Influence of Thermal Perturbation of the Oligomeric Size of ?-Crystallin on its Chaperone Function – A Biophysical Study

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By

Biswajit Laha

Under the Guidance of

Dr. Ashis Biswas

DEPARTMENT OF CHEMISTRY
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA – 769008, ORISSA

CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis/ dissertation entitled "**Influence of Thermal Perturbation of the Oligomeric Size of ?-Crystallin on its Chaperone Function – A Biophysical Study**" submitted by **Sri / Smt. Biswajit Laha**, M.Sc. 2nd year student (Roll No. **408CY113**) of National

institute of technology Rourkela, Orissa, India for the award of **Master of Science degree** of **National Institute of technology Rourkela**, is absolutely based upon his/her own work under the supervision of Dr. Ashis Biswas, Assistant Professor, Department of Chemistry, School of Basic Sciences, Indian Institute of technology Bhubaneswar (formerly Assistant Professor of Department of Chemistry, NIT Rourkela) and that neither this thesis nor any part of its has been submitted for any degree/diploma of any other academic award anywhere before.

Dated:
IIT Bhubaneswar
Bhubaneswar

(Dr. Ashis Biswas)
(Signature of the supervisor(s) &
date with official seal)

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Rourkela
Date: (Biswajit Laha)

Contents

Chapter 1

A Brief Review of Origin, Structure, and Molecular Chaperone Function of (-Crystallin and Scope and Objective of the Thesis

1.1 Introduction

The main constituents of the mammalian eye lens fiber cells are crystallin proteins namely

(-, (- and (-crystallin (Bloemendal, 1981). Among all the crystallins, (-crystallin is the major protein and responsible for maintaining the transparency and refractive properties of the eye lens (Groenen et al., 1994; Hoenders and Bloemendal, 1981; Horwitz, 1993). The term "crystallin" means the structural proteins. In birds and reptiles, there is another major lens protein called (-crystallin (Piatigorsky, 1984; Rabaey, 1962). There are many other crystallins which are known as taxon-specific crystallins (Bloemendal and de Jong, 1991; Piatigorsky, 1992; Wistow and Piatigorsky, 1988). There are two (-crystallin genes, giving rise to (A- and (B-crystallin. In the mammalian lens, the molar ratio of (A to (B is about 3:1 (Bloemendal, 1981). In humans, (A-crystallin has 173 and (B-crystallin has 175 amino acid residues. There is about 57% amino acid sequence identity between (A- and (B-crystallin (Bloemendal and de Jong, 1991).

Although it was believed for many years that (-crystallin was strictly lens-specific protein, both (A- and (B-crystallin have now been found in many non-lenticular tissues (Iwaki et al., 1989; Lowe et al., 1992; Sax and Piatigorsky, 1994; Srinivasan et al., 1992). (A- and (B-Crystallin have been linked to various neurological diseases such as Alexander's disease (Iwaki et al., 1989), Creutzfeldt-Jacob disease (Iwaki et al., 1992; Renkawek et al., 1992), Alzheimer's disease (Lowe et al., 1992), Parkinson's disease (Iwaki et al., 1992), Lewy body disease (Lowe et al., 1990). Furthermore, (-crystallin is a key member of the small heat shock protein (sHSPs) family and its structural and functional similarities are conserved from bacteria to humans (Ingolia and Craig, 1982; Klemenz et al., 1991; MacRae, 2000; Merck et al., 1993; Narberhaus, 2002). (B-Crystallin is known to be inducible by various stress conditions such as heat shock or oxidative stress (Klemenz et al., 1991; Dasgupta et al., 1992) and (A- and (B-crystallin are able to confer cellular thermoresistance (Aoyama et al., 1993; van den Ijssel et al., 1994). Finally, (-crystallin like other sHSPs has been found to act as molecular chaperone *in vitro* by preventing the aggregation of other proteins under thermal stress or other stress conditions (Das and Surewicz, 1995 b; Farahbakhsh et al., 1995; Horwitz, 1992; Horwitz, 1993; Jacob et al., 1993; Raman and Rao, 1994; Wang and Spector, 1994). Although the chaperone activity of (-crystallin *in vivo* is well known (Horwitz, 1992), the molecular mechanisms of the chaperone function of (-crystallin still remains unknown.

(-crystallin is present in the lens in a very concentrated form. In the center of a human lens the concentration of protein is about 450 mg ml^{-1} (Fagerholm et al., 1981). The crystallins generally form 90% or more of the total protein mass in which the contribution of (-crystallin is only 45%. The other crystallins such as (-crystallin contributes 20% and (-crystallin 35%. A unique property of the lens is the lack of protein turnover in the differentiated fiber cells. The lens does not have any mechanism to dispose the damaged proteins, whereas the other tissues in the body, most of the enzymes in the liver, for example, turnover in minutes, hours or days. The lens fiber cells and their proteins must survive for life. The mechanism by which the protein mass remains transparent over such a long period has thus become a subject of intense investigations.

1.2 Origin of ?-crystallin

The lens is a remarkably specified product of evolution. Crystallins have been recruited as lens protein simply because of its stable structure (Wistow and Piatigorsky, 1987). In vertebrates,

the most common crystallins are ?, ? and ? families. Recent years many other crystallins have been identified in various species, those are known as taxon specific crystallins (Horwitz, 1993) such as ?, ?, ? etc.

Crystallins seem to have originated by gene duplication and divergence specializing for lens environment. The major taxon specific crystallins of vertebrates and invertebrates are enzymes or closely related to enzymes (Wistow and Piatigorsky, 1987). For instance, duck ?-crystallin is identical to lactate dehydrogenase (Wistow *et al.*, 1987), ?-crystallin is closely related to argininosuccinate lyase (urea cycle enzyme), ?-crystallin is closely related to ?-enolase (Wistow and Piatigorsky, 1987). The dual function of these proteins that can serve as structural protein or enzymes led to the concept of "gene sharing". It implies that the same protein can have more than one function (Horwitz, 1993). Here natural selection occurs for further use of stable molecules that are expressed for various cellular functions in new structural notes in a specified tissue.

Ingolia and Craig (1982) showed that mammalian ?-crystallin is related to a family of sHSPs in *Drosophila* which reflects a common evolutionary ancestry. ?-crystallin belongs to the small heat shock protein superfamily, sHSPs. The amino acid sequence of ?-crystallin was found to be similar to that of sHSPs over ~ 40% of their lengths. The same conserved region has also been observed in p40 egg antigen of *S.mansoni*, several mycobacterial surface proteins and numerous plant proteins. This indicates that they are originated by gene duplication from a common ancestral gene.

The phylogenetic tree (de Jong *et al.*, 1998) based on the "?-crystallin domains" of ?-crystallin-small heat-shock protein (?-HSP) superfamily indicates that animal ?-HSPs might derive from one of the multiple types of ?-HSP genes that are already present at the time of prokaryote-eukaryote divergence (Fig. 1.1). Alignment of certain prokaryote ?-HSPs (e.g. Bradyhizobium, Escherichia, Buchnera, Legionella) show specific resemblances with animal ?-HSPs. Descendents of this particular gene should of course have been present in the last common ancestor of animals, plants and fungi.

1.3 Structure of (-Crystallin

The exact secondary and tertiary structure of ?-crystallin is not known due to non availability of crystallographic or other high resolution data. However, circular dichroism spectroscopy (CD) studies indicated a mostly ?-sheet structure with little or no ?-helical content (Li and Spector, 1974; Horwitz, 1976; Liang and Chakrabarti, 1982; Siezen and Argos, 1983). More recent CD studies suggest that ?-crystallin possesses ~ 50% ?-sheet and ?-turns and 10-15% ?-helical structure (Horwitz *et al.*, 1998; Koretz *et al.*, 1998). Similar results were also obtained by Fourier-transform infrared spectroscopy (Lamba *et al.* 1993). Reddy *et al.* (2000) showed that the secondary structure of ?A-crystallin is slightly different from that of ?B-crystallin. ?A-Crystallin was found to have 43% ?-sheet and 18% ?-helix compared to 48% ?-sheet and 12% ?-helix for ?B-crystallin. Recently Mornon *et al.* (1998) suggested that the conserved C-terminal ?-crystallin domain of all the small heat shock proteins mainly consists of ?-sheet. With the help of molecular modelling, they also showed that the ?-strands in ?-crystallin and other small heat shock proteins adopt 1g fold symmetry. Site-directed spin labeling studies of ?-crystallin and Hsp 27 also

supports this view (Koteiche and Mchaourab, 1999).

By probing the environments of various amino acids or the location of hydrophobic sites, Stevens and Augusteyn (1997) gave the information about the tertiary structure of ?-crystallin. The tertiary structure of lens crystallins, studied by absorption, fluorescence and near UV-CD spectroscopy (Liang et al, 1985a, b; Liang and Rossi, 1989; Messmer and Chakraborti, 1998). The tertiary structure of ?-crystallin changes with age. These studies also showed that ?-crystallin has a buried tryptophan residue, with exposed hydrophobic sites. Some other studies revealed that the N-ternimal domain is hydrophobic in nature, whereas the C-terminal domain is hydrophilic (Carver *et al.* 1993; Carver and Lindner, 1998).

The quaternary structure of ?-crystallin has been a subject of intense debate for many years. ?-Crystallin generally exists as a heterogeneous multimeric assembly with a molecular weight distribution varying between 300 kDa to ~1000 kDa. The molecular weight of each subunit is 20 kDa. The oligomeric structure of individual small heat shock protein super family is different from each other. The oligomeric structure of ?-crystallin, Hsp 25, Hsp 27 is heterogeneous in nature. On the other hand, the oligomeric structure of Hsp 16.5, Hsp 16.9 is homogeneous in nature (Kim *et al.*, 1998; van Montfort *et al.* 2001). Several studies revealed that the molecular weight of ?-crystallin depends on concentration, pH, temperature, ionic strength etc. Due to poly disperse nature of ?-crystallin; its crystal structure is not available. Due to this reason, various hypothetical models have been proposed for the quaternary structure of ?-crystallin (Groenen *et al.*, 1994; Harding, 1997). Other models propose a “protein-micelle” like structure [Fig. 1.2A] (Augusteyn and Koretz, 1987; Walsh *et al.*, 1991), a rigid cubic and rhombic dodecahedron model [Fig.1.2B] (Wistow, 1993), a GroEL double toroid structure [Fig. 1.2C] (Carver *et al.*, 1994b), a micellar model with elongated subunits (Groth-Vasseli *et al.*, 1995), and a “pitted-flexible” model [Fig. 1.2D] (Smulders *et al.*, 1998). Cryo-electron microscopic studies revealed that the recombinant ?B-crystallin has an asymmetric variable quaternary structure (Haley *et al.*, 1998). The variable quaternary structure of ?B-crystallin is consistent with polydisperse size of ?-crystallin assembles. Other studies show that the quaternary structure of ?-crystallin is dynamic nature in solution. For instance, NMR studies by Carver and Linder (1998) showed that C-terminal of ?-crystallin is flexible. Van den Oetelaar *et al.* (1990) first observed that there is an intermolecular exchange of the ?-crystallin subunits between assemblies. Using fluorescence resonance energy transfer (FRET), Bova *et al.* (1997) also reexamined this point and found that subunit exchange depends on temperature, pH and ionic strength.

1.4 Stability of (-Crystallin

The stability of proteins is related to covalent or conformational changes and these changes occur due to denaturation by heat or chemical reagents. (-Crystallin is readily denatured by heat and denaturant like urea or Guanidine hydrochloride (Gu-HCl).

1.4.1 Stability against thermal stress

(-Crystallin is known to have a very high thermal stability (Maiti et al., 1988; Walsh et al.,

1991). Even upto 100^{0}C, (A-crystallin remains soluble, although (B-crystallin is precipitated out around 65(C (Liang et al., 2000; Sun and Liang, 1998; van Boekel et al., 1999). (-Crystallin undergoes a conformational transition between approximately 38 and 50(C indicating an increase in exposure of hydrophobic sites. This tertiary structural change is irreversible (Das and Surewicz, 1995b). Infrared spectroscopy showed a thermal transition between 60 and 62(C together with a massive loss of native secondary structure (Surewicz and Olesen, 1995). Both infrared and circular dichroism spectroscopy demonstrated the thermal transition of (-crystallin around 61(C (Das and Liang, 1997). Even though there was a destruction in secondary structure with increase in temperature, secondary structure fully recovered after cooling to 25(C indicating that high thermal stability and reversibility of (-crystallin (Lin et al., 1998). The recovery of secondary structure but irreversibility of tertiary structure of (-crystallin was shown by several workers (Clauwaert, 2000; Das et al., 1997; Lee et al., 1997c; Maiti et al., 1988). The secondary structure of (B-crystallin is more stable than that of (A-crystallin (Clauwaert, 2000). There occurs also an irreversible temperature-induced quaternary structural change of (-crystallin (Das et al., 1997; Raman and Rao, 1994; Surewicz and Olesen, 1995). From the functional point of view, for maintaining the transparency of the eye lens, high stability of (-crystallin is necessary in absence of protein turnover. A detailed understanding of the stability of (-crystallin is required to better understand the cataract problem.

1.4.2 Stability against different kinds of denaturant

Quaternary, tertiary and secondary structures are all broken down at high concentration of denaturant like urea (non-ionic) and guanidine hydrochloride (ionic). The final state, possibly a random coil or fully extended polypeptide is often called an unfolded state. Experimental measurements of stability by various techniques often reflect different things. For example, fluorescence emission spectra revealed that 1 M Gu-HCl was required for 50% unfolding of bovine (-crytallin (Das and Liang, 1997; santini et al., 1992). On the other hand, far-UV CD measurements indicated that (-crystallin was 50% unfolded at approximately 1.5 M Gu-HCl (Das and Liang, 1997) or 2.6 M Gu-HCl (santini et al., 1992). This is due to the fact that fluorescence emission spectra only reflect the unfolding of N-terminal region, since (A-crystallin contains only a single Trp (at position 9) and (B-crystallin contains two (at positions 9 and 60). In case of denaturation with urea, fluorescence spectroscopy showed 50% unfolding at about 2.9 M urea (santini et al., 1992; van den Oetelaar and Hoenders, 1989) whereas it was 3.2 M in case of far-UV CD spectroscopy (santini et al., 1992). The stabilities of (A- and (B-crystallin were quite different. In case of bovine (A-crystallin about 3 M urea required for 50% unfolding, while (B-crystallin was 50% unfolded at about 1 M urea (van den Oetelaar and Hoenders, 1989). In Gu-HCl, the (G for human (A-crystallin was reported to be around 27 KJ/mole and 21 KJ/mole for (B-crystallin (Sun et al., 1999). Abgar et al. (2001) reported the high stability for (A-crystallin at the level of tertiary and quaternary sructure, although (B-crystallin is somewhat more stable than (A-crystallin at the level of secondary structure (Clauwaert, 2000). From these data, it is clear that, (A-crystallin is more stable than (B-crystallin and (B-crystallin is stabilized when it is complexed with (A-crystallin in the (-crystallin assembly (Sun and Liang, 1998). The rapid refolding from 8 M urea showed that the refolded (-crystallin has native-like secondary, tertiary and quaternary structure (Raman et al., 1995 b). Although the structural change of (-crystallin against denaturant has been studied extensively, the role of this structural stability on the chaperone activity of (-crystallin still remains unknown.

1.5 Function of ?-crystallin: Molecular Chaperone activity

Investigations on (-crystallin structure and function took a new turn when Horwitz (1992) reported for the first time that (-crystallin has molecular chaperone-like properties. He showed that (-crystallin was able to prevent the heat-induced aggregation of a number of proteins including (- and (-crystallin. He proposed that this is a physiological function of this protein, which plays vital role in maintaining the transparency of the lens *in vivo*. Later, it was shown that (-crystallin not only prevented thermal aggregation of proteins, but also prevented disulphide bond cleavage and UV-light exposure-induced aggregation of proteins as well. The chaperone function of (-crystallin has been the subject of interest and functional models have been suggested (Abgar et al., 2001; Carver et al., 1994; Derham and Harding, 1999; MacRae, 2000).

1.5.1 Substrate specificity of ?-crystallin

Damage of proteins may occur in the lens due to various reasons such as oxidative stress, ultraviolet radiation or any other kind of stress. When proteins are damaged, they may aggregate forming large insoluble particles. This is detrimental to the transparency of the lens. (-Crystallin, the major protein of the eye lens, captures these denaturing proteins, keeping them in soluble form. The disulphide bond of insulin gets cleaved in presence of DTT and insulin B-chain gets aggregated. In presence of (-crystallin, this aggregation is prevented indicating that insulin B-chain is bound by (-crystallin. The substrate is bound by (-crystallin only in stress condition. (-Crystallin does not bind any substrate in its native state; it binds substrate when it tends to aggregate. The heat induced aggregation of many enzymes such as (-glucosidase, enolase, alcohol dehydrogenase, carbonic anhydrase, citrate synthase, lactate dehydrogenase, aldolase, phosphoglucose isomerase is prevented in presence of (-crystallin. These substrates may have implications for *in vitro* studies, but substrates of *in vivo* relevance to eye lens (-crystallin are (-crystallin, (-crystallin, aldose reductase etc. whose aggregation under various conditions are also prevented by (-crystallin (Andley et al., 1996; Bhattacharyya and Das, 1998; Borkman et al., 1996; Das and Surewicz, 1995 a; Horwitz, 1992; Lee et al., 1998; Marini et al., 1995; Raman and Rao, 1994; Wang and Spector, 1994).

The hydrophobic pockets present in the surface of (-crystallin have been suggested to be prerequisite for the chaperone function of (-crystallin (Raman and Rao, 1994). The chaperone activity of (-crystallin is largely studied using thermal aggregation of protein as assay system. The aggregation experiments were performed with different substrates using the temperature range between approximately 45 and 65^0C (Horwitz, 1992; Takemoto et al., 1993; Wang and Spector, 1994). There must be some conformational change of (-crystallin at elevated temperature along with denaturation of substrate. (-Crystallin undergoes a conformational transition between approximately 38 and 50^0C. Bis-ANS binding study showed that this transition was due to an increase in exposure of hydrophobic surfaces. It was seen that in presence of 3 M urea, quaternary structure of (-crystallin was perturbed and its chaperone activity against photoaggregation of (-crystallin was enhanced (Raman and Rao, 1994). Thus not only the thermotropic changes, quaternary structural perturbation of (-crystallin by nonthermal mode also results in enhancement of chaperone activity. Raman et al. (1995 b) also studied the refolding properties of (-, (- and (-crystallins by the method of rapid refolding which was achieved by diluting the sample with

refolding buffer. It was shown that when (-crystallin was refolded together with (- or (-crystallin, the recovery of (- or (-crystallin in the solution increased significantly whereas the native (-crystallin was less effective in preventing the aggregation of (- or (-crystallin. It was suggested that an intermediate state of (-crystallin was formed in its refolding pathway and this state was more effective in chaperone activity compared with the native state.

Besides aggregation prevention, (-crystallin has also been found to reactivate enzymes, refolding from unfolded conditions (Biswas and Das, 2004a; Muchowski and Clark, 1998; Rawat and Rao, 1998; Wang and Spector, 2000; Wang and Spector, 2001). Reactivations occur under extremely dilute condition of the enzyme, where its self aggregation is minimized. Reactivations occur over a period of about one hour with maximum activity recovered being in the range 10-30% in most cases (Muchowski and Clark, 1998; Rawat and Rao, 1998; Wang and Spector, 2000; Wang and Spector, 2001). Although ATP has been reported to yield marginally higher activity (Biswas and Das, 2004a; Muchowski and Clark, 1998; Wang and Spector, 2000; Wang and Spector, 2001), the requirement of ATP hydrolysis has been questioned (Biswas and Das, 2004a; Rawat and Rao, 1998). It has been found that unlike GroEL-substrate complex, ATP did not dissociate (-crystallin-substrate complex, but instead it enhanced the association and improved chaperone activity (Biswas and Das, 2004a). Whether the refolding activity of (-crystallin has any physiological role in eye lens is not yet known.

1.5.2 Substrate-binding sites:

The hydrogen-deuterium study identified that ?A32-37 and ?B28-34 are the possible substrate binding sites at the N-terminal domain of ?-crystallin (Smith *et al.*, 1996). Binding of ANS to the N-terminal domain decreases the chaperone activity of ?-crystallin (Smulders and de Jong, 1997). Moreover, ?A^{INS}-crystallin has less chaperone activity that may be due to its decreased substrate binding capacity (Smulders *et al.*, 1995). Crosslinking method identified two possible binding regions of ?B-crystallin to alcohol dehydrogenase (Sharma *et al.*, 1997), one is ?A57-69 and the other is ?B93-107. Chaperone activity of ?-crystallin is greatly influenced by these two above mentioned regions. A synthetic 19-residue peptide from the (-crystallin domain (residues 70-88) of human (A-crystallin prevented thermal aggregation of ADH (Sharma *et al.*, 2000). This peptide was also shown to bind bis-ANS. Results indicate that multiple regions are involved in substrate binding. Whether different substrates are sequestered at different binding sites is still an open question.

Mutational study is used to identify the substrate binging in the N-terminal domain of (-crystallin. Substitution of Gly in (B-crystallin reduced the chaperone function slightly (Plater *et al.*, 1996). Removal of methionine from the N-terminal region is an age dependent process and is also the main cause of diminished chaperone function of (-crystallin with age (Karnei *et al.*, 1997). However, some mutations do not alter the chaperone function of (-crystallin. For instance, mutation of Trp^{9} and Leu^{39} do not affect the chaperone activity of (-crystallin. The Asp^{2}, Phe^{24} and Phe^{27} mutants exhibit reduced chaperone activity *in vitro* (Plater *et al.*, 1996). These results show that N-terminal region of (-crystallin contributes to substrate binding.

C-Terminal domain of (-crystallin is also functionally important. Cleavage of the amino

acids from the C-terminal regions decreased the aggregation prevention ability of (A-crystallin (Takemoto *et al.*, 1993). Similarly, when 42 amino acids from the C-terminal end of HSP27 deleted, its chaperone function reduced significantly (Mehlen *et al.*, 1993). The C-terminus is electropositive in nature and may interact with unfolded substrate protein via charge-charge interaction. Mutations of charged residues at the C-terminal extensions of (B-crystallin significantly reduce the chaperone function (Plater *et al.*, 1996). However, galactose modification of C-terminal region of (-crystallin did not affect the chaperone function, but the flexibility lost significantly (Blakytny *et al.*, 1997).

1. Effect of Temperature on Chaperone Activity

Binding of ANS to the N-terminal domain of (-crystallin increases with temperature (Carver *et al.*, 1995; Das and Surewicz. 1995a). The heat induced conformational change was characterized by an increase in surface hydrophobicity (Das and Surewicz, 1995a). High resolution calorimetry study revealed two thermal transitions of (-crystallin, one between 35-57^{0}C and other between 49-73^{0}C (Walsh et. al, 1991). Complete loss of secondary structure was observed above 70^{0}C, whereas tertiary structure was lost around 62^{0}C (Lee *et al.*, 1997b). But ^{1}H-NMR spectroscopy provided evidence for sufficient residual structure of ?-crystallin even at high temperatures (Carver *et al.*, 1993). Oligomeric size of (-crystallin varied with temperature (Burgio *et al.*, 2000; Burgio *et al.*, 2001; Vanhoudt *et al.*, 2000). Chaperone activity also increased with temperature and pre-incubation (Bhattacharyya and Das, 1998; Burgio *et al.*, 2000; Das *et al.*, 1997; Das and Surewicz, 1995a; Datta and Rao, 1999; Raman and Rao, 1994; Reddy *et al.*, 2000). The subunit exchange kinetics increased significantly with temperatures (Bova *et al.*, 1997) implicating the enhancement of dynamic interactions in chaperone function.

1.6 Damage of ?-crystallin with age

Cellular ageing is thought to be the result of the passive accumulation of damaged cellular constituents such as DNA, RNA, proteins and lipid. The heat aggregation prevention ability of HSP70 decreases significantly with age in rats (Shpund and Gershon, 1997). Genetic factors also contribute to ageing. Some studies show that the magnitude of induction of heat shock protein is significantly reduced with age (Liu et al., 1989a, b).

?-Crystallin is usually found as a hetero aggregate of subunits with a molecular mass ~ 800 kDa. With ageing, high-molecular weight ?-crystallin was formed with a concomitant decrease in the amount of low-molecular weight ?-crystallin (Stauffer *et al.*, 1974). The high-molecular mass species of several million Daltons, is responsible for the cataract formation (Benedek, 1971). Increased amount of crosslinked proteins were found in the aged cataract lenses. Tryptic digestion study of young and old lenses revealed that sequences were modified during ageing (Horwitz *et al.*, 1992). CD, absorption and fluorescence of high-molecular weight ?-crystallin had different tertiary structure, which was more unfolded compared to ?-crystallin (Liang and Rossi, 1989; Liang *et al.*, 1985a; Messmer and Chakrabarti, 1998). The fewer hydrophobic regions were observed in high-molecular weight ?-crystallin (Liang and Li, 1991) which is the main cause of the age related decrease in chaperone activity of ?-crystallin (Takemoto and Boyle, 1994; Carver

et al., 1996). The ?-crystallin from young lens more effectively prevented heat-induced aggregation than from older lens (Horwitz *et al.*, 1992; Cherian and Abraham, 1995a).

Deamidation of the C-terminal of ?A-crystallin was observed with age (Dilley and Harding, 1975; Takemoto, 1995). Also residue in human ?B-crystallin undergoes *in vivo* deamidation with age (Srivastava and Srivastava, 2003). Another major modification reported for ?-crystallin with age is the truncation of residues from the c-terminus. In bovine lens, it was noted that a stepwise degradation of ?A-crystallin at residues 169, 168, 151 and 101, and ?B-crystallin at residue 170, occurred post-synthetically (Van Kleef *et al.*, 1975). Subsequently, in the aged human cataract lens, c-terminal truncations have been reported for ?A-crystallin at residues 101, 162, 168 and 172 (Land *et al.*, 1996; Ma *et al.*, 1998: Takemoto, 1995; Thampi *et al.*, 2002; Colvis and Garland, 2002), whereas no such degradation appears to occur in ?B-crystallin. The cleavage sites, on the carboxyl side residues 168, 169 and 172 suggested that the reported *in vivo* truncation of ?A-crystallin was due to the action of proteases present in the lens.

1.7 Scope and Objective of the present thesis

From the above discussion, it is seen that despite the enormous number of studies on the structure of (-crystallin in absence of high resolution data, the tertiary and quaternary structure of (-crystallin is still the subject of much debate and speculation.

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As ?-crystallin is an oligomeric protein, whether the oligomerization is absolutely required for its chaperone function is not clearly established. Detailed analysis of perturbation of oligomeric structure of ?-crystallin under stress conditions and its relationship with altered chaperone activity is virtually non-existant.

Objective of the thesis:

To explore the quaternary structural changes due to the thermal perturbation of (-crystallin; to investigate the effect of these structural changes on the chaperone activity of (-crystallin.

Chapter 2

Effect of Thermal Perturbation on the Quaternary Structure and Chaperone Activity of (-Crystallin

Introduction

The bovine eye lens consists of three types of soluble proteins: (-, (- and (-crystallin (Augusteyn and Stevens, 1998). The intricacy of quaternary association of (-crystallin still remains unclear because of its high molecular mass and polydispersity. Numerous models have been proposed for the quaternary structure of (-crystallin on the basis of known physico-chemical properties. These models are mainly of three types – three-layer assemblies, micelle like structures and assemblies of tetrameric building blocks (Augusteyn and Stevens, 1998; Groenen et al., 1994). Models based on rhombododecahedral structures has also been proposed (Wistow, 1993). There is no general agreement among the various models. X-ray structure of (-crystallin is not known. Cryo-electron microscopic investigations of bovine (-crystallin and human (B-crystallin led to re-constructed 3D-images that reveal spherical structures with inner cavities (Haley et al., 1998; Haley et al., 2000). There seems to be a general agreement that under physiological conditions the quaternary assembly is characterized by a relatively fast subunit exchange indicating a certain degree of conformational flexibility of (-crystallin (Abgar et al., 2000; Bova et al., 1997; Datta and Rao, 2000; Ehrnsperger et al., 1999; Putilina et al., 2003).

(-Crystallin is the major protein component of the vertebrate eye lens and is composed of two subunits ?A- and ?B-, 20 kDa each having 57% sequence homology between them. Both subunits associate to form large oligomer of 800 kDa average molecular weight. It is a key member of the small heat shock protein (sHSP) superfamily having a conserved core '?-crystallin domain' (de Jong *et al*., 1998; de Jong *et al*., 1993). While ?A- and ?B-crystallin are expressed at high levels in the lens, both proteins have been identified in small amounts in non-lens tissues as well (Srinivasan *et al*., 1992: Bhat and Nagineni, 1989; Dubin *et al*., 1989). An important finding that shed light on the understanding of the function of ?-crystallin has been the discovery of its molecular chaperone-like function a decade ago (Horwitz, 1992; Horwitz, 1993). (-Crystallin, like other sHSPs acts like a molecular chaperone in preventing the aggregation of other proteins (Jacob *et al*., 1993; Wang and Spector, 1994; Raman and Rao, 1994). An intense research activity on the structure and function of ?-crystallin has grown since then (Boyle and Takemoto, 1994; Das and Surewicz, 1995a; Das and Surewicz, 1995b; Raman *et al*., 1995b; Das *et al*., 1996; Rawat and Rao, 1998; Muchowski and Clark, 1998; Das *et al*., 1999; Derham *et al*., 2001; Cobb and Petrash, 2002) and subject matter has been extensively reviewed in recent years (van Montfort *et al*., 2001; Narberhaus, 2002; Horwitz, 2003). Regardless of subunit composition, all the diverse assemblies efficiently protect other proteins from aggregation. It has been found that temperature assisted dissociation leads to efficient chaperone activity (Haslbeck et al., 1999). It is believed that oligomeric association of (-crystallin occurs through exposed hydrophobic groups. The hydrophobic exposures of (-crystallin is highly depend on temperature (Das and surewicz, 1995b; Raman et al., 1995 a; Raman and Rao, 1994). The quaternary structure of (-crystallin also varied with thermal perturbation (siezen et al., 1980). ?-Crystallin's oligomeric size can also be varied by varying temperature (Burgio et al., 2000; Burgio et al., 2001; Vanhoudt et al., 2000). Temperature

and pre-incubation affect the chaperone activity of ?-crystallin drastically (Bhattacharyya and Das, 1998; Burgio et al., 2000; Das et al., 1997; Das and Surewicz, 1995b; Datta and Rao, 1999; Raman and Rao, 1994; Reddy et al., 2000). This gives us a great opportunity to study the effect of temperature on the quaternary structure of ?-crystallin and its effect on its chaperone function Therefore, we have, undertaken a study of the oligomeric and chaperone properties of pre-incubated (A- and ?B-crystallin.

Experimental Procedure

Materials:

Molecular weight markers such as Thyroglobulin (mol. wt. 669,000), Apoferritin (mol. wt. 443,000), (-Amylase (mol. wt. 200,000), Alcohol dehydrogenase (mol. wt. 150,000), Bovine serum albumin (mol. wt. 66,000) and Carbonic anhydrase (mol. wt. 29,000) were purchased from Sigma. Dithiothreitol (DTT), trypsin and all buffer salts (tris, phosphate etc) were from Sisco Research Laboratories, India. Other chemicals used in this study were all of analytical grade.

Expression and purification of recombinant (A- and (B-crystallin:

Plasmid DNA of human ?A-crystallin construct in pAED4 vector was a gift from Dr. W.W. de Jong of Katholic University, The Netherlands. Plasmid for human ?B-crystallin in pET20b+ expression vector was provided as a gift by Dr. J. Horwitz of Jules Stein Eye Institute, Los Angeles, CA, USA. For overexpression, both the plasmids were separately introduced into *Escherichia coli* strain BL21-DE3. Cultures were grown in LB medium at 37^{o}C with IPTG induction. Cells were centrifuged, subjected to freeze-thaw treatment and then extracted with DNase and lysozyme. Proteins were dialyzed in 20 mM Tris buffer, pH 7.2 containing 0.5 mM EDTA and 0.5 mM DTT (buffer-A), concentrated in Amicon stirred cell, applied to DEAE anion exchange column and eluted with linear 0-0.5M NaCl gradients. ?A- and ?B-crystallin fractions were then applied to Sephacryl S-300 HR size exclusion column (1.5 cm x 90 cm) and eluted with buffer-A containing 0.1M NaCl. Main peak fractions were concentrated and dialyzed against buffer A or 50 mM phosphate buffer pH 7.2, containing 0.5 mM DTT and stored in aliquots at –70^{o}C. SDS-PAGE of both ?A- and ?B-crystallin showed single band around 20 kDa. Concentration of recombinant proteins was determined spectrophotometrically by measuring absorbance at 280 nm using extinction coefficients of 0.83 $(mg/ml)^{-1}.cm^{-1}$ and 0.95 $(mg/ml)^{-1}.cm^{-1}$ for ?A- and ?B-crystallin respectively (Horwitz *et al*., 1998). Molar concentration of all ?-crystallins was always expressed in subunit basis.

Effect of pre-incubation on the oligomeric structure of ?-crystallin:

***(a) Gel filtration method*:**

Individual solutions of (A- and ?B-crystallin (1 mg/ml; in 50 mM sodium phosphate buffer, pH 7.2) were incubated at different temperatures between 25-70(C for 1 hr and was cooled to room temperature for 1 hr. The each sample was loaded on to a superose 6 column (1 x 40 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.2. After loading, the column was eluted with the loading buffer at 0.25 ml/min. The outlet from the column was fed to an on-line UV

detector and recorder, which automatically determined the chromatogram by measuring absorbance at 280 nm. Standard molecular weight markers were separately run through the column using same conditions. From the elution volume (V_e) of the individual peaks of the marker proteins, a calibration graph of log MW against V_e/V_0, where V_0 is the void volume, was generated. Molecular mass of pre-incubated (A- and ?B-crystallin was obtained from this calibration plot using the elution volume.

***(b) Light scattering method*:**

Light scattering of (A- and ?B-crystallin solutions were measured using a Hitachi F4500 spectrofluorimeter. For measuring light scattering at respective temperature, 0.2 mg/ml (A- and ?B-crystallin (600 (l) in 50 mM sodium phosphate buffer, pH 7.2, was placed at a thermostatic cell holder. The solution was heated at respective temperature for 5 min. The light scattering of the solution at each temperature was measured with an excitation wavelength at 400 nm and emission wavelength 400 nm. The bandpass of both excitation and emission monochromators were 2.5 and 5 nm respectively. The scattering values were corrected for appropriate blanks. All the solutions were filtered through 0.22 (m membrane filter to avoid the dust particles.

Chaperone activity of ?-crystallin under different temperatures:

Chaperone activity of (A- and ?B-crystallin, pre-incubated at different temperatures was measured at 25(C using insulin B-chain aggregation assay. 0.35 mg/ml insulin in 50 mM phosphate buffer, pH 7.2 in absence and presence of 0.175 mg/ml ?B-crystallin which was preincubated at different temperatures for 1 hr. Aggregation was initiated by adding freshly prepared DTT to a final concentration of 20 mM and the apparent absorbance at 400 nm was monitored at the kinetic mode using a Shimadzu UV-2401PC spectrophotometer maintained at 25^{o}C. The assay was also performed in presence of 0.35 mg/ml (A-crystallin, pre-incubated at different temperatures.

Results

The purity of ?A- and ?B-crystallin was checked by SDS-PAGE and Western blotting (with a monoclonal antibody to ?A-/?B-crystallin) (Figure 2.1). Change of oligomeric size due to pre-incubation of ?A- and ?B-crystallin at various temperatures was monitored by gel filtration experiment. Freshly prepared ?A- and ?B-crystallin roughly correspond to 800 kDa average molecular mass. However, upon storage at -20^{0}C for sometime both proteins starts to form high molecular weight (HMW) aggregates having molecular mass of several million dalton. Consequently stored (A-crystallin showed two peaks, one corresponding to HMW species and the other corresponding to low molecular weight (LMW) species (Figure 2.2). The molecular mass corresponding to these two peaks were calculated to be approximately 14,000 kDa and 801 kDa. The first peak (14,000 kDa) was due to HMW species and the second peak (801 kDa) was due to LMW species. With increase in pre-incubation temperature the area of the first peak decreased while that of the second peak increased. This trend contained up to 57^{0}C after which the first peak completely disappeared. Only the second peak was observed, but its position slightly shifted towards the right. With further increase in temperature to 70^{0}C, the peak broadened and the position further shifted to somewhat lower molecular mass range. Thus, the results indicated that

with increase in temperature (A-crystallin was gradually converted to LMW species. Another set of gel filtration experiments were carried out starting with (B-crystallin at 25^{0}C, and pre-incubating it at various temperatures as before. We found that pre-incubation at 70^{0}C, ?B-crystallin completely converted to LMW species (data not shown)

These gel filtration experiments carried out with pre-incubated ?A- and ?B-crystallin revealed two important points. Firstly, storage of ?A- and ?B-crystallin leads to gradual formation of high molecular weight aggregates. Secondly, on heat treatment, the HMW aggregates dissociate into lower oligomer.

All of the above measurements were done at room temperature, after cooling the samples pre-heated at various temperatures. The results, therefore, did not reflect upon the oligomeric size of (-crystallin at the respective temperatures. In order to gain information about their relative oligomeric size at various temperatures, we performed light scattering measurement as a function of temperature. Light scattering by macromolecules reflects upon the size of a macromolecular species in solution (Tanford, 1961) and qualitatively, smaller macromolecules scatter less light than the larger molecules. Figure 2.3 showed the light scattering readings of ?A- crystallin solution as a function of temperature. It was seen that with increase in temperature, scattering value decreased meaning that oligomeric size of ?A-crystallin decreased. We have also done the similar experiment with ?B-crystallin (Figure 2.4). The trend shown by both sets of data were similar. The results clearly demonstrated that oligomeric size decreased upon heat treatment.

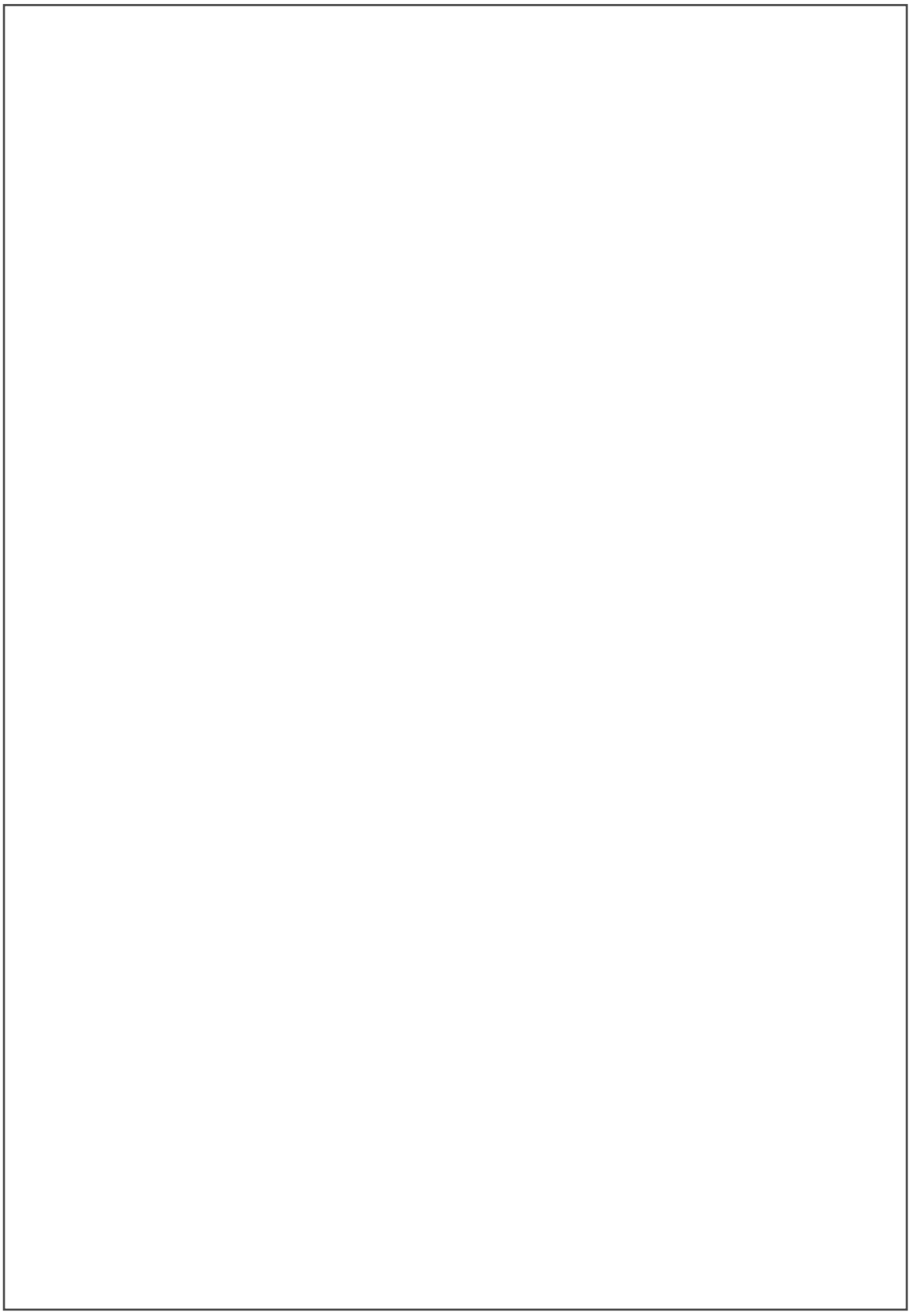
The effect of pre-incubation of (-crystallin at various temperatures on its chaperone activity has been studied by utilizing the insulin assay system. We deliberately chose the ratio of insulin & ?A- or ?B-crystallin 1:1 or 1:0.5 respectively, so that at 25^{0}C about 25% protection against insulin B-chain aggregation can be achieved in case of both proteins. When (B-crystallin was pre-incubated at 37^{0}C, the protection increased to 60%. The inset in Figure 2.5 showed the percentage protection as a function of temperature. It was seen that at 60^{0}C nearly full protection could be achieved. Similar experiment has also been done using (A-crystallin. The results were very similar (Figure 2.6). Thus (A-crystallin whose chaperone activity at 25^{0}C was low compared to ?B-crystallin (< 20%), on pre-incubation at 70^{0}C became fully active.

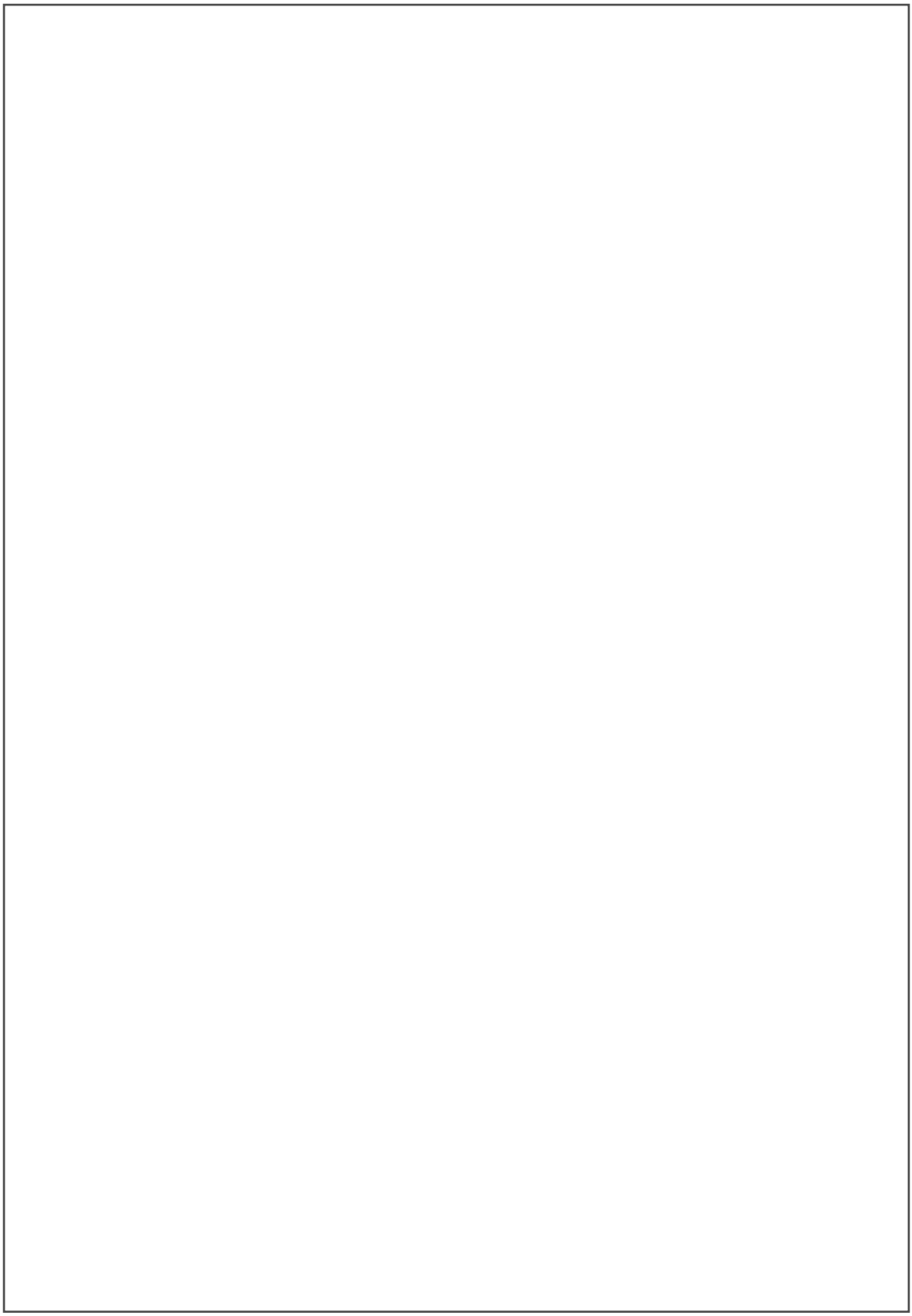
Discussion

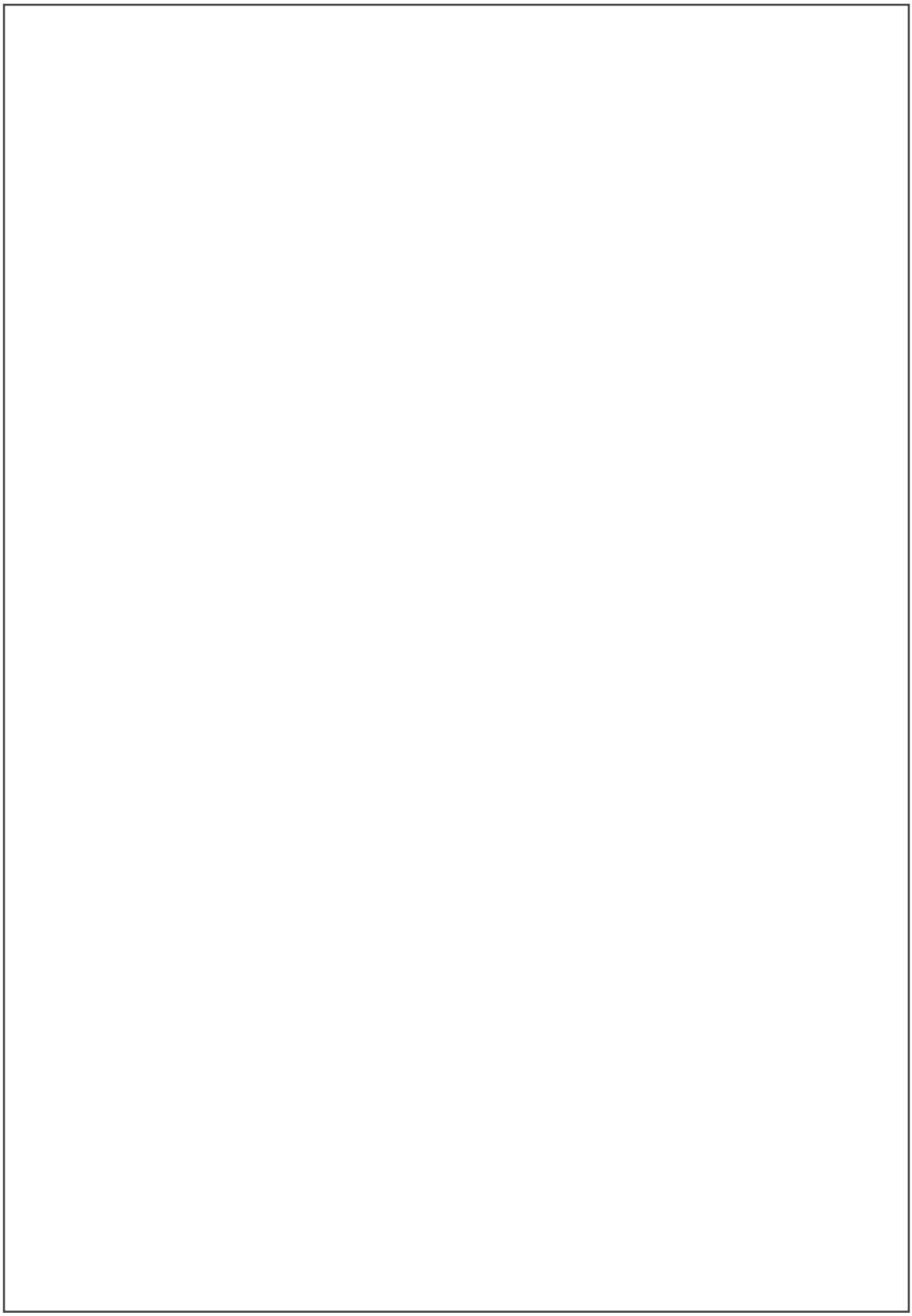
Results presented in this Chapter showed that pre-incubation of (-crystallin reduced its oligomeric size. Both light scattering and gel filtration experiment revealed the same finding. Gel filtration experiment showed that ?A- and ?B-crystallin existed as low and high molecular mass species. Progressive pre-incubation to higher temperatures dissociated the high molecular mass species to the low molecular mass species. Previous studies on the effect of heating on the oligomeric size reported somewhat different results (Putilina et al., 2003; Siezen et al., 1980). Siezen et al. (1980) reported that oligomeric size decreased on increasing temperature from 25^{0}C to 37^{0}C. Beyond this temperature, they reported an increase in size due to aggregation. Putilina et al. (2003) demonstrated from small angle X-ray scattering data an irreversible doubling of

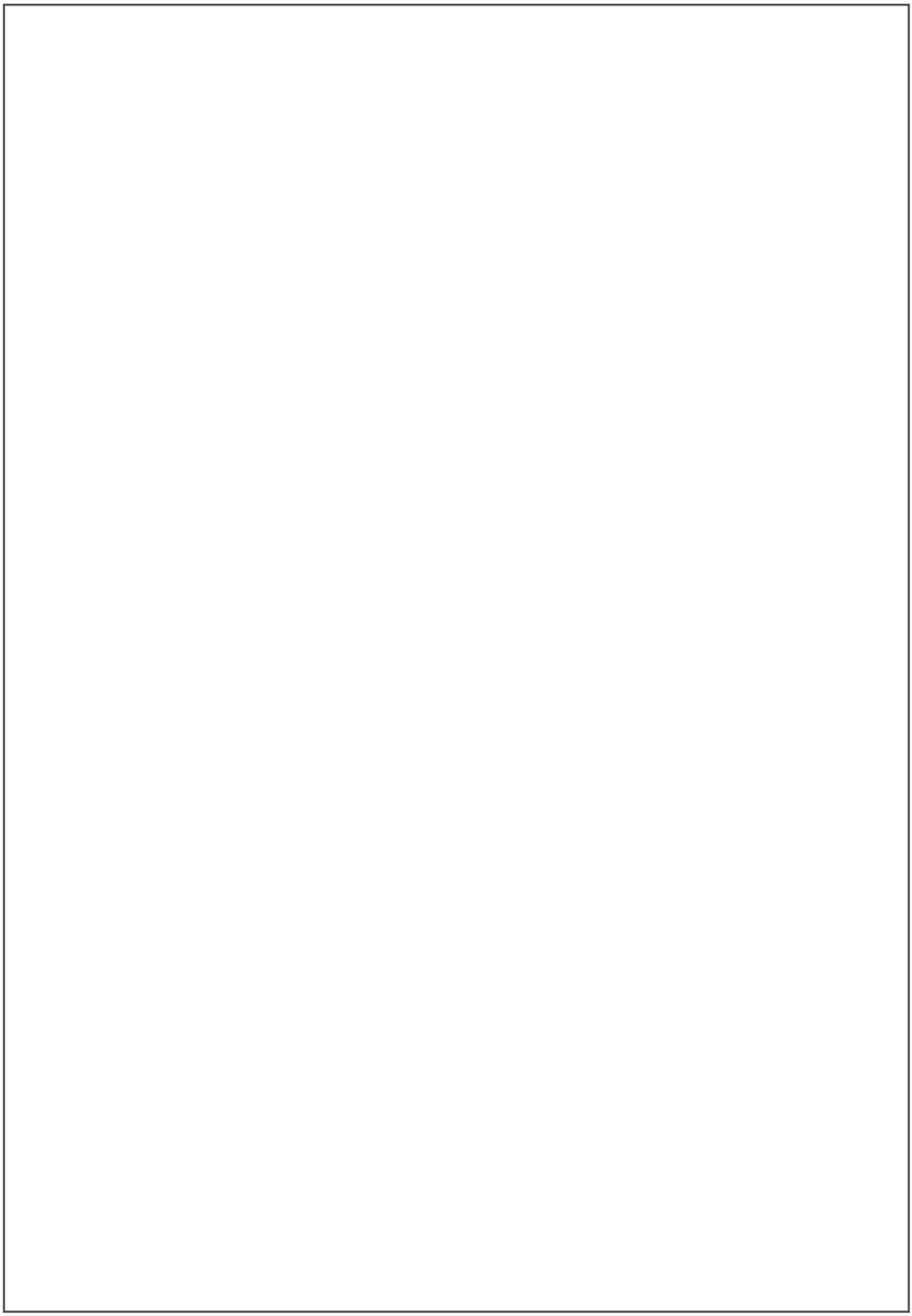
molecular weight and a corresponding increase in size of (-crystallin at temperatures above 60^0C. A similar observation has also been reported by Burgio et al. (2000). Such a trend was not observed in our study. Our data showed that in terms of heating and cooling on a broad time scale, high and low molecular mass aggregates seem to be interconvertible. However, an increased activity of (-crystallin was reported by Burgio et al. (2000) after a cycle of heating and cooling, which was attributed to the conformation and packing parameters of (-crystallin in the oligomeric assembly.

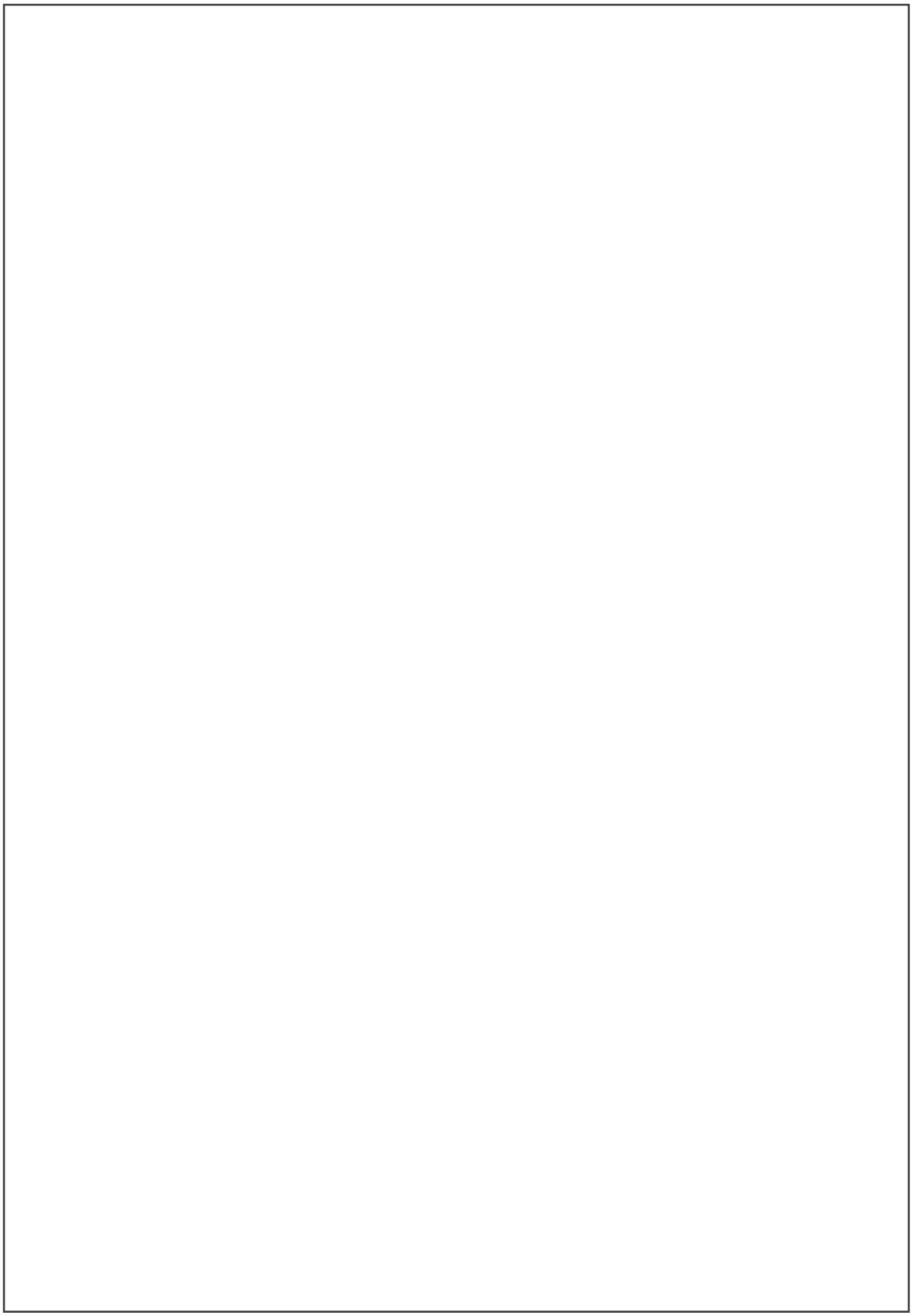
Our main objective in this Chapter was to study the chaperone activity of ?A- and ?B-crystallin whose quaternary structure was perturbed by pre-incubation. Both gel filtration as well as light scattering experiments indicated reduction in the size of ?A-/?B-crystallin oligomer with increase of pre-incubation temperature. Our chaperone assay study indicated increase of chaperone activity with increase of temperature. Our results also indicate that apart from other age related reasons, simple storage of ?A- and ?B-crystallin may lead to formation of high molecular weight aggregated (-crystallin which has very low chaperone activity. One way to prevent the loss of chaperone activity of (-crystallin would be to prevent its further association into larger oligomer.

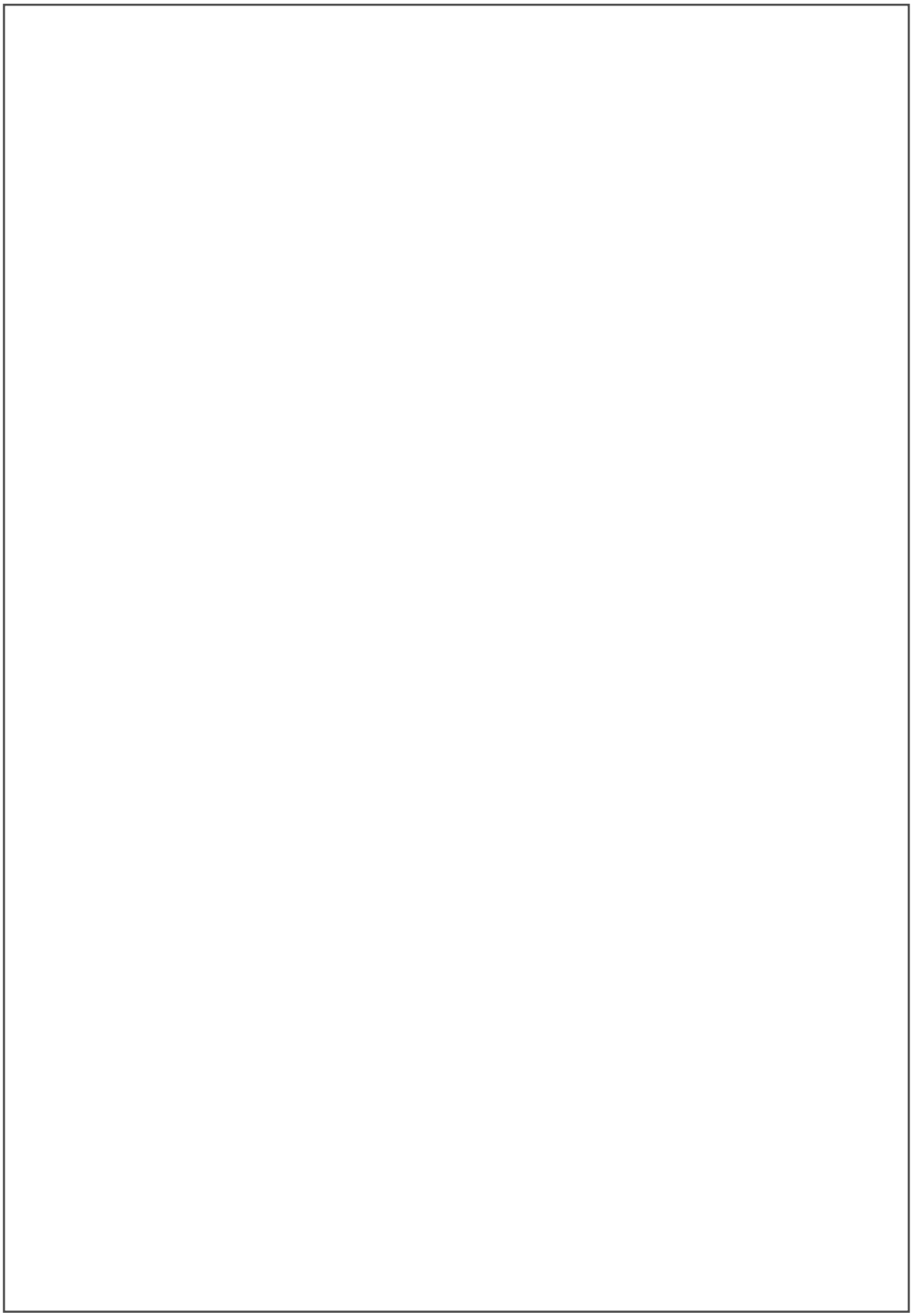












Summary

This thesis presents a structural study of (-crystallin during structural alterations caused by externally applied stress. In Chapter 1, we present a brief account of literature on the origin, structure and chaperone function of (-crystallin. It's substrate specificity, degradation due to aging and nature and temperature-chaperone function relationship is discussed.

In Chapter 2, we explored the relationship between oligomeric size and chaperone activity using human recombinant (A- and ?B-crystallin. We used thermal pre-incubation as a tool to vary the oligomeric size of (A- and ?B-crystallin and study its effect on chaperone activity. With increase in pre-incubation temperature, the oligomeric size of both (A- and ?B-crystallin was reduced but the chaperone activity of both proteins was enhanced. This observation leads us to conclude that chaperone activity of (-crystallin is inversely proportional to its oligomeric size. Our results, also showed that the high molecular weight aggreated species was formed on simple storage of both (A- and ?B-crystallin which has very low chaperone activity. The result implies that loss of chaperone activity of (-crystallin can be prevented by preventing its further association into larger oligomer.

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