to observe in the NMR spectrum due to its low concentration relative to the α B subunits.

The α -CH ^1H chemical shifts for dogfish α -crystallin were very similar to random coil values of Wishart et al. (1991) (Figure 7.4). This is consistent with a highly flexible region of the polypeptide chain that adopts no defined structure flexible (Wishart et al. 1991). Similar behaviour is observed for α -CH ^1H chemical shift in bovine α B-crystallin (Wishart et al. 1991).

The chaperone activity of dogfish α -crystallin was assessed under both heat and reduction stress of target proteins and compared to that of bovine α -

crystallin. It was found that dogfish α -crystallin was a comparable chaperone to bovine α -crystallin in a heating assay with β_L -crystallin as the target protein but was a significantly better chaperone towards reduced insulin as a target protein. The greater chaperone activity of dogfish α -crystallin at 37°C with reduced insulin compared to bovine α -crystallin could be due to longer regions of flexibility in the C-terminal extension in dogfish α -crystallin. In dogfish α -crystallin, the last 17 amino acids of the predominant B subunits have significant flexibility and are highly exposed to solvent, which is longer than the extension in bovine αA - and αB -crystallin.

7.4 Conclusion

In this preliminary study, NMR and visible absorbance spectroscopy investigate of dogfish α -crystallin it has been proved that bovine and dogfish α -crystallin share very similar structural and functional properties. Thus, they share the common feature of having a very mobile and unstructured C-terminal extension, a region that is important in the chaperone action of all mammalian sHsp (Muchowski et al. 1997, Muchowski et al. 1999, Derham et al. 2001, Bhattacharyya et al. 2002, Pasta et al. 2002). Furthermore, bovine and dogfish α -crystallin had a comparable chaperone action in a heating assay but were slightly different in a reduction assay. These studies imply that the structure and function of α -crystallin have not changed significantly during evolution. Thus, the chaperone ability of α -crystallin has been around for a very long time suggesting that the protein has had a role as a molecular chaperone for this period.

Appendix

Buffers

S-300 column buffer (pH 7.5)

Tris-HCl	50 mM
Tris base	50 mM
EDTA	1mM
NaN ₃	0.05% (w/v)

Sephadex G-75 column buffer (pH 2.5)

Glycine	0.1 M
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Sephadex G-25 column buffer (pH 7.5)

DTT	2 mM
NaH ₂ PO ₄ · 2H ₂ O	50 mM

NMR sample buffer

NaH ₂ PO ₄ · 2H ₂ O	50 mM
NaCl	100 mM
MilliQH ₂ O	100%(v/v)
NaN ₃	0.05% (w/v)

For reduced α-lactalbumin, NMR samples buffer also contained:

NaCl	100 mM
EDTA	1mM

For dogfish α-crystallin, NMR samples contained:

NaH ₂ PO ₄ · 2H ₂ O	10 mM
NaN ₃	0.03% (w/v)

SDS-Page reagents

SDS-Page running buffer (pH 8.3)

Tris base	15 g/L
Glycine	72 g/L
SDS	5 g/L
Sample cracking buffer	
Tris-HCl (pH 6.8)	60 mM
SDS	1% (w/v)
β-mercaptoethanol	1% (w/v)
Glycerol	10% (v/v)
Bromophenol blue	0.01% (w/v)
Rapid Coomassie blue staine	
Coomassie blue	R-250 1g
Methanol	200 mL
Glacial acetic acid	50 mL
Distilled H ₂ O	500 mL
Rapid destain	
Methanol	400 mL
Glacial acetic acid	100 mL
Distilled H ₂ O	1 L
Final destaine	
Glacial acetic acid	100 mL
Glycerol	40 mL
Distilled H ₂ O	1L

Subunit exchange experiment buffer

NaCl	100 mM
MOPS	20 mM
NaH ₂ PO ₄ · 2H ₂ O	50 mM
DTT	2 mM
HPLC buffer	
NaH ₂ PO ₄ · 2H ₂ O	50 mM
NaN ₃	0.05% (w/v)
EDTA	1 mM
CD buffer	
NaH ₂ PO ₄ · 2H ₂ O	10 mM
Thioflavin T buffer	
NaH ₂ PO ₄ · 2H ₂ O	50 mM
ThT	0.4 μM
NaN ₃	0.04% (w/v)
Glycine	50 mM

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