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## Temperature-dependent structural and functional properties of a mutant (F71L) $\alpha$ A-crystallin: Molecular basis for early onset of age-related cataract

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### Abstract

Previously we identified a novel mutation (F71L) in the  $\alpha$ A-crystallin gene associated with early onset of age-related cataract. However, it is not known how the missense substitution translates into reduced chaperone-like activity (CLA), and how the structural and functional changes lead to early onset of the disease. Herein, we show that under native conditions the F71L-mutant is not significantly different from wild-type with regard to secondary and tertiary structural organization, hydrophobicity and the apparent molecular mass of oligomer but has substantial differences in structural and functional properties following a heat treatment. Wild-type  $\alpha$ A-crystallin demonstrated increased CLA, whereas the F71L-mutant substantially lost its CLA upon heat treatment. Further, unlike the wild-type  $\alpha$ A-subunit, F71L-subunit did not protect the  $\alpha$ B-subunit in hetero-oligomeric complex from heat-induced aggregation. Moreover, hetero-oligomer containing F71L and  $\alpha$ B in 3:1 ratio had significantly lower CLA upon thermal treatment compared to its unheated control. These results indicate that  $\alpha$ -crystallin complexes containing F71L- $\alpha$ A subunits are less stable and have reduced CLA. Therefore, F71L may lead to earlier onset of cataract due to interaction with several environmental factors (e.g., temperature in this case) along with the aging process.

### Keywords

Age-related cataract; F71L-mutation;  $\alpha$ A-crystallin; Chaperone-like activity; Thermal stability

## 1. Introduction

Crystallins are the major structural proteins of the eye lens and whose short ordered arrangement provides the physical basis for the lens transparency [1,2]. Impaired lens function, due to partial or complete loss of transparency is called cataract, a leading cause of

blindness worldwide [3].  $\alpha$ -Crystallin, a small heat-shock protein (sHSP<sup>1</sup>) characterized by the presence of a conserved ‘ $\alpha$ -crystallin domain’ at its C-terminal region, is one of the three major crystallins and constitutes about 40% of the total soluble proteins of the vertebrate eye lens [1,4]. Eye lens  $\alpha$ -crystallin is composed of two subunits,  $\alpha$ A and  $\alpha$ B, encoded by *CRYAA* and *CRYAB* genes, respectively. Chaperone-like activity (CLA) of  $\alpha$ -crystallin is considered to be critical for the maintenance of eye lens transparency (reviewed in [1,4,5]). Studies with single and double knockout animals of  $\alpha$ A and  $\alpha$ B have highlighted the importance of  $\alpha$ -crystallin in lens transparency [6–8]. Further, certain point mutations in  $\alpha$ A- and  $\alpha$ B-crystallin genes are linked with non-syndromic, hereditary human cataracts [reviewed in 1,4]. However, many of these early onset cataracts are inherited by autosomal dominant mechanism that result in early onset either congenitally or relatively early in life.

Despite the availability of effective surgical treatment, cataracts still comprise a significant risk for visual impairment all over the world, particularly in older individuals. Cataract accounts for an estimated 16 million cases of blindness worldwide, with approximately half of all cases originating from Africa and Asia [3]. Age-related cataract (ARC) is generally considered as a multifactorial disease. While, epidemiological research has been focused mostly on the role of environmental risk factors, recent studies indicated a contribution of genetic factors in the pathogenesis of ARC [9,10]. Genetic predisposition in association with other etiological factors may contribute to ARC. Although, evidence for the genetic component to the development of ARC is increasing, genetic variations associated with ARC are very few compared to those for congenital and hereditary cataracts. Recently we identified a novel point mutation (F71L) in exon-2 of *CRYAA* ( $\alpha$ A-crystallin) gene associated with early onset of age-related cataracts [11]. The age-at-onset of nuclear cataract in F71L patients was about 10 years earlier (50 years) when compared to the mean age-at-onset of nuclear cataract cases (60 years) in individuals without the mutation.

The effect of missense mutations on  $\alpha$ -crystallin structure and function have been extensively studied [12–17]. In general, point mutations in  $\alpha$ A-crystallin result in altered structure, loss of chaperone-like activity, and appear to lead to cataract formation and abnormal lens development [13–17]. While the F71L- $\alpha$ A-crystallin generally displayed a loss of CLA that varied (10–90%) depending upon the client proteins and assay conditions the mutation did not significantly affect the apparent molecular mass, secondary and tertiary structure and hydrophobicity of  $\alpha$ A-crystallin [11]. Sharma et al. [18] using a synthetic peptide (mini- $\alpha$ A-crystallin), have demonstrated the importance of sequence 70–88 in the chaperone-like function of  $\alpha$ A-crystallin. A later study demonstrated that Phe-71 is critical for CLA [19]. It is intriguing to note that the missense mutation (F71L) in  $\alpha$ A-crystallin did not cause major structural changes, but substantially lowered CLA [11]. Hence, the early age-at-onset of cataract in the individuals carrying F71L mutation in  $\alpha$ A-crystallin might be due to a partial loss of in vivo CLA or enhanced susceptibility to structural alterations induced by deleterious environmental factors along with aging. Structural reorganization of  $\alpha$ -crystallin due to thermotropic and other factors is known to modulate the CLA [1]. In this study we report temperature-dependent structural and functional alterations in F71L- $\alpha$ A-crystallin and the thermal sensitivity of the mutant protein that may provide a molecular basis for the early on-set of cataract due to the F71L mutation.

## 2. Materials and methods

### 2.1. Expression and purification of recombinant wild-type and F71L mutant $\alpha$ A-crystallins

The recombinant wild-type and F71L mutant  $\alpha$ A-crystallins were overexpressed in *Escherichia coli* and the proteins were purified to homogeneity according to previously reported methods [11,20]. The concentration of wild-type and F71L mutant  $\alpha$ A-crystallin proteins was estimated by Lowry method.

### 2.2. Preheat treatment

Structural perturbation in case of  $\alpha$ -crystallin generally refers (but not exclusive) to treatment of mild (low) concentrations of denaturants or heating to high temperatures followed by cooling it to ambient temperature (preheated). To understand thermotropic mediated structural and functional properties, the wild-type and F71L  $\alpha$ A-crystallins (1 mg/ml) were heated at 65 °C for 15 min in a water bath and allowed to cool-down to room temperature and referred henceforth as preheated wild-type  $\alpha$ A- and preheated  $\alpha$ A-F71L- $\alpha$ A-crystallin.

### 2.3. Size-exclusion chromatography

The apparent molecular mass of wild-type and F71L mutant  $\alpha$ A-crystallins under normal conditions and after preheating was determined by size-exclusion chromatography on a 600  $\times$  7.5 mm TSK-3000 SW column (Tosoh Co., Japan) using a Shimadzu HPLC system [21]. The column was equilibrated with 0.1 M sodium phosphate buffer, pH 6.9 containing 0.1 M sodium sulfate at a flow rate of 1 ml/min. Column was calibrated using standard molecular weight markers (thyroglobulin-669,  $\gamma$ -globulin-160, BSA-67, ovalbumin- 45 kDa).

### 2.4. ANS fluorescence studies

The surface hydrophobicity of normal and preheated  $\alpha$ A-crystallins was measured using a hydrophobic probe, 8-anilino-1-naphthalene sulfonic acid (ANS). 50  $\mu$ M ANS solution in 10 mM of sodium phosphate buffer was added to protein samples of 0.1 mg/ml and incubated in the dark at room temperature for 30 min. The samples were excited at 385 nm and the emission spectra were recorded between 400 and 600 nm using a Jasco-FP-6500 spectrofluorometer [11].

### 2.5. Circular dichroism studie

Secondary and tertiary structural changes of normal and preheated  $\alpha$ A-crystallins were investigated by far- and near-UV CD spectra in a Jasco-810 spectropolarimeter [11,21,22]. Protein concentrations of 0.1 mg/ml and 1 mg/ml were used for recording the far- and near-UV CD spectra respectively. The reported CD spectra are the average of five accumulations.

### 2.6. Chaperone-like assays

The ability of normal and preheated  $\alpha$ A-crystallins to prevent protein aggregation was assessed by using different target proteins under different assay conditions. Heat-induced aggregation of  $\beta$ L- and  $\gamma$ -crystallins at 60 °C and DTT-induced aggregation of insulin at 37 °C were monitored by measuring light scattering as a function of time at 360 nm using a

Lamda-35 spectrophotometer (Perkin– Elmer) according to previously described methods [20,22].

### 3. Results

The secondary and tertiary structures of wild-type and F71L mutant  $\alpha$ A-crystallins were determined under normal conditions and upon preheat treatment by far- and near-UV CD spectral analysis. The far-UV CD spectra of wild-type and F71L- $\alpha$ A-crystallins were similar suggesting no major differences in secondary structure (Fig. 1A). While the secondary structure of preheated wild-type  $\alpha$ A-crystallin is essentially unchanged, a significant alteration in secondary structure was observed with preheated F71L  $\alpha$ A-crystallin as evidenced by an increase in negative ellipticity in the region 210–220 nm indicating formation of beta-sheet rich structure (Fig. 1A). Although, the near-UV CD spectra for the two proteins were similar under normal conditions the near-UV CD spectra of preheated wild-type  $\alpha$ A-crystallin revealed alteration at tertiary levels as reported previously [22]. However, compared to preheated wild-type  $\alpha$ A-crystallin, preheated F71L- $\alpha$ A-crystallin showed dramatic changes in near-UV region, particularly loss of intensity between 270 and 290 nm region (Fig. 1B) which indicates a change in the chiral environment of tryptophan residues.

As reported previously elution profile of wild-type and F71L- $\alpha$ A-crystallin was found to be similar with an approximate oligomeric mass of 650 kDa (Fig. 2A). The preheated wild-type  $\alpha$ A-crystallin eluted just before its respective unheated control indicating an increase in the aggregate molecular mass (approx. 850 kDa) upon structural perturbation. However, F71L- $\alpha$ A-crystallin eluted even before the preheated wild-type  $\alpha$ A-crystallin (Fig. 2A). This indicates that preheat treatment of F71L- $\alpha$ A-crystallin led to formation of a predominantly large soluble aggregate of molecular mass >1000 kDa and suggests that temperature disrupts the integrity of the oligomeric assembly of the F71L mutant and thereby affects its stability. These observations are consistent with the previous studies that show thermally disintegration of the oligomeric structure with diminished chaperone activity for R116C and R116H  $\alpha$ A-crystallin mutants [23–25]. We have also shown earlier that  $\alpha$ B-homo-oligomer but not the hetero-oligomer with (3:1)  $\alpha$ A to  $\alpha$ B precipitated upon preheating indicating that  $\alpha$ A imparts thermal stability to the hetero-oligomer by preventing the aggregation of  $\alpha$ B-crystallin at higher temperature [21]. Therefore, we determined the mass of oligomeric complexes of normal and preheated hetero-oligomer with (3:1)  $\alpha$ A to  $\alpha$ B or  $\alpha$ A-F71L to  $\alpha$ B. Consistent with reported studies, preheating the hetero-oligomer of  $\alpha$ A and  $\alpha$ B prevented the aggregation of  $\alpha$ B-crystallin and eluted before its respective unheated heteropolymer (Fig. 2B). However, in contrast to wild-type  $\alpha$ A,  $\alpha$ A-F71L was not only able to protect  $\alpha$ B upon preheating of the hetero-oligomer of  $\alpha$ A-F71L and  $\alpha$ B, but it too precipitated along with  $\alpha$ B substantiating the role of  $\alpha$ A moiety in stabilizing the  $\alpha$ B-crystallin (Fig. 2B).

Consistent with the previous studies, the intensity of ANS fluorescence emission of preheated wild-type  $\alpha$ A-crystallin was higher than its corresponding unheated variant (Fig. 3). However, the ANS-binding to preheated F71L  $\alpha$ A-crystallin increased significantly than the preheated wild-type  $\alpha$ A-crystallin (Fig. 3) implying that the available hydrophobic

surface is increased appreciably. Notably, this increase in hydrophobicity did not result in a corresponding increase in CLA. Instead, heat-treatment caused a loss of CLA, suggesting that at higher temperatures the structural stability of F71L is altered due to unfolding and exposure of hydrophobic sites.

Previously we reported a conditional loss of CLA for F71L compared to  $\alpha$ A-crystallin, where the degree of loss was dependent on the aggregation assay conditions. We observed particularly greater losses of CLA when measurements were carried out using thermal aggregation assays [11]. It is therefore, important to study the CLA of preheated versions in the context of temperature-induced conformational changes of wild-type and F71L-mutant. Thus, the CLA of preheated wild-type and F71L- $\alpha$ A-crystallin was investigated by a battery of thermal and chemical aggregation assays. As reported previously by many studies, in the heat-induced aggregation of  $\beta$ L- and  $\gamma$ -crystallin, the preheated wild-type  $\alpha$ A-crystallin showed enhanced protection compared to normal wild-type  $\alpha$ A-crystallin (Fig. 4). In contrast, the preheated F71L-mutant showed a decreased CLA in suppressing the heat-induced aggregation of  $\beta$ L- and  $\gamma$ -crystallin compared to normal F71L- $\alpha$ A-crystallin (Fig. 4). Similar results were found in the heat-induced aggregation of citrate synthase assay even at 60 °C (data not shown). In the DTT-induced aggregation assay of insulin, the unheated F71L-mutant showed a marginal (10%) decrease in CLA compared to that of unheated wild-type  $\alpha$ A-crystallin, while the preheated wild-type protein exhibited enhanced CLA over its unheated variant (Fig. 5). However, the preheated F71L-mutant completely lost its chaperone-like activity and co-aggregated with the client protein in DTT-induced aggregation assay of insulin (Fig. 5). The increase in light scattering of preheated F71L- $\alpha$ A-crystallin due to co-aggregation in the insulin assay might be a result of non-specific hydrophobic interactions between F71L-mutant and reduced insulin B-chain. From the aggregation assays, it is apparent that the CLA of F71L-mutant is dependent on client protein but more so on temperature. Though F71L is a homozygous missense mutation [11], the in vitro studies described above were carried out on subunits of  $\alpha$ A and  $\alpha$ B separately. However, the situation in the eye lens is not same where the  $\alpha$ -crystallin is known to exist as a heteropolymer with  $\alpha$ A and  $\alpha$ B in 3:1 ratio [21,22,26]. Further, we have shown earlier very clearly that  $\alpha$ A subunit plays an important role in the heteropolymer in that it also provides stability to  $\alpha$ B [21,27]. Therefore, we have combined F71L- $\alpha$ A with  $\alpha$ B in 3:1 ratio and allowed subunits to exchange to equilibrium. Then, we investigated the effect of thermal stability on CLA. CLA of preheated (3:1)  $\alpha$ A to  $\alpha$ B was significantly increased compared to unheated (3:1)  $\alpha$ A to  $\alpha$ B in both  $\gamma$ -crystallin (Fig. 6) and  $\beta$ -crystallin assays (not shown). In contrast, CLA of preheated (3:1) F71L- $\alpha$ A to  $\alpha$ B was significantly lower compared to unheated (3:1) F71L- $\alpha$ A to  $\alpha$ B in both  $\gamma$ -crystallin (Fig. 6) and  $\beta$ -crystallin assays (not shown).

#### 4. Discussion

We have identified a novel mutation (F71L) in the exon-2 of CRYAA gene associated early-onset of ARC and reported that despite insignificant structural changes, the F71L mutant  $\alpha$ A-crystallin displayed significant loss of CLA, particularly in thermal aggregation assays performed at elevated temperatures [11]. Nevertheless, the reasons for the time lag shown by this mutation in the expression of a cataract phenotype around 50 years of age compared to

60 years without the mutation are not clear. It is plausible that some genes may be involved in adult cataract with late expression of phenotype due to interaction with several environmental factors during aging.

Eye lens  $\alpha$ -crystallin is a hetero-oligomer composed of two similar subunits,  $\alpha$ A and  $\alpha$ B, present in a 3:1 molar ratio in most vertebrates [26]. Previously, we showed that despite high sequence similarity, there is a dichotomy in structure and function of  $\alpha$ A- and  $\alpha$ B-crystallins that is consistent with a molecular basis for the existence of  $\alpha$ -crystallin hetero-oligomer with 3:1  $\alpha$ A to  $\alpha$ B ratio [20–22,27]. Our studies showed that  $\alpha$ A-crystallin is not only more stable but also imparts stability to the hetero-oligomer by preventing the aggregation of  $\alpha$ B-crystallin at higher temperatures. Interestingly,  $\alpha$ -crystallin complexes containing F71L subunits instead of wild-type  $\alpha$ A subunits were not only able to protect  $\alpha$ B-crystallin but also unable to suppress  $\alpha$ B-mediated co-aggregation of lens proteins [27]. In fact, F71L mutant precipitated upon heating to high temperatures and contributed to increased light scattering at high temperature in these studies. Generally, upon heating,  $\alpha$ -crystallin undergoes structural changes resulting in increased exposure of additional hydrophobic sites associated with increased CLA [1,20–22,28,29]. Once exposed to high temperatures, the protein upon cooling does not return to its original conformational state but adopts a conformation characterized by increased surface hydrophobicity and molecular mass (aggregate size) [1,29–31]. This increase in CLA of  $\alpha$ -crystallin upon structural perturbation is mediated by  $\alpha$ A-but not  $\alpha$ B crystallin. Therefore, in the present study we investigated alterations in the molecular chaperone-like function of  $\alpha$ -crystallin, and the associated structural changes, upon structural perturbation (preheat treatment at 65 °C for 15 min followed by cooling) of wild-type and F71L- $\alpha$ A-crystallins to understand the mechanism of early-onset cataract due to F71L mutation in  $\alpha$ A-crystallins.

On comparison of human recombinant wild-type and F71L- $\alpha$ A-crystallin by size-exclusion chromatography, fluorescence and far- and near-UV CD studies it appears that  $\alpha$ A-F71L missense mutation did not significantly affect the apparent molecular mass, secondary and tertiary structures or hydrophobicity of  $\alpha$ A-crystallin under normal conditions. However, the F71L- $\alpha$ A-crystallin displayed significant loss of CLA in thermal treatment but little to no loss or insignificant loss in chemical aggregation assays [11]. These observations were consistent with the previous studies made on replacement of Phe-71 with Gly (F71G) which did not indicate structural changes, but CLA was severely impaired in  $\alpha$ A-F71G crystallin [19]. It is reported that the Phe-71 contributes to the chaperone-like action of  $\alpha$ A-crystallin and the 70–88-region in  $\alpha$ A-crystallin is identified as the functional chaperone-like binding site in  $\alpha$ A-crystallin [19]. However, temperature-dependent studies on both the wild-type and the F71L mutant revealed an increase in the oligomeric mass, shift in tryptophan fluorescence emission wavelength, alteration in secondary and tertiary structure, and further loss of CLA in F71L-mutant protein suggesting that the heat stability of the protein was affected by the mutation.

The preheated wild-type  $\alpha$ A-crystallin exhibited a better protection in chaperone-like assays compared to wild-type  $\alpha$ A-crystallin. These observations support the hypothesis that the regions of  $\alpha$ A-crystallin become more exposed with temperature; a structural transition above 50 °C might therefore be important for its CLA [20,28–30]. In contrast, the preheated

F71L mutant exhibited a decreased CLA in all the aggregation assays despite a slight increase in hydrophobicity. Based on our and others studies, it has been shown that hydrophobicity plays an important role but it is not the sole determinant of  $\alpha$ -crystallin CLA [1,20,32]. Therefore, it is unlikely that all the exposed hydrophobic patches on F71L are involved in suppressing the substrate protein aggregation. The increase in ANS intensity might also be due to ANS-binding to residues other than those involved in chaperone-like activity [32,33]. Other factors like charge and structural integrity may influence the functional property to different extents. The preheated F71L mutant  $\alpha$ A-crystallin showed almost complete loss of chaperone-like activity in all the aggregation assays and it co-aggregated along with insulin in DTT-induced aggregation. These observations support the idea that the chaperone-like activity of the mutant was dependent on client protein properties along with environmental factors that enhanced aggregation of substrate proteins might be directly related to the onset of cataract [34].

Considering that the preheated F71L mutant has a larger hydrophobic exposure when compared to wild-type  $\alpha$ A-crystallin protein, the increase in amount of aggregation might be caused due to non-specific hydrophobic interactions between F71L mutant and the substrate protein. These results suggests that the impaired CLA of preheated F71L mutant might be due to altered specificity towards potential substrate proteins, since there is an alteration in its structure as evidenced by larger oligomers and changed CD spectra. Other mutations are known to decrease the overall structural stability of  $\alpha$ A-crystallin such as the G98R mutation [15] and other important proteins [34,35]. The temperature-induced conformational studies on F71L- $\alpha$ A-crystallin also indicated alterations in both structural stability and chaperone-like activity.

Heat stability studies corroborated the effect of temperature on the dynamic quaternary structure of the F71L mutant similar to that R116C mutant [36]. This study indicates that Phe-71 is not only essential for the chaperone-like activity, but is also important in maintaining the structural integrity of the protein at higher temperatures (it plays a role in thermal stability of the protein). These data suggest that the F71L mutant has a compromised structural stability, and in course of time (due to alterations in body temperatures and environmental factors) loses its native structural conformation, which effects its protein-protein interactions resulting in defective CLA and aggregation of potential client proteins. It might also be more susceptible to age-related modifications and contribute to the early-onset-cataract formation in individuals carrying the mutation. Size-exclusion chromatography studies with preheated hetero-oligomer with  $\alpha$ A-F71L to  $\alpha$ B in 3:1 ratio indicate that  $\alpha$ A-F71L precipitated along with  $\alpha$ B instead of stabilizing it.

Together the data presented in this study indicate that the F71L mutant is somewhat unstable even under physiological conditions. Considering that the protein, under the best of conditions, should last several decades of time, it seems likely that the early onset cataract at 5 decades may be reflecting the slow but insidious loss of the  $\alpha$ A subunit over time. Further, the presence of the mutant  $\alpha$ A subunit destabilized the wild-type  $\alpha$ B subunits, which resulted in a reduction in CLA and solubility following heat treatment. In vivo, this could be equivalent to an acceleration of  $\alpha$ -crystallin loss over time, which would slowly predispose the lens to cataract formation. From the present study, it is apparent that the molecular basis

for the development of cataract in the affected individuals might be loss of thermal stability of the protein due to substitution of Phe-71 which results in the formation of highly oligomerized  $\alpha$ A-crystallin with altered structural stability and defective chaperone-like activity.

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## Abbreviations

<b>ARC</b>	age related cataract
<b>ANS</b>	8-anilino-1-naphthalene sulfonic acid
<b>CLA</b>	chaperone-like activity
<b>PrHt</b>	preheated
<b>sHSP</b>	small heat shock proteins

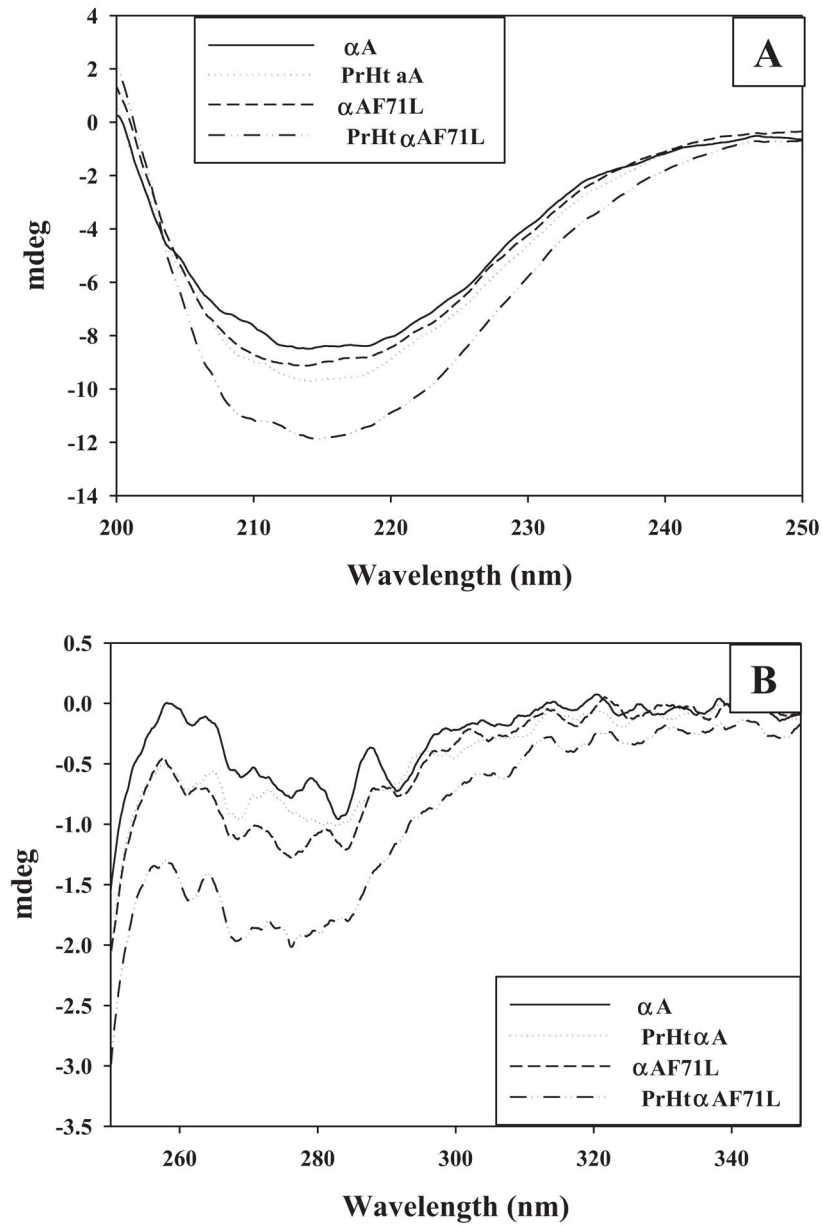
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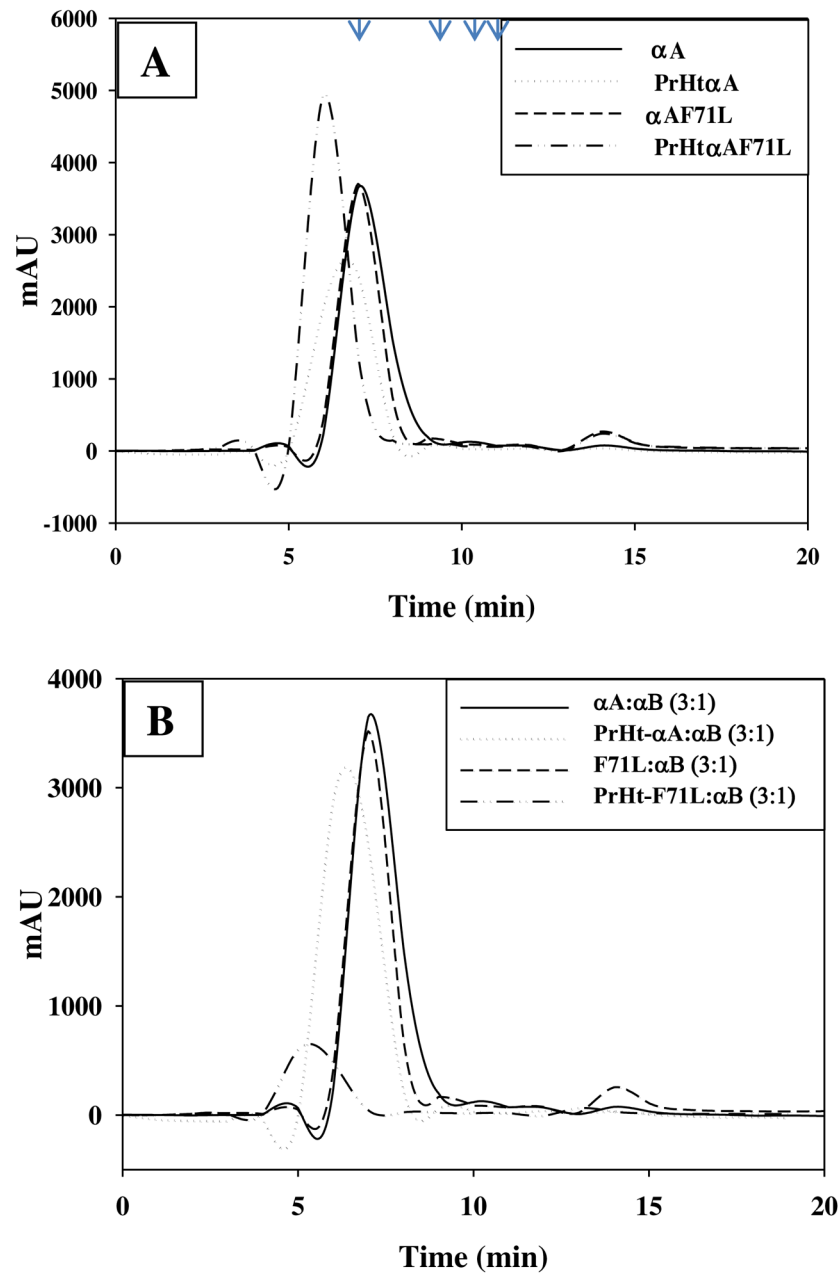


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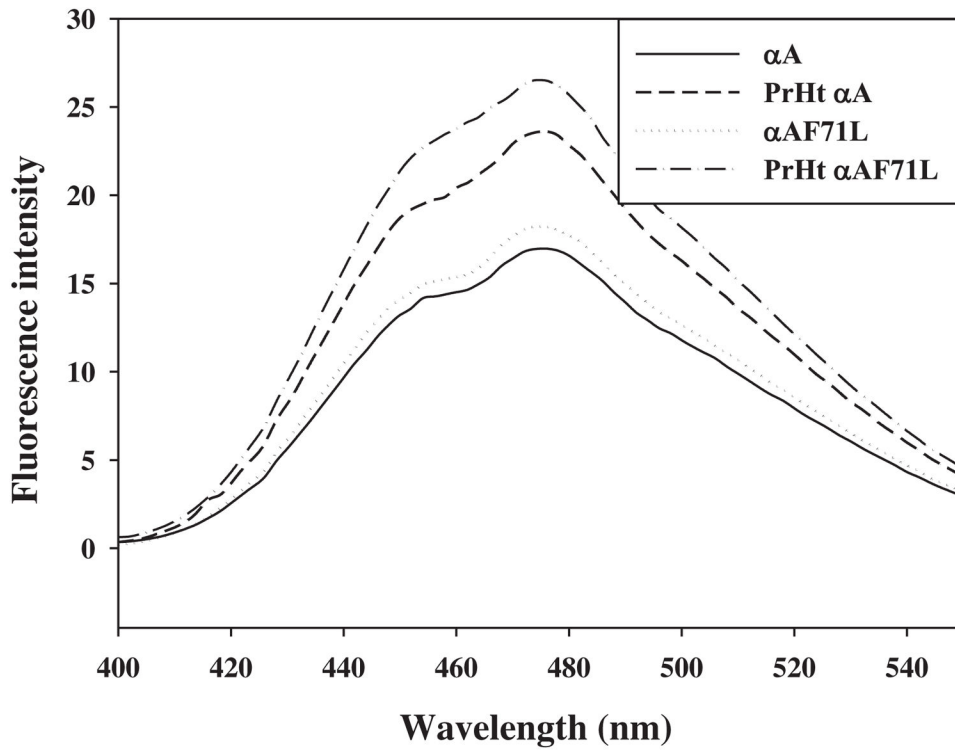
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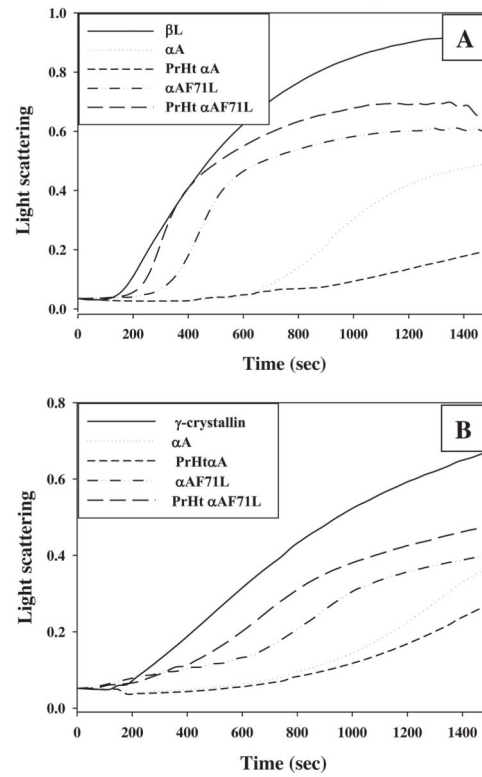
**Fig. 1.** Secondary and tertiary structure. Far-UV (A) and near-UV CD spectra (B) of normal and preheated wild-type and F71L- $\alpha A$ -crystallin. Data are representative of three independent experiments.



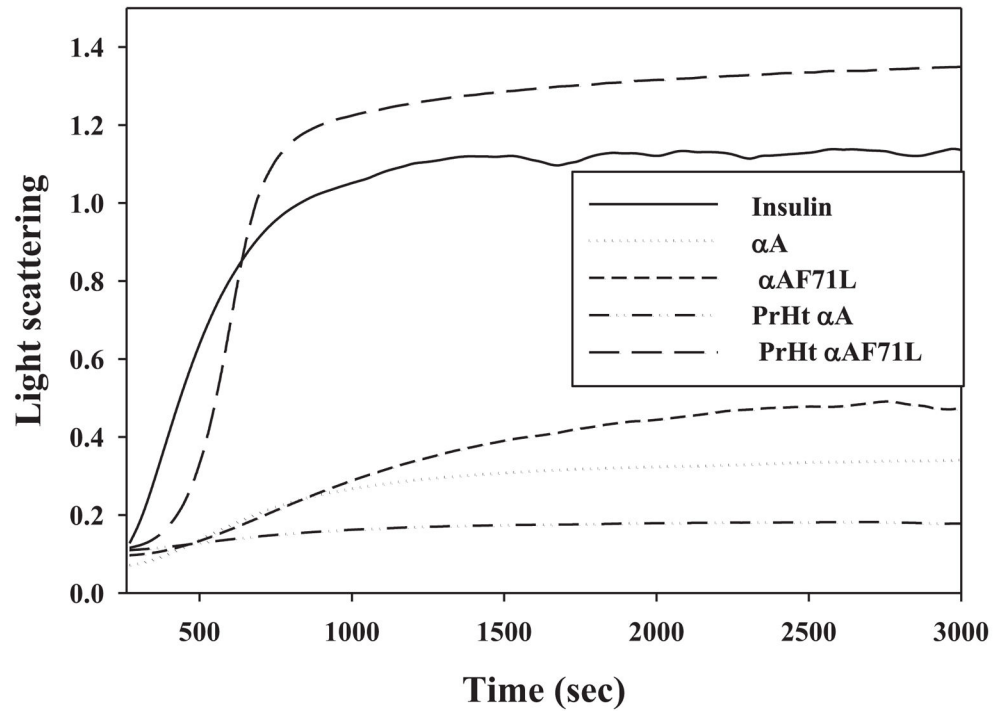
**Fig. 2.** Molecular mass. Size-exclusion chromatography profiles of normal and preheated wild-type and F71L mutant  $\alpha$ A-crystallin on a  $600 \times 7.5$  mm TSK-3000 SW column (A). Positions of molecular weight markers is shown in top with arrows; thyroglobulin-669,  $\gamma$ -globulin-160, BSA-67, ovalbumin-45 kDa. Data are representative of three independent experiments. Size-exclusion chromatography profiles of normal and preheated heteropolymer of  $\alpha$ -crystallin with (3:1)  $\alpha$ A to  $\alpha$ B or F71L- $\alpha$ A- to  $\alpha$ B (B). Data are representative of three experiments.



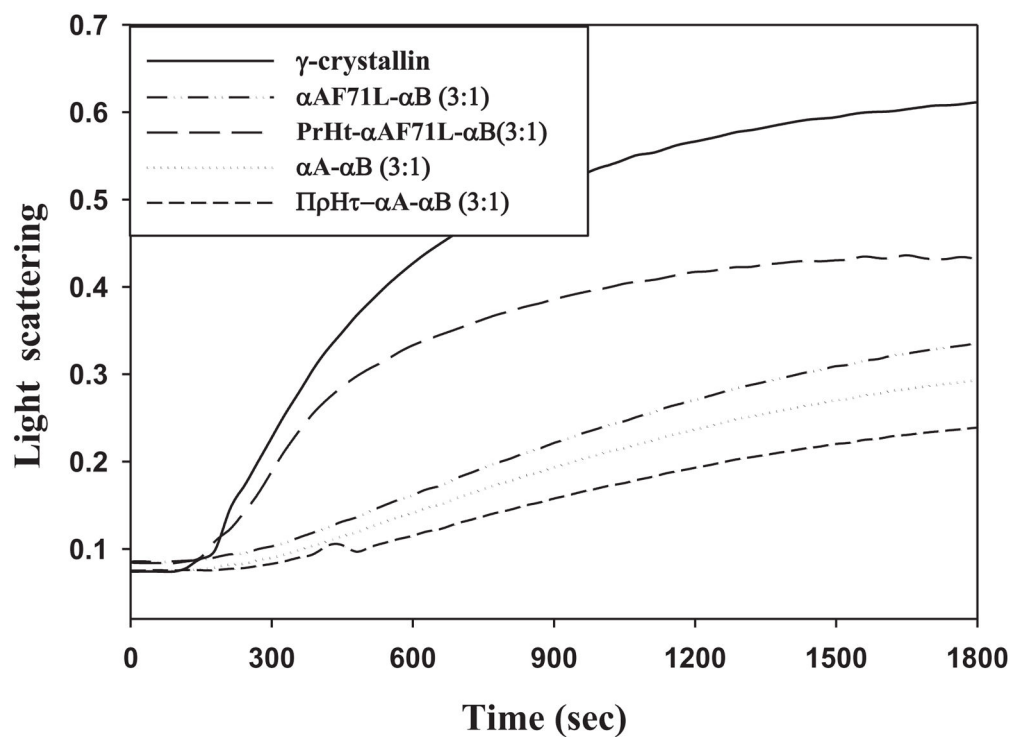
**Fig. 3.** Hydrophobicity. ANS-fluorescence spectra of normal and preheated wild-type and F71L- $\alpha A$ -crystallin. Data are representative of three independent experiments.



**Fig. 4.** Chaperone-like activity. Chaperone-like activity of normal and preheated wild-type and F71L- $\alpha$ A-crystallin (0.05 mg/ml) in heat-induced aggregation of  $\beta$ L-crystallin (0.25 mg/ml) (A) and  $\gamma$ -crystallin (0.25 mg/ml) assay (B) at 60 °C. Data are representative of three independent experiments.



**Fig. 5.** Chaperone-like activity. Chaperone-like activity of normal and preheated wild-type and F71L- $\alpha$ A-crystallin (0.4 mg/ml) in DTT-induced aggregation of insulin (0.3 mg/ml) assay at 37 °C. Data are representative of three independent experiments.



**Fig. 6.** Chaperone-like activity. Chaperone-like activity of normal and preheated hetero-oligomer of  $\alpha$ -crystallin with  $\alpha$ A to  $\alpha$ B or with F71L- $\alpha$ A- $\alpha$ B in 3:1 ratio (0.03 mg/ml) in heat-induced aggregation of  $\gamma$ -crystallin (0.25 mg/ml) assay at 60 °C. Data are representative of three independent experiments.