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ALPHA-CRYSTALLIN: A SMALL HEAT SHOCK PROTEIN WITH CHAPERONE ACTIVITY

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Protein folding is a major unsolved problem in biology. Protein misfolding and aggregation are shown to be the molecular basis for a growing number of diseases. Many proteins can fold to their native forms *in vitro* without any external assistance. However, the situation may not be similar in the crowded environment of a living cell. Nascent proteins, *in vivo*, have neither space nor time to fold correctly. Molecular chaperones and heat shock proteins assist their folding and assembly *in vivo*. Thus, an understanding of chaperone activity appears to be necessary for designing strategies to mitigate protein aggregation-related problems in biotechnology and in medicine. α-Crystallin is a major protein of the mammalian eye lens, which has homology with small heat shock proteins. Homology with "α-crystallin domain" is now considered as a characteristic feature of small heat shock proteins. Recent studies, including our own, show that α-crystallin possesses chaperone activity in preventing aggregation of other proteins, offering protection against heat-induced inactivation of enzymes and helping in refolding enzymes to their active state. In addition to the eye lens, α-crystallin is expressed in several other tissues and appears to play an important role in neurodegenerative diseases such as Alzheimer's and Parkinson's, neuroblastoma, congenital cataract, desmin related myopathy and renal insufficiency. We have been investigating the mechanistic aspects of the chaperone-like activity of α-crystallin with an aim to understand the structure-function relationship and to design strategies to enhance its activity. Since loss of activity has been clearly associated with diseases, enhancement of activity should prove useful in the therapeutic context. Our studies show that structural perturbation can lead to enhanced activity. We have cloned α-crystallin and introduced several point mutations including disease-causing mutations. The study, in addition to providing structural insight, has demonstrated molecular basis for pathology. Swapping domains resulted in chimeric proteins with enhanced activity. This review describes our efforts and major achievements in understanding the structure-function relationship of α-crystallin in the context of protein aggregation related diseases.

Key Words: α-Crystallin; Small Heat Shock Protein; Chaperone; Refolding; Zeta-Crystallin; Citrate Synthase; Reactivation; Chimeric Protein; Domain Swap

1 Introduction

Molecular mechanism of protein folding is a major unsolved problem in biology. A large number of proteins can fold to their native forms *in vitro* without any external assistance. However, the situation may not be similar in the crowded environment of a living cell. Nascent proteins *in vivo* have neither space nor time to fold correctly. "Defective protein folding" has recently been recognized as the molecular basis for a growing list of human diseases[1-4]. Several neurodegenerative diseases such as Alzheimer's, scrapie (mad cow disease in cattle) appear to be caused due to protein aggregation[1-4]. Molecular chaperones, a group of unrelated proteins, assist protein folding and assembly *in vivo*[5-8]. Thus, an understanding of chaperone activity is necessary for designing strategies for mitigating protein aggregation-related problems in biotechnology and medicine.

α-Crystallin is a major protein of the mammalian lens. It is a multimeric protein comprised of two gene products, αA- and αB-crystallin, both of which have molecular masses of 20 kDa[9,10]. Studies over the past few years have shown that αB-crystallin is also expressed at significant levels in several non-lenticular tissues such as heart, brain, and kidney[11-15] Trace amounts of αA-crystallin have been reported in the spleen and the thymus[16]. The expression of αB-crystallin is enhanced several fold during stress and disease conditions[14,17,18]. Both αA- and αB-crystallin form homomultimers and upon mixing they exchange

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to form a heteromultimer[19-22]. Both the subunits have been shown to have homology with small heat shock proteins (sHsps)[23-26]. Fig.1 shows the sequence comparison between αA-, αB- crystallin and Hsp27, as an example. Horwitz showed that α-crystallin can prevent thermal aggregation of β- and γ-crystallins and a few other proteins like a molecular chaperone[27]. Demonstration of chaperone-like activity of α-crystallin has provided an excellent opportunity to investigate the mechanistic aspects of chaperone function in general and the role of α-crystallin under stress conditions in particular. It is possible that, in the lens, α-crystallin may chaperone the formation of transparent and appropriately refracting ensemble and may also keep it that way by interacting with damaged proteins. αB-Crystallin may have a similar function of interacting with aged or damaged proteins in brain of patients with Creutzfeld Jakob Disease[28] and in the ischemic heart tissue[29]. αB-Crystallin may even play a regulatory role in cytomorphological rearrangements during development[30]. A mutation (R116C) in αA-crystallin is known to lead to congenital cataract[31] and a missense mutation (R120G) in αB-crystallin was shown to cause desmin related myopathy[32].

Recently, Hatters *et al.* have shown that α-crystallin can inhibit the amyloid formation by apolipoprotein C-II[33]. Small heat shock protein 27 (sHsp27) can inhibit the *in vitro* amyloidogenesis of $A\beta^{1-42}$ peptide, but α-crystallin (hetero multimer) made very little difference in this situation[34]. However, a later fluorescence energy transfer study showed that αB-crystallin interacts with $A\beta^{1-42}$ peptide[35]. Interestingly, αB-crystallin is shown to suppress the tubulin aggregation through complex formation with monomers[36]. Iwaki *et al.* have shown, using sense and anti-sense cDNA approach that αB-crystallin alters the stress fiber formation and confers thermoresistance[37]. Recently, αB-crystallin was shown to disaggregate the filamentous cytoplasmic inclusions formed by glial fibrillary acidic protein in cells overexpressing αB-crystallin[38].

Study on subcellular distribution of ectopically expressed αB-crystallin shows that, in addition to being in the cytoplasm, the protein resides in the nucleus[39]. This observation suggests that αB-crystallin can cross the membrane. Indeed, a study of the lacrimal glands shows that αB-crystallin is present in the lacrimal gland duct cells and in the tear fluid indicating the possibility of secretion[40].

Clearly, αB-crystallin has a major role to play in several tissues and in several processes. Since loss of its activity is associated with disease, maintaining or enhancing its activity should have therapeutic value. Enhanced chaperone activity should also be of value

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αA-Crystallin   MDVTIQHPWFKRTLGPFY-PSRLFDQFFGEGLFEYDLLPFLSSTISPYYR-QSLFRT--V
αB-Crystallin   MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPTSTSLSPFYLRPPSFLRAPSW
Hsp27           -------MFGRDPFDSLF--ERMFKEFFATPMTGTTMIQSSTGIQISGKG----FMP---
                       : : .: .:.  .*:*.:**.  :    ::   :.           : .

                LDSGISEVRSDRDKFVIFLDVKHFSPEDLTVKVQDDFVEIHGKHN-------ERQDDHGY
                FDTGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHE-------ERQDEHGF
                ----ISIIEGDQ-HIKVIAWLPGVNKEDIILNAVGDTLEIRAKRSPLMITESERIIYSEI
                    :* :. :: :: :   :  .. *:: ::. .* :*::.*:.       **

                ISR-EFHRRYRLPSNVDQSALSCSLSADGMLTFCGPKIQTGLDATHAERAIPVSREEKP-
                ISR-EFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRKQ----VSGPERTIPITREEKPA
                PEEEEIYRTIKLPATVKEENASAKFEN-GVLSVILPKAE-----SSIKKGINIE------
                 .. *::*  ::*: *.    :..:.  *:*:.  *: :     :  :: * :

                -TSAPSS
                VTAAPKK
                -------
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Fig. 1 Sequence alignment of αA-crystallin, αB-crystallin and Hsp27 using the program CLUSTAL X (1.5b)

in biotechnology. Thus, several years ago, we set out to investigate the mechanistic aspects of its chaperone activity and its structure-function relationship. We have used non-thermal aggregation systems and found that the chaperone-like activity of α-crystallin is temperature-dependent[41-44]. Our studies with photoaggregation of γ-crystallin[41], thermal aggregation of ξ-crystallin and DTT-induced aggregation of insulin[42] together with the rapid refolding of crystallins[45] and the role of α-crystallin in these processes resulted in a hypothesis that sheds some light on the chaperone-like activity of α-crystallin. These studies show that α-crystallin prevents the aggregation of non-native structures by providing appropriately placed hydrophobic surfaces. A structural transition above 30°C enhances the protective ability perhaps by increasing or reorganising hydrophobic surfaces. We have also shown that tertiary structural changes precede quaternary structural changes[43].

As stated earlier, α-crystallin is a hetero multimer of two gene products, αA- and αB-crystallins. αA- and αB-crystallins have 57% sequence homology with each other[46] and have nearly 40% sequence homology with other small heat shock proteins[25]. Both the homo-multimers function as chaperones, albeit to different extents[47,48]. We have observed that they also show different temperature-dependent changes in structure and activity[48]. Subunits can exchange to form a hetero multimer; our studies showed that the packing of the hetero multimer induces conformational and functional changes[49]. A point mutation R116C in αA-crystallin leads to congenital cataract and R120G in αB-crystallin leads to congenital cataracts and desmin related myopathy[31,32]. We have cloned human αA- and αB-crystallin and introduced several mutations including the disease-causing ones mentioned above. We found that both the mutations lead to structural changes (secondary, tertiary and quaternary) and result in significant loss of chaperone-like activity[50]. This observation provides the molecular basis for the pathology.

We could successfully engineer a chimeric protein by swapping the N-terminal domains of αA- and αB-crystallin that is several-fold more active than the wild type αB-crystallin in preventing aggregation of other proteins[51]. In order to understand the interaction of α-crystallin with its target protein and the conformational requirements of target proteins, we have investigated the interaction of α-crystallin with carbonic anhydrase, α-lacatalbumin, RNase A and lysozyme. Our studies showed that α-crystallin interacts with the aggregation prone-molten globule state of target proteins[52-54].

On the strength of the knowledge accumulated over the years as a result of our biophysical and molecular biological efforts, we have been testing a few candidate molecules that can interact with α-crystallin and enhance its activity. Our preliminary results indicate that one such molecule indeed enhances the chaperone-like activity. It also appears to convert the disease-causing mutant, α-R120G, to its active form, an observation likely to have significant therapeutic value. This review documents our studies, over the years, on the structure and chaperone function of α-crystallin in conjunction with the developments in the field.

2 Structural Perturbation and Chaperone-Like Properties of α-Crystallin

The chaperone-like activity of α-crystallin was first demonstrated using heat-induced aggregation of other crystallins and some other enzymes at temperatures above 50°C[27]. We initiated studies to investigate whether α-crystallin could prevent the aggregation of γ-crystallin upon irradiation with UV light, which has relevance in the context of eye-lens transparency and cataract. To our surprise, we found that α-crystallin was not very effective in preventing aggregation at 25°C. However, when we investigated the effect of α-crystallin on the photoaggregation of γ-crystallin as a function of temperature, we observed dramatic increase in the extent of protection above 30°C[41]. These results showed that α-crystallin is active at physiologically relevant temperatures. Since the extent of protection increased in a temperature-dependent manner, we studied the chaperone-like activity of α-crystallin using other model systems, where also aggregation is induced by non-thermal means.

Matured insulin consists of two polypeptide chains, A and B, which are linked through two interchain disulfides. Upon reducing the disulfide bonds with reducing agents such as dithiothreitol (DTT), the B-chain of insulin aggregates and precipitates out leaving the A-chain in solution[55]. Farahbaksh *et al.*[56] showed that α-crystallin could completely prevent the aggregation of the insulin B-chain at 25°C. This was possible with the use of very high concentrations of α-crystallin. As in the case of the photoaggregation of γ-crystallin, we have shown temperature-dependent

increase in the activity of α-crystallin in preventing the aggregation of insulin B-chain[42]. α-Crystallin provided almost complete protection even at a very low ratio at 40.6°C. Our studies using aggregation of the guinea pig lens protein zeta-crystallin – an NADPH-binding protein[57] - also supported the temperature-induced enhancement of chaperone-like activity[42]. These results suggest the involvement of a structural perturbation in α-crystallin in the enhancement of its chaperone-like activity. We perturbed the structure of α-crystallin using 3 M urea and found that this mode of structural perturbation also led to enhancement of its protection. While γ-crystallin aggregated in the presence of 3 M urea upon exposure to UV light at 20°C, α-crystallin in 3 M urea completely prevented this aggregation[41].

Hydrophobic interactions play an important role in the interaction between chaperones and partially folded proteins. Proteins in their partially folded states, expose hydrophobic surfaces leading to hydrophobic collapse and aggregation in the absence of chaperones. We have shown that γ-crystallin, upon irradiation by UV-light, exposes its hydrophobic surfaces, as measured by enhanced ANS-binding[41]. Thus, appropriately placed hydrophobic surfaces appear to play an important role in the prevention of aggregation of partially folded/unfolded proteins by α-crystallin. We have investigated the accessibility of the hydrophobic surfaces of α-crystallin as a function of temperature by solubilizng the hydrophobic fluorophore, pyrene[41] and observed that the solubility of pyrene, measured by the absorbance at 338 nm, increased significantly between 30 and 40°C. The solubility did not change much between 40 and 50 °C but increased markedly beyond 50°C. Thus, our results showed that the temperature-induced increase in the chaperone-like activity of α-crystallin can be well-correlated with the temperature-induced exposure of its hydrophobic pockets or target protein-binding sites[41,44]. Differential calorimetry studies by Walsh *et al.*[58] had shown that α-crystallin undergoes two transitions as a function of temperature – the first around 40°C and the second around 60°C. Several other studies have also shown that the quaternary structure of α-crystallin can be perturbed by temperature, pH of the medium and ionic strength[59,60]. The high molecular mass (800 kDa) multimer of α-crystallin was shown to be irreversibly converted into a 360 kDa multimer upon incubating at 37°C[59,60]. We have shown that tertiary structural changes precede quaternary structural changes in the temperature-induced perturbation of α-crystallin[43]. Thus, these studies indicated a structural perturbation of α-crystallin around 30°C, accompanied by an increase in the surface hydrophobicity that led to its enhanced chaperone-like activity in preventing the aggregation of target proteins[41-44].

We have studied the chaperone-like activity of α-crystallin towards the refolding of β_L and γ-crystallins[45]. We have shown that β- and γ-crystallin aggregate even at low concentrations upon refolding from 8 M urea, whereas α-crystallin does not aggregate even at very high concentrations. α-Crystallin regains its native structure upon refolding from its urea-denatured state. Our results showed that when α-crystallin was co-refolded with β- or γ-crystallin, it completely prevented their aggregation, but it could prevent the aggregation of β- or γ-crystallin only marginally when it was present in the refolding buffer without any structural perturbation[45]. Since an increase in temperature brings about a structural alteration in α-crystallin, we also investigated the effect of α-crystallin on the refolding-induced aggregation of β_L-crystallin as a function of temperature[43]. In the absence of α-crystallin the extent of aggregation of β_L-crystallin refolded from its guanidinium chloride (GdmCl) unfolded state increased as a function of temperature. When α-crystallin was present in the refolding buffer, at temperatures below 30°C, there was only a marginal decrease in the aggregation of β_L-crystallin. But beyond 30°C, the extent of aggregation decreased significantly in a temperature-dependent manner with two distinct transitions[43] – one at 30°C and the other at 55°C similar to those we observed in the solubilization of pyrene[41]. Our results were also supported by studies from other laboratories. Das and Surewicz[61] also observed similar temperature-dependent exposure of hydrophobic surfaces using bis 8-anilinonaphthalene-1-sulfonate. Smith *et al.*[62] used hydrogen-deuterium exchange of amide protons to study conformational changes of α-crystallin as a function of temperature. They observed that hydrophobic regions around the residues 32-37 and 72-75 of αA-crystallin and 28-34 of αB-crystallin become exposed to the solvent. Increased chaperone-like activity of α-crystallin has also been observed upon perturbation of its structure with low concentrations (<1.5M) of GdmCl[63]. Thus, all these studies supported our earlier hypothesis[41] that α-crystallin prevents aggregation of other proteins by providing

appropriately placed hydrophobic surfaces; a structural transition above 30°C enhances this chaperone-like activity of α-crystallin several-fold.

We investigated the availability and specificity of hydrophobic sites that become available at elevated temperatures[64]. We found that α-crystallin complexed with insulin at 37°C was able to prevent the thermal aggregation of β_L-crystallin at 60°C but could not prevent DTT-induced aggregation of α-lactalbumin at 37°C. On the other hand, α-crystallin complexed with β_L-crystallin at 60°C could not prevent the DTT-induced aggregation of insulin or α-lactalbumin at 37°C. Our results suggest that more target protein binding sites become available at elevated temperatures, the sites available at 37°C being a subset of the total sites available at elevated temperatures. It is still not clear whether different target proteins have different binding sites in α-crystallin. Sharma *et al.*[65,66] used alcohol dehydrogenase and melittin as target proteins to identify target protein binding sites in αA- and αB-crystallin. They found that ADH-binding sites encompass residues 57-69 and 93-107, while melittin bound to residues 75-82 in αB-crystallin. They also carried out bis-ANS binding studies and found that bis-ANS and target protein interacted with similar sites[67].

Growing evidence has accumulated over the years that temperature regulates the chaperoning process, in general, through structural perturbation of the chaperone molecules. Hsp70 has been shown to exhibit enhanced chaperone activity above 40°C involving a conformational change[68,69]. Wheat Hsp16.9, one of the members of α-crystallin-like small heat shock proteins, whose X-ray crystal structure has recently been solved[70], is a dodecamer under normal temperature exhibits dissociation at 40°C[70]. The substrate-bound dimers of Hsp16.9, in temperature-dependent equilibrium with higher assembly forms, have unfolded N-terminal arms and exposed hydrophobic binding sites on the conserved α-crystallin domain[70]. The small heat shock protein, Hsp16.3 from *Mycobacterium tuberculosis*, is a nonamer at normal temperatures. It dissociates at elevated temperatures, accompanied by a greatly enhanced chaperone-like activity[71]. The dissociation of the nonameric structure appears to be a prerequisite for Hsp16.3 to bind to denaturing substrate proteins[71]. Structurally perturbed state of Hsp16.3 at low concentrations of GdmCl has also been shown to exhibit enhanced chaperone activity[72]. Yonehara *et al.*[73] showed that Hsp90 acquires chaperone activity which is latent under normal conditions but is heat-induced. Upon heat-treatment at 43°C, Hsp90, which is dimer under normal conditions, is converted to discrete higher oligomers which are active[73]. Tanaka *et al.*[74] have shown disruption of the intramolecular interaction - i.e., the liberation of the N-terminal substrate-binding domain from the middle suppressor domain - is involved in the mechanism of heat-induced activation of Hsp90. Calreticulin, a molecular chaperone functions in the recognition of misfolded MHC class I heavy chains in the endoplasmic reticulum[75]. Mancino *et al.*[75] have shown that calreticulin itself is converted to oligomeric species on exposure to 37°C or higher temperatures, and that oligomeric forms of calreticulin are active in inhibiting thermal aggregation of peptide-deficient HLA-A2. In some cases, the chaperone function of the bacterial GroEL/ES machinery has been shown to be modulated by temperature[76,77]. Thus, temperature-induced activation or enhancement of chaperone function appears to be a general mechanism by which cell tackles the demands at heat shock or non-permissible temperature.

3 Chaperone-like Activity of αA- and αB-Crystallin

As mentioned earlier, eye lens α-crystallin is composed of two types of subunits - αA- and αB-crystallin – present at a molar ratio of 3:1[9]. They are encoded by evolutionarily related genes and have 57% sequence identity[46]. They share high homology in the middle region of the sequence called "α-crystallin domain" while the flanking regions are variable. In fact, small heat shock proteins contain the highly conserved stretch of about 80-100 amino acids of the "α-crystallin domain"[46,78-80]. The α-crystallin domain is flanked by a non-conserved, hydrophobic, N-terminal domain and a small, unstructured, C-terminal extension[46,78-80]. Both, the N-terminal domain and the C-terminal extension, vary in length and sequence among the sHsps[78-80]. The sequence variation in the N-terminal domain and the C-terminal extension between αA- and αB-crystallin suggests that they may be expected to exhibit subtle differences in structure and function.

The demonstration of their independent expression in other tissues and the overexpression of αB-crystallin under stress and in disease condition[11-18] suggests that αA- and αB-crystallin also play individual roles. Thomson and Augusteyn[81] purified αA- and αB-crystallin subunits from bovine α-crystallin by

chromatofocussing in urea and demonstrated that the individual subunits could reassemble into homoaggregates of αA- and αB-crystallin with molecular masses of 360 and 420 kDa respectively. Sun *et al.*[47] used recombinant DNA technology to clone αA- and αB-crystallin and studied their structure and chaperone activity. In a parallel study, we have also investigated purified calf eye lens αA- and αB-crystallin and studied their structure and chaperone function[48]. αA- and αB-crystallin exhibit very different near-UV CD spectra, suggesting that they have different tertiary structure[47,48]. The major secondary structure of these proteins is the β-conformation (β-sheet and β-turns). Studies using the hydrophobic fluorescent probe, 8-anilinonaphthalene-1-sulfonic acid (ANS) demonstrated that αB-crystallin exhibits more accessible hydrophobic surfaces than αA-crystallin[47,48]. αB-crystallin was found to be more effective as a chaperone in preventing the aggregation of target proteins than αA-crystallin[47,48]. αB-crystallin exhibits 3-4 fold higher chaperone-like activity against the DTT-induced aggregation of insulin than αA-crystallin[47,48]. However, both the homo- and hetero-multimers of αA- and αB-crystallin exhibited comparable activity against the thermal aggregation of β_L-crystallin at 58°C[48].

We have studied the temperature-dependent chaperone activity of αA- and αB- crystallin individually[48]. Comparison of the chaperone-like activities of αA- and αB-crystallin towards DTT-induced aggregation of insulin at temperatures between 25°C and 42°C showed that they differ in their chaperone activity at temperatures below 37°C. At about 40°C, both the homo multimers exhibit comparable activities[48]. αB-Crystallin displays significant chaperone-like activity at temperatures below the physiological temperature, whereas αA- or lens α-crystallin worked efficiently only at or above the physiological temperature[48]. A subsequent study by Reddy *et al.*[82] have also showed similar results with recombinant human αA- and αB-crystallin.

We investigated the temperature-dependent structural changes by CD and fluorescence spectroscopy[48]. Near-UV CD spectra showed significant alteration in the tertiary structure of αA-crystallin only above 50°C, whereas αB-crystallin showed considerable loss of structure by 45°C. Far-UV CD spectra between 25 and 65°C also showed significant changes in secondary structure of αB-crystallin with a gradual increase in ellipticity being observed at 217 nm with increase in temperature. On the other hand αA-crystallin did not show significant changes with temperature, except an enhanced signal at 207 nm at higher temperature. These results show that αB-crystallin is structurally less stable and shows structural alterations by 45°C, whereas αA-crystallin is stable even at 55°C. Thus, αB-crystallin has greater chaperone-like activity but lower structural stability whereas αA-crystallin has lower chaperone-like activity but greater structural stability[48]. Our studies thus suggest that the properties of the heteromultimeric lens α-crystallin is a compromise between structural stability and chaperone-like activity[48]. Similar conclusions regarding the stability of αA- and αB-crystallin were arrived by Sun *et al.*[83] using GdmCl-induced unfolding of the proteins.

4 Dynamic Nature of α-Crystallin Assembly and Subunit Exchange

Except sHsp12.6 from *C. elegans* which is a monomeric protein, all sHsps known so far form multimeric assemblies[84]. The quaternary structure of homo- or the hetero-multimeric assembly of αA- and αB-crystallin is not understood so far and is a matter of controversy. Several models that have been proposed for the quaternary structure of α-crystallin include micellar model[85], concentric three layer model[58] cubic and rhombic dodecahedral model[86] toroid model[87], disc-like model[88], open-loose model[89] and pitted-flexiball model[90]. However, none of them explains the properties of α-crystallin completely. Some sHsps such as Hsp16.9 from wheat[70], Hsp16.5 from *Methanococcus janaschii*[91] and Hsp16.3 from *Mycobacterium tuberculosis*[71] form discrete oligomeric assemblies of 12, 24 and 9 subunits respectively. However, many sHsps including α-crystallin form polydisperse assemblies[79,80]. The assembly size of α-crystallin is known to vary with temperature, pH and ionic strength[59,60]. This polydispersity may be one of the possible reasons for the unsuccessful efforts to crystallize either the homo- or hetero-multimers of αA- αB-crystallin so far. The dynamic nature of the assembly of α-crystallin is known. Siezen and Owen[92] showed that calf lens α-crystallin, that is isolated into five sub-populations with various molecular masses, returns to its original distribution upon mixing, indicating a dynamic equilibrium between α-crystallin oligomers. Homomultimers of αA- and αB-crystallin are also known to exchange subunits among themselves and with the α-crystallin heteromultimers[19-22]. Subunits

exchange between assemblies of αA- or αB- crystallins and that of Hsp27 have been demonstrated[93]. Although some sHsps form mono-disperse assemblies, they are known to exchange subunits. For example, wheat Hsp16.9 and an orthologous class I pea Hsp18.1 dodecamer readily engage in subunit exchange in solution[70]. Subunit exchange occurs readily between the Hsp16.3 oligomers, even at a temperature as low as 4°C[71]. Increasing temperature is known to increase the rate of exchange between αA- and αB-crystallin homo-multimers to form a hetero-multimeric assembly, however, the exchange does not take place significantly at 4°C[19-21]. Whether the subunit exchange between αA- and αB-crystallin affects structural and functional properties of the assembly, was not paid much attention to earlier. The few general questions that are relevant in this context are: Are the interactions between the subunits non-specific, dictated mainly by hydrophobic interactions, or are there any specific, preferential interactions? Are the subunits packaged in the hetero multimer like rigid spheres or do they exhibit a degree of structural flexibility? We, therefore, undertook studies to investigate the subunit exchange between αA- and αB-crystallin and its influence on the structure and chaperone activity of the assembly[49].

If there are no specific interactions between the subunits in the hetero multimers, the near-UV CD spectrum should be an algebraic sum of the spectra of αA- and αB-crystallin homo-multimers. The near-UV CD spectra of αA- and αB-crystallin homo multimers differ in the 270-290 nm region, where tyrosine and tryptophan contribute to the signal. The near-UV CD spectrum of a 3:1 (w/w) mixture of αA- and αB-crystallin did not overlap with that of the hetero multimer of α-crystallin immediately after mixing. When the mixture was incubated at 4°C for 24h, no exchange took place as revealed by the near-UV CD spectrum. However, after incubating the subunits at 37°C for 24 h to allow the exchange to take place, the near-UV CD spectrum of the exchanged 3:1 mixture was identical to that of the α-crystallin hetero multimer. The effect of subunit-exchange on the secondary structure was also studied. The far-UV CD spectrum of the 3:1 (w/w) mixture of αA- and αB-crystallin homo multimers was almost similar to that of α-crystallin. Upon subunit exchange at 37°C for 24 h, no significant changes were observed. Our results showed that a change from homologous subunit packing to heterologous subunit packing altered the tertiary but not the secondary structure. It is known that the secondary structure of α-crystallin is very stable against perturbation by temperature.

Subunit exchange also resulted in an increase in the quaternary structure as monitored by gel-filtration chromatography. The molecular masses of the purified calf eye lens αA- and αB-crystallin homo multimers were 600 and 450 kDa respectively, while that of lens α-crystallin is 800 kDa. Incubation of a 3:1 (w/w) mixture of αA- and αB-crystallin homo multimers at 4°C did not show any change in the elution volume. However, upon incubation at 37°C to facilitate subunit exchange, there was a significant decrease in the elution volume after 4 h; and after 24h, the hetero multimers generated, eluted as a sharp peak with molecular mass larger than those of the homo multimers. Since subunit exchange between homo multimers led to changes in tertiary and quaternary structure, we investigated the functionality of the hetero multimer. Interestingly, the chaperone-like activity of the hetero multimer decreased in a time-dependent manner and fell to ~50% after 5-6 hours of subunit exchange. On further incubation, the protective ability decreased slightly and upon complete subunit exchange was comparable to that of the native lens α-crystallin hetero multimers. Thus, our studies showed that packing of subunits in the variable oligomer assembly leads to tertiary and quaternary structural changes as well as changes in chaperone-like activity[49]. This dynamic nature of α-crystallin may provide a mechanism for the modulation of chaperone-like activity[49]. It is also known that the ratio of αA- and αB-crystallin in eye lens hetero multimeric α-crystallin varies with age and during differentiation of lens epithelial cells to fibre cells[94-96]. It also varies between species[97,98]. In a mixture, αA and αB subunits completely exchanged regardless of their individual ratios[49]. This result suggests the equivalence of αA and αB subunits in the oligomeric assembly. Sun and Liang[21] found that at a 3:1 ratio of αA/αB, αA-crystallin is most efficient in the prevention of thermal aggregation of a target protein. This suggests that packing induced alterations with different ratios of αA and αB-crystallin may lead to different extents of chaperone activities of the hetero multimers.

5 Conformational Status of Target Protein Recognized by α-Crystallin : Formation of Stable Complexes

The chaperone-target protein interactions appear to be non-specific with respect to the target proteins but

specific in terms of the conformational states of the target protein which interact with the chaperone. For example, GroEL interacts with the molten-globule state of target proteins[99]. The molten-globule state of a protein is characterized by the presence of native-like or significant amount of secondary structural elements with little or no tertiary structural packing and enhanced exposure of buried hydrophobic surfaces[100-102]. Molecular chaperones from the Hsp70 family are known to bind target proteins in the extended conformation[103]. What are the conformational features of the target proteins that α-crystallin recognizes in preventing the aggregation of proteins? In order to address this question, we studied the interaction of several proteins with α-crystallin.

We investigated the aggregation properties of human and bovine carbonic anhydrase which is a metalloprotein having Zn^{2+} ion as a co-factor. Inspite of sharing a high degree of sequence homology, we found that human carbonic anhydrase (HCA) aggregated at 60°C whereas the aggregation of bovine carbonic anhydrase (BCA) at this temperature was negligible. Upon addition of metal chelators such as EDTA and ortho-phenanthroline, BCA also aggregated at 60°C with a biphasic transition. On the other hand, the presence of EDTA, had almost no effect on the thermal aggregation of HCA. These results suggested that removal of Zn^{2+} had a significant effect on the stability of BCA but not on that of HCA. Fluorescence and CD studies showed that removal of Zn^{2+} by a chelator such as EDTA enhanced the propensity of the enzyme to adopt the molten-globule state. The presence of α-crystallin prevented the aggregation of BCA as well as that of HCA. Taken together, these results suggested that α-crystallin recognises the molten-globule state of the enzyme and prevents its aggregation. In an earlier study, Rao *et al.*[104] had shown that when carbonic anhydrase is heat-denatured in the presence of α-crystallin, it forms a stable complex with the enzyme. We have shown the HCA bound to α-crystallin in the complex exhibits molten-globule-like properties by fluorescence, ANS-binding and near and far UV-CD studies[52]. Das *et al.*[105] have also shown earlier that the conformational state of γ-crystallin bound to α-crystallin is compact in nature.

Though these results demonstrated that carbonic anhydrases bind α-crystallin in their molten-globule form[52], it was not clear whether the molten-globule state was the only structural requirement that target proteins must possess for interaction of α-crystallin or if other non-native states would also be recognised by α-crystallin. α-Lactalbumin (LA) partially denatures under different conditions to form intermediates having varying amounts of secondary and tertiary structures[106]. α-LA is a calcium-binding protein. The unfolding properties of α-LA have been extensively studied and it has been used as a model protein exhibiting three-state unfolding properties[107,108]. It can partially denature into distinct intermediates[100, 106-108], which can be obtained by (i) removal of the bound calcium ion at low ionic strength or above room temperature, (ii) reduction of the disulfide bonds using a reducing agent like DTT, (iii) acid-denaturation of α-LA.

The calcium-free form of α-LA exhibits a conformational state which has reduced tertiary structure and native-like secondary structure. However, this molten-globule structure does not interact with α-crystallin. The disulfide-reduced α-LA which also adopts a molten-globule-like state, but which differs from the calcium-removed state, also did not interact with α-LA. We observed that reduced apo- α-LA at pH 6.0 and in the presence of 150 mM NaCl aggregated readily and the presence of α-crystallin prevented this aggregation[53]. Lindner *et al.*[109] also observed an interaction between α-crystallin and an aggregation-prone, disordered molten-globule state of a single disulfide-reduced (cys 6-cys 120) and carboxymethylated α-LA. Our fluorescence and CD spectroscopic and gel-filtration studies showed that only the aggregation-prone molten-globule state of α-LA interacted with α-crystallin[53].

Das and Surewicz[110] have investigated the effect of α-crystallin on the thermal and refolding-induced aggregation of rhodanese and concluded that α-crystallin only recognizes intermediates on the unfolding pathway of proteins, but not refolding intermediates. We investigated the effect of α-crystallin on the refolding of denatured-reduced and denatured-disulfide intact hen egg white lysozyme and bovine pancreatic RNase A[54]. We had earlier shown that lysozyme forms an aggregation-prone refolding-competent intermediate on its refolding pathway[111]. α-Crystallin prevented the aggregation and oxidative refolding of denatured-reduced lysozyme while it had no effect on the refolding of denatured-reduced RNase A[54]. As mentioned earlier, we also found that α-crystallin prevented the refolding-induced aggregation of β_L-crystallin[43,45]. These results clearly showed that

α-crystallin can recognize aggregation-prone molten-globule like intermediates not only in the unfolding pathway but also on the refolding pathways. The fact that it does not affect the oxidative refolding of RNase A, shows that it does not interact with proteins in randomly coiled conformation[54].

Based on our own studies[52-54] as well as those from other laboratories[105, 109, 112], we concluded that (i) α-crystallin-bound target proteins exhibit properties like those of molten globule states of proteins; it does not interact with extended states or randomly coiled conformations of target proteins which exhibit less hydrophobicity; (ii) α-crystallin does not interact with compact, non-aggregating molten-globule states of target proteins but interacts only with the aggregation-prone molten-globule states of target proteins to form stable complexes; and (iii) α-crystallin interacts with molten-globule like states of proteins not only on their unfolding pathways but also on their refolding pathways.

6 Transient or Reversible Interactions: Effect of α-Crystallin on Thermal Protection and Reactivation of Enzymes

α-Crystallin has been shown to protect some enzymes such as catalase[113], sorbitol dehydrogenase[114], glyceraldehyde-3-phosphate dehydrogenase[115] and also some labile restriction enzyme such as NdeI[116] from heat-inactivation. However, the molecular mechanisms involved in preventing thermal inactivation have not been completely understood. Though molecular chaperones exhibit certain common properties such as binding to partially unfolded states and preventing their aggregation, they differ in their ability to confer protection against heat-induced inactivation of enzymes. For example, Hsp90 stabilizes citrate synthase in heat-induced inactivation, while GroEL accelerates the inactivation process at high concentrations[117,118]. The reason for such differences is also not known so far.

We, therefore, studied the thermal inactivation and aggregation of citrate synthase and the role of recombinant human αA- and αB-crystallin in preventing the aggregation and reactivation[119]. Citrate synthase gets inactivated at 43°C. The intermediates formed during its thermal denaturation have been well-characterized[117,118]. The enzyme, on thermal denaturation, initially forms reactivatable intermediates that do not aggregate to an appreciable extent. It forms aggregation-prone late intermediates. Citrate synthase is, therefore, an ideal system to study the interactions of α-crystallin with each of these intermediates. We have shown that aggregation-prone partially unfolded state(s) of the enzyme is significantly populated at 43°C and at higher temperatures. We found that both αA- and αB-crystallin prevented the aggregation of citrate synthase at 43°C, αB-crystallin being more potent in preventing aggregation than αA-crystallin[119].

We investigated the inactivation kinetics of the enzyme at 43°C and the effect of αA- and αB-crystallin on it[119]. The enzyme lost 70% activity in 5 min while it did not show significant aggregation at this time. Both αA- and αB-crystallin reduced the rate of inactivation and thus conferred thermal protection to the enzyme. αA- and αB-crystallin thus protect enzyme activity as well as prevent aggregation, αB-crystallin being more effective than αA-crystallin. The temperature-induced unfolding pathway of citrate synthase has been shown to involve two early unfolding intermediates which are (i) dimeric, (ii) less aggregation-prone and (iii) able to bind to the substrate oxaloacetate and refold to the native state[117,118]. These intermediates kinetically partition to late unfolding intermediates that are aggregation-prone. Gel-filtration studies showed the early unfolding intermediate(s) of citrate synthase did not form a stable complex with α-crystallin while the late aggregation-prone intermediate(s) of citrate synthase formed a stable complex. In earlier studies, the complex between α-crystallin and glyceraldehyde-3-phosphate dehydrogenase, as well as that between α-crystallin and sorbitol dehydrogenase exhibited some enzyme activity[114,115]. In our study, the fractions corresponding to the citrate synthase-α-crystallin complex did not exhibit enzyme activity. This suggested that α-crystallin can transiently or reversibly interact with the early unfolding intermediates of citrate synthase. Since the early unfolding intermediates are known to bind oxaloacetate and such binding facilitates the refolding of those intermediates to the native state, we have investigated whether αB-crystallin increases the populations of these early unfolding intermediate states, which can be refolded to active state by oxaloacetate. Our results showed that the population of the early unfolding intermediates is indeed increased significantly by αB-crystallin.

Based on these results, we propose a mechanism for the chaperone-like activity of α-crystallin towards the heat-induced denaturation of citrate synthase (Fig 2)[119]. Transient and reversible interactions of αB-crystallin with the early unfolding intermediates

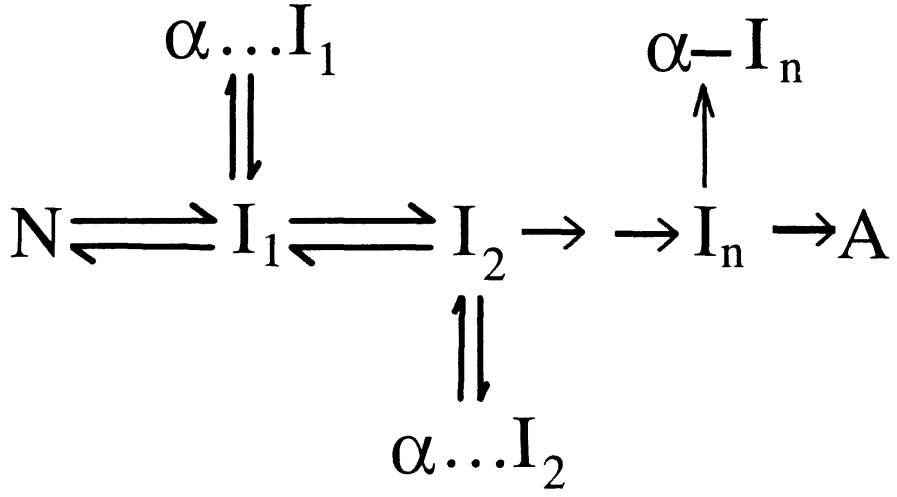

Fig. 2 Schematic representation of the possible mechanism by which αA- and αB-crystallin protect citrate synthase from thermal denaturation. N represents native enzyme, I_1 and I_2 represent the early unfolding intermediates, which can be reactivated by the addition of oxaloacetate, I_n represents the late aggregation-prone intermediate, while A represents the aggregated protein. α...I_1 and α...I_2 represent transient/reversible interaction. α—I_n represents a stable complex[119].

(I_1 and I_2) of citrate synthase inhibit their partitioning to late aggregation-prone intermediates (I_n). This leads to the observed increase in the equilibrium population of the early unfolding intermediate at any given time. αB-crystallin binds stably to the late aggregation-prone intermediates and prevents their aggregation. It is, therefore, evident that the chaperone-like activity of α-crystallin towards heat-induced denaturation of citrate synthase involves two distinct mechanisms. (i) The chaperone molecule interacts transiently with the early unfolding intermediates, which are in reversible equilibrium with the native state. This limits the conversion of these early unfolding intermediates to the aggregation-prone intermediates. These intermediates refold to their native state on removing the denaturing conditions, which is observed as prevention of heat inactivation by α-crystallin. (ii) The chaperone molecule interacts with the late unfolding, aggregation-prone intermediates to form a stable complex, thereby preventing these intermediates from aggregating out of solution. These mechanisms might be involved in the chaperone-like activity of α-crystallin towards heat-induced denaturation of proteins, in general.

We have also studied the heat inactivation of two other enzymes, malate dehydrogenase and catalase[119]. Both αA- and αB-crystallin confer protection on all the three enzymes during thermal inactivation, αB-crystallin being more effective than αA-crystallin. However, the extent of protection offered by these homomultimers, differs significantly between the enzyme systems. The reasons for these observed differences in protection between different enzyme systems are not clear. However, factors that dictate the extent to which these enzymes get inactivated in the presence of these homomultimers may include the following: (i) Whether or not the heat-induced unfolding pathway of the enzyme is significantly populated by early unfolding intermediate states in equilibrium with the native state that are capable of interacting with α-crystallin. α-Crystallin may not affect the inactivation kinetics in those cases, where the unfolding pathway involves a highly co-operative transition from the native state to the aggregation-prone intermediate states, as it would form stable complexes with those intermediates. (ii) The rate at which the early unfolding intermediate(s) partitions to the aggregation-prone intermediate states which leads to irreversible aggregation. It, therefore, appears that the nature of the intermediate states of the target proteins and their differential interactions with α-crystallin is the determining factor for the observed effect of thermo-protection of the chaperone offered to enzymes[119].

Despite the demonstration of the anti-aggregation property of α-crystallin and sHSPs, there are only a few reports, which indicate that sHSPs can reactivate proteins. Unequivocal evidence for its role in refolding enzymes to their native state has not been obtained earlier. α-Crystallin was, therefore, referred to as chaperone-like or junior chaperone[14]. Ganea and

Harding[115] studied the refolding of glyceraldehyde-3-phosphate dehydrogenase from its GdmCl-denatured state, both in the absence and in the presence of α-crystallin. They showed that the presence of α-crystallin in the refolding buffer increased the recovery of the activity of glyceraldehyde-3-phosphate dehydrogenase. Rawat and Rao[120] demonstrated that xylose reductase, which could not be refolded from its GdmCl-denatured state, could be refolded to its active state in the presence of α-crystallin. They have shown that α-crystallin interacts with an intermediate state of xylose reductase with properties of a molten-globule[120].

We have studied the refolding of ζ-crystallin from its different denatured states in urea[121]. Zeta-crystallin, a 140 kDa tetrameric protein comprising four identical subunits of molecular mass 35 kDa[122], can serve as a good model target protein to investigate chaperone property of α-crystallin because (i) it aggregates at 42°C – close to the physiological heat-shock temperature, (ii) it possesses quinone oxidoreductase activity, which can be easily assayed, and (iii) it binds the nucleotide co-factor NADPH which can be used as a spectroscopic reporter to assess changes in its structure[57].

Urea-induced inactivation profile of the enzyme showed that the enzyme is almost completely inactivated by 2 M urea[121]. On refolding the enzyme from various concentrations of urea, we found that the enzyme regained almost complete activity when refolded from urea concentrations upto 2 M. However, when the enzyme was refolded from urea concentrations above 2 M urea, the extent of activity regained dropped sharply and was negligible beyond 2.5 M urea. We investigated the role of α-crystallin in refolding zeta-crystallin. Since our studies had shown that structural perturbation enhances the chaperone-like activity of α-crystallin[41,42,44], and co-refolding α-crystallin with β- and γ-crystallin is more effective in preventing their aggregation[45], we co-refolded zeta-crystallin and α-crystallin from different concentrations of urea. While the activity regained when zeta-crystallin was refolded from 2.5 M urea was <10%, upon co-refolding with α-crystallin, the activity regained was ~54%. Enhancement of reactivation by α-crystallin was seen at 2.25 M and 3 M urea, however, α-crystallin could not improve the reactivation yield at urea concentration beyond 3M. αA- and αB-crystallins also show an enhancement in the activation of zeta-crystallin. However, αB-crystallin is more efficient than αA-crystallin in this process. These results reinforce again that αB-crystallin is more efficient as a chaperone than αA-crystallin.

Since α-crystallin improved the reactivation yields upon refolding ζ-crystallin only from its denatured states in certain concentrations of urea, we have investigated the unfolding pathway of ζ-crystallin[121]. Fig. 3 schematically describes the unfolding pathway of ζ-crystallin and the effect of α-crystallin. Our study demonstrated that α-crystallin assists the refolding and assembly of the monomeric, partially unfolded ζ-crystallin with properties of a molten-globule to its native tetrameric state, by inhibiting the partitioning of this intermediate to aggregation.

Taken together, our studies show that α-crystallin interacts with partially unfolded states with molten-

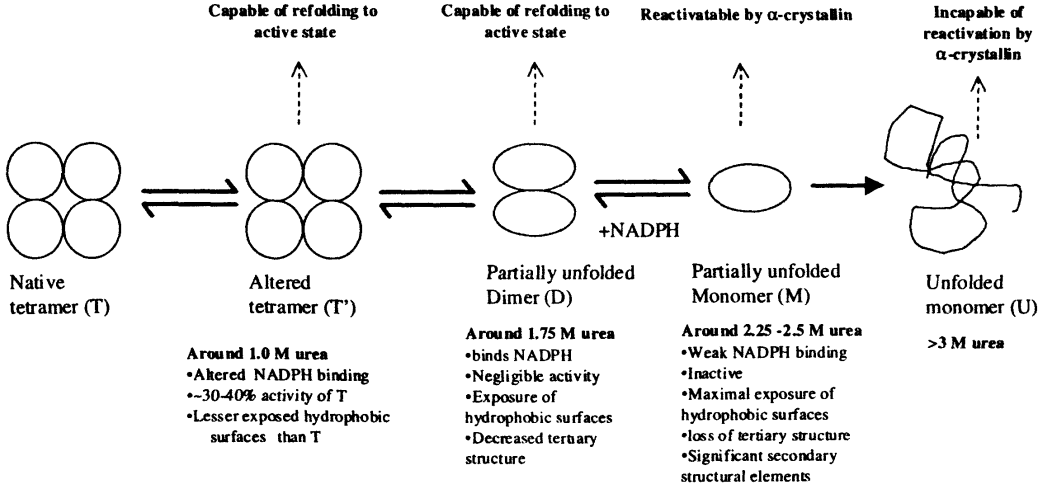

Fig. 3Schematic representation of the urea-induced intermediates formed on the unfolding pathway of ζ-crystallin showing the states capable of refolding and those requiring the assistance of α-crystallin[121].

globule-like properties. However, such intermediate states of some proteins, depending on the extent of unfolding and/or the extent of exposed hydrophobicity can interact with α-crystallin either transiently/ reversibly, which leads to regain of their activity or permanently to form stable complexes. Whether the permanently bound target protein could be released by some means to allow the refolding of the target protein is not well-established. Although α-crystallin has been shown to bind ATP[123,124] like other molecular chaperones, the role of ATP-binding in its activity is poorly understood. The reactivation of xylose reductase by α-crystallin was found to be facilitated by the presence of ATP, however, the non-hydrolysable ATP analogue, AMP-PNP could also facilitate reactivation suggesting that hydrolysis of ATP in not a prerequisite[120]. ATP-binding appears to increase the chaperone activity of αB-crystallin in the refolding of citrate synthase; however, in this case the non-hydrolysable ATP analogues had no effect on its reactivation[125]. Considering the properties of α-crystallin in aggregation and reactivation of proteins, it is appropriate to describe α-crystallin, not just as a junior chaperone or chaperone-like but as a heat shock protein with molecular chaperone activity. However, the role of ATP-binding and/or hydrolysis on the chaperone activity of α-crystallin still remains to be explored.

7 α-Crystallin and Disease

αB-Crystallin is known to interact with intermediate filaments and probably helps in maintaining the integrity of these filaments[126,127]. Under conditions of ischemic stress where there is acidification in the cytosol, the binding affinity of αB-crystallin to desmin increases considerably[128]. Patients suffering from DRM show skeletal muscle weakness and hypertrophic cardiomyopathy. A point mutation in αB-crystallin (R120G) has been shown to be associated with DRM as well as cataract[32]. This arginine residue is conserved through out the small heat shock family of proteins. The mutation of the corresponding arginine (R116) to cystine in αA-crystallin is shown to cause congenital cataract [31]. In order to understand the molecular basis of these diseases, we have studied the structural and functional consequences of these mutations. We cloned human αA- and αB-crystallin genes and mutated the conserved Arg residue to Cys and Gly respectively, by site-directed mutagenesis[50]. Near-UV CD spectroscopy of mutant αA-crystallin (R116C αA) revealed changes in the 275-295 nm region of the spectrum, contributed by tryptophan residues. The near-UV CD spectrum of R120GαB also showed alteration in this region. Intrinsic fluorescence spectroscopy showed that the emission maxima of the mutant protein did not change (337 nm) relative to those of wild-type αA- and αB-crystallin. However, there was a significant decrease in the fluorescence intensity suggesting that the solvent accessibility of the tryptophan residues did not change but the tryptophans were in a more flexible environment. Far-UV CD spectra of mutant αA- and αB-crystallin showed distinct changes in the region around 208 nm. Such changes had earlier been attributed to alteration in the packing of secondary structural elements. Taken together, the studies showed significant tertiary structural changes including altered packing of secondary structural elements. Gel permeation chromatography revealed polydispersity for both the mutant proteins. We observed an appreciable increase in the molecular mass of R116C αA-crystallin. The apparent molecular mass of the R120GαB mutant was ~720 kDa; the profile suggested the presence of species having larger and smaller molecular masses than wild-type αB-crystallin. However, the extent of polydispersity was much lesser than that observed for R116CαA mutant. Interestingly, the mutations, R116C and R120G, decreased to a great-extent the chaperone-like activity of both αA- and αB-crystallin respectively[50] as shown in Fig. 4. Similar studies by Bova *et al.*[129] and Perng *et al.*[130] also showed that the aberrant R120G mutant protein forms bigger and more polydisperse multimers than the wild-type molecule, has reduced chaperone-like activity and also alters its interaction with intermediate filaments. Shroff *et al.*[131] studied the R116C mutation in αA-crystallin and found that the mutant protein formed large multimers with intermolecular disulfide bonds, altered structure and loss of chaperone-like activity. These results showed that mutation of the conserved arginine residue impaired the structure and the chaperone-like activity of αA- and αB-crystallin. Loss of chaperone activity and consequent protein precipitation appears to be the molecular basis of the pathology.

Expression of αB-crystallin is also enhanced in the case of Alexander's disease, which is characterized by the presence of Rosenthal's fibres[13]. Its expression has also been observed in scrapie-infected hamster brain cells[132] and in fibroblasts from patients with the Werner syndrome[133]. αB-Crystallin and sHsp27 occur at increased levels and colocalize with amyloid plaques in Alzheimer's disease[18,134,135]. The interaction

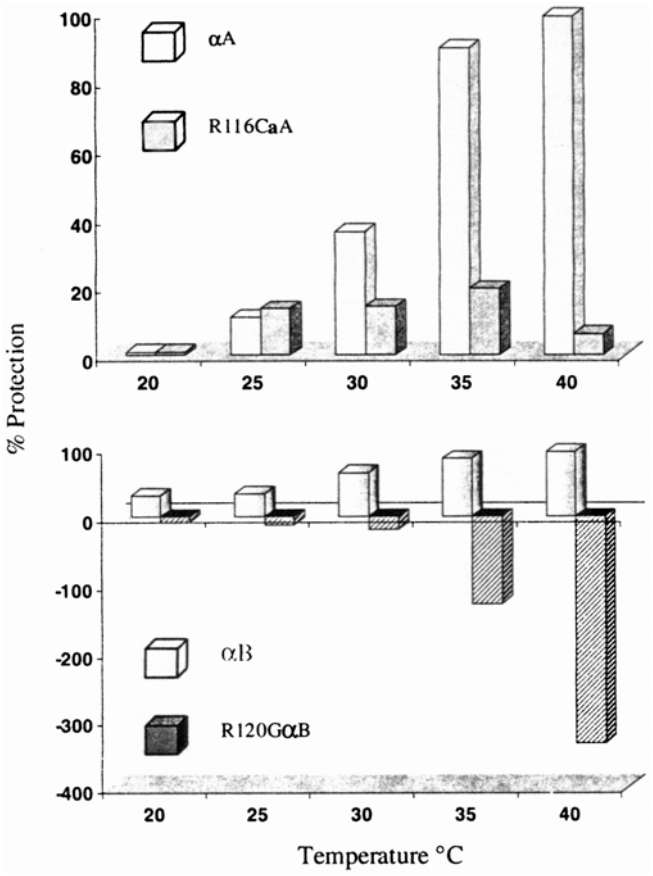

Fig. 4 The chaperone activity of αA- and αB-crystallin and the R116CαA and R120GαB mutants towards the DTT-induced aggregation of insulin as a function of temperature. Percentage protection represents the percentage prevention of aggregation of insulin

of αB-crystallin with the amyloidogenic peptide,Aβ 1-42, appears to be controversial: Stege *et al.*[136] have reported that αB-crystallin prevents the fibril formation while Liang[137] has reported that αB-crystallin promotes fibril formation. αB-Crystallin has been shown to inhibit amyloid formation by apolipoprotein C-II - it interacts with the partially structured amyloidogenic precursors and inhibits nucleation of amyloid formation but cannot inhibit elongation of fibrils[33]. αB-Crystallin is known to be present in heart tissues and its expression is increased under ischemic conditions[29]. Although limited studies showed αB-crystallin's role in protection of tissues, the exact role and the molecular mechanism are not understood. Future studies in the field should throw light on such aspects.

8 Domains of α-Crystallin and Their Significance

As mentioned earlier, the members of the sHSP family proteins contain a very conserved stretch of about 80-100 amino acids called the "α-crystallin domain". The α-crystallin domain is flanked by a non-conserved, largely hydrophobic N-terminal domain and a small, unstructured C-terminal extension[78-80]. Both, the N-terminal domain and the C-terminal extension vary in length and sequence[78-80]. Understanding of the role of individual domains on the overall structure and chaperone function would be useful in the absence of high resolution structural details of α-crystallin. The "α-crystallin domain" is involved in inter-subunit interactions[138-139] as well as in target protein binding[65,66]. The N-terminal domain is believed to help in the assembly of functional, high molecular mass oligomers[140,141].

The N-terminal domains and the C-terminal extensions of αA- and αB-crystallin differ significantly. As seen earlier, these two proteins differ significantly in their chaperone activity. To investigate the role of the N-terminal domains in the differential structural and functional properties of αA- and αB-crystallin, we swapped their N-terminal domains coded by exon 1[51]. We used a unique Xmn I restriction site at the beginning of nucleotide sequence encoding the "α-crystallin domain" in the αA-and αB-crystallin genes to create chimeric proteins with swapped N-terminal domains of αA- and αB-crystallin, αANBC and αBNAC[51]. Far-UV CD studies showed β-sheet structures for the chimeric proteins. The far-UV CD spectrum of αANBC was comparable to that of wild-type αA- and αB-crystallin, while αBNAC showed increased ellipticity. The near-UV CD spectrum of αANBC was comparable to that of αB-crystallin, with increased chirality for αANBC, whereas the near-UV CD spectrum of αBNAC was similar to that of αA-crystallin. Gel-filtration studies showed a large increase in oligomer size of αBNAC while the oligomer size of αANBC was comparable to that of wild type αB-crystallin. The most significant outcome of these studies is an engineered protein, αBNAC, that has several-fold higher activity compared to the wild-type proteins[51] as shown in Fig. 5. This engineered protein with considerably higher activity should be of value in biotechnology.

The role of the C-terminal extensions in structure and chaperone-activity is not clear. It is polar, unstructured, and flexible and is believed to function as a 'solubilizer' of the α-crystallin-target protein complex[142] as deletion of 17 residues from the C-terminal of αA-crystallin (R 157STOP) was shown to

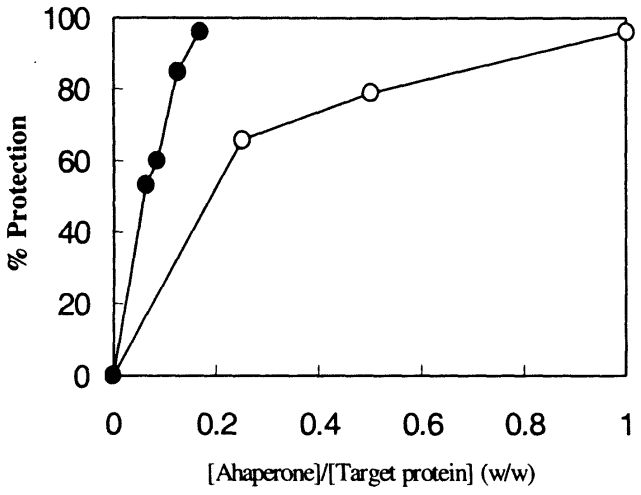

Fig. 5 The chaperone activity of αB-crystallin (o) and the N-terminal domain-swapped chimeric protein, αBNAC (•) towards the DTT-induced aggregation of insulin at 37°C with different ratios (w/w) of the chaperone to target protein. αB-Crystallin exhibits almost 100% protection at the ratio of 1 whereas the chimeric protein exhibits almost 100% protection at the ratio of 0.17 itself.

have marked decrease in chaperone-like activity[143]. We have swapped the C-terminal extensions between αA- and αB-crystallin and studied the chimeric proteins, αABce and αBAce, with respect to their wild-type counterparts (unpublished results). αBAce exhibited dramatically enhanced chaperone activity compared to the wild-type proteins. The other chimera, however, lost most of its activity. Significant changes in tertiary and quaternary structures and exposed hydrophobic surfaces were observed upon swapping the C-terminal extensions. Our study demonstrates that the C-terminal extensions play a crucial role in the structure and chaperone activity of α-crystallins. It is noteworthy that the two sHSPs whose crystal structures are available, *M.jannaschii* Hsp16.5 and wheat Hsp 16.9, suggest that the C-terminal extension is involved in subunit interactions.

Thus, our domain swapping experiments show that the N-terminal swapped chimeric molecule, αBNAC, and the C-terminal extension swapped chimeric molecule αBAce exhibited several fold increased chaperone activity compared to wild-type counterparts. Presently, we are trying out the possibility of using these molecules in biotechnological applications.

9 Enhancing the Chaperone Activity of α-Crystallin by External Agents

Our studies showed that temperature and low concentrations of urea can perturb the structure and enhance the chaperone activity of α-crystallin[41-43]. Our protein engineering approach yielded domain-swapped chimeric molecules of αA and αB-crystallin such as αBNAC and αBAce which exhibit enhanced activity. Structural perturbation of α-crystallin appears to be important for enhancement of its chaperone-like activity. If biologically compatible small molecules could be used to cause a structural perturbation and enhancement of chaperone-like activity of α-crystallin, they would be of therapeutic importance. Clark and Huang[144] studied the effects of two cellular metabolites – glutathione and pantethine – on the chaperone-like activity of α-crystallin and showed that these could enhance its apparent anti-aggregation activity. We have tested several biologically compatible molecules and have found at least one compound that enhances the chaperone-like activity of α-crystallin significantly (unpublished data). We believe that these findings would be expected to yield promising strategies in general health and disease management.

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