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Characterization of an N-terminal mutant of α A-crystallin α A–R21Q associated with congenital cataract

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Abstract

Several mutations associated with congenital cataracts in human beings target conserved arginine residues in α A-crystallin. The N-terminal region of α A-crystallin is a “mutational hotspot,” with multiple cataract-related mutations reported in this region. Two mutations at arginine 21 in the N-terminal domain of α A-crystallin — α A–R21L and α A–R21W have been associated with congenital cataract. A third mutant of R21, α A–R21Q, was recently identified to be associated with congenital cataract in a South Australian family. The point mutation was reported to compromise the quaternary structure of α A-crystallin by preventing its assembly into higher ordered oligomers. To assess the effect of the α A–R21Q mutation on α A-crystallin function, recombinant α A–R21Q was expressed, purified and characterized *in vitro*. Compared to wild-type α A-crystallin, the recombinant α A–R21Q exhibits enhanced chaperone-like activity, increased surface hydrophobicity, lesser stability in urea and increased susceptibility to digestion by trypsin. α A–R21Q demonstrated increased binding affinity towards unfolding ADH and bovine lens fiber cell membranes. α A–R21Q homo-oligomers and hetero-oligomers also prevented H₂O₂–induced apoptosis in ARPE–19 cells. Taken together, α A–R21Q exhibited a gain of function despite subtle structural differences as compared to wild-type α A-crystallin. This study further validates the involvement of arginine 21 in regulating α A-crystallin structure and function.

1. Introduction

The refractive properties and transparency of the eye lens are a result of the ordered arrangement of its constituent fiber cells and the short-range interactions of their proteinaceous content (Benedek, 1971; Boettner and Wolter, 1962; Tardieu, 1988). The crystallins – α , β and γ , account for 90% of the total soluble protein content of the eye lens, where they serve both structural and functional roles. Of the soluble proteins, α -crystallins (α A– and α B–) make up ~40% of the protein content and play a major role in mechanisms involved in promoting and preserving lens transparency (Jaffe and Horwitz, 1992). In the

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eye lens, α -crystallins exist as a hetero-oligomer of α A- and α B-subunits mixed in a molar ratio of 3:1 (de Jong WW.; Lubsen, 1994). As small heat shock proteins (sHSPs), both α A- and α B-crystallins have been shown to exhibit chaperone-like activity, and are believed to be responsible for suppressing non-specific aggregation of other lens proteins (Jaffe and Horwitz, 1992). The chaperone-like activity of α -crystallins is a subject of intrigue, as the eye lens maintains its transparency for the better part of an individual's lifetime despite the lack of blood supply and protein turnover. Structurally, both α A- and α B-crystallins comprise of three distinct domains – the N-terminal domain which approximately spans the first sixty residues, followed by the highly conserved α -crystallin domain (ACD) (~90–100 residues long), and the C-terminal extension (Derham and Harding, 1999). The chaperone-like activity of α -crystallins has been mapped to the ACD (Santhoshkumar et al., 2016; Sharma et al., 1998; Sharma et al., 2000), and studies have shown that the variable N- and the C-terminal domains are associated with maintaining the oligomerization and solubility of the proteins (Derham and Harding, 1999; Poulain et al., 2010).

Mutations in α -crystallins result in the development of cataracts. Genetic mutations in α -crystallins have been associated with the development of both congenital and age-related cataracts (ARC). In addition, studies on human lens crystallins from different age groups have shown an age-dependent increase in the accumulation of post-translational modifications on α -crystallins (Hains and Truscott, 2007; Hanson et al., 2000). The modifications seem to compromise their stability and chaperone-like properties, causing them to aggregate and precipitate, leading to ARC. Many α A-crystallin mutations associated with congenital cataract target conserved arginine residues. The N-terminal domain of α A-crystallin possesses four arginine residues that are associated with six different congenital cataract mutations. One arginine residue at position 21 has been associated with two distinct mutations, namely α A-R21L (Graw et al., 2006) and α A-R21W (Devi et al., 2008; Hansen et al., 2007). Recently, a third mutation α A-R21Q was identified as a basis for the development of congenital heterozygous cataract in a South Australian family (Javadiyan et al., 2016; Laurie et al., 2013). Among the different family members tested, the phenotype of the opacities varied from lamellar to dense nuclear. Western blot analysis of isolated protein content from an affected individual suggested the mutation affected oligomerization of α A-crystallin, a feature critical to its structural and functional stability (Kundu et al., 2007). To better establish a relationship between the mechanistic effects of α A-R21Q with the observed cataract phenotype, we subjected recombinantly synthesized α A-R21Q to multiple structural and functional analyses. Our study reveals that α A-R21Q displays an increased chaperone-like activity towards EDTA induced aggregation of Alcohol Dehydrogenase (ADH) and TCEP induced aggregation of Bovine Pancreatic Insulin. Size exclusion chromatography analyses reveal that α A-R21Q exhibited an increased affinity towards unfolding protein substrate. The mutant protein also exhibited an increased affinity towards bovine lens fiber cell membranes and prevented apoptosis in ARPE-19 cells subjected to oxidative stress. The R21Q point mutation exerts subtle effects on the structure of α A-crystallin as observed in near and far-UV CD spectra of the protein. The structural changes increase the surface hydrophobicity of α A-R21Q, promote faster unfolding in urea, and rapid degradation in the presence of trypsin. Taken together, the R21Q mutation greatly

influences the functional aspect of α A-crystallin. The N-terminal domain R21, therefore, is critical for governing the chaperone-like activity of α A-crystallin.

2. Materials and Methods

2.1. Expression and Purification of Recombinant α A-Crystallins

The point mutation was introduced in a pET23d+ vector containing the cDNA sequence for wild-type α A-crystallin using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The recombinant plasmid was scaled up in XL-1 Blue cells (Agilent Technologies). Plasmid sequence was confirmed by DNA sequencing at the University of Missouri DNA Core Facility and was used to transform BL21(DE3)pLysS cells (Invitrogen). Protein purification of wild-type α A-crystallin (α A-WT), α B-WT and α A-R21Q was accomplished using methods described earlier (Horwitz et al., 1999). Following purification, all proteins were stored at -20°C in PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 , 1.5mM KH_2PO_4 pH 7.4) until use.

2.2. Size exclusion Chromatography Analysis

α A-WT (0.2 ml of 1 mg/ml) and α A-R21Q in PBS pH 7.4 were incubated at 37°C for 3 hours in a water bath. Following incubation, 100 μl of the sample was injected on a TSKG5000PW_{XL} analytical size exclusion column (Tosoh Bioscience, King of Prussia, PA) connected to an HPLC system, coupled with a MALS DLS detectors (Wyatt Technology, Goleta, CA). Hetero-oligomeric nature of α -crystallin was investigated by mixing recombinant α A-WT, α A-R21Q and α B-WT in the predetermined molar ratios of 3:1 (α A-WT: α B-WT — henceforth referred as α L) or 1.5:1.5:1 (α A-WT: α A-R21Q: α B-WT — henceforth referred as α R). The samples were pre-incubated for 3 hours at 37°C to allow subunit exchange prior to SEC analysis. MALS measurements for molar mass and DLS measurements for hydrodynamic radius were determined using the ASTRA 6.1 software package (Wyatt Technology). Mass distribution and polydispersity analysis was carried out using ASTRA 6.1 for a selected peak following SEC elution to determine the nature of oligomer assemblies within that peak. SEC runs were repeated thrice per sample for statistical significance.

2.3. Circular Dichroism Measurements

Near- and far-ultraviolet (UV) circular dichroism (CD) measurements were recorded at 25°C using a Jasco J-815 spectropolarimeter (Jasco Analytical Instruments). α A-crystallin (2 mg/ml) in 50 mM phosphate buffer (pH 7.4) was used for near-UV CD measurements using a one cm quartz cuvette. For far-UV CD, 0.16 mg/ml of purified protein in 5 mM phosphate buffer (pH 7.4) was analyzed in a 0.2 cm quartz cuvette. The bandwidth was maintained at 5 nm. The reported spectra are an average of five scans after they were smoothened and subtracted for buffer blank.

2.4. Intrinsic Tryptophan Fluorescence and Bis-ANS Binding Studies

Intrinsic tryptophan and Bis-ANS based fluorescence studies were carried out with 0.2 mg/ml of α A-WT and α A-R21Q in PBS, pH 7.4. Tryptophan fluorescence spectra were recorded from 300–450 nm using 295 nm as the excitation wavelength. Surface

hydrophobicity measurements for the two proteins were taken after incubating 0.2 mg/ml of the proteins with 10 μ M Bis-ANS for 30 minutes at room temperature in the dark. Fluorescence spectra were recorded from 400–600 nm using 395 nm as the excitation wavelength. The emission spectra during the two studies were recorded in a Jasco FP750 spectrofluorometer (Jasco Inc., Easton, MD). The spectra shown are an average of three independent recordings.

2.5. Chaperone-Like Activity Assays

Purified α -crystallin homo- and hetero-oligomers were incubated with aggregating client proteins such as ADH and bovine pancreatic insulin to analyze their chaperone-like activity. Unfolding of ADH (250 μ g) was carried out in PBS + 50 mM EDTA pH 7.2, and that of insulin (150 μ g) was facilitated in PBS containing 250 μ M TCEP. Chaperone-like activity of α L, α M (α A–R21Q: α B–WT mixed in molar ratios of 3:1) and α R hetero-oligomers was assessed after pre-incubating the crystallin mixtures described above for 3 hours at 37 °C to facilitate subunit exchange and equilibration. Defined concentrations of the mixture were then incubated in the presence of aggregating substrate proteins to measure the efficiency of chaperone-like activity. The kinetic assays were monitored at 360 nm in a Shimadzu UV-VIS spectrophotometer equipped with a temperature controlled multi-cell transporter. All assays were performed thrice for statistical significance.

2.6. ADH binding assays

The relative binding of unfolding, aggregation-prone substrate protein to α A–crystallin was assessed by co-incubating 75 μ g of α A–WT or α A–R21Q with 250 μ g of ADH in PBS of pH 7.4 for different time intervals, ranging from 0–60 minutes at 37 °C. Aggregation of ADH was initiated with EDTA at a final concentration of 18.75 mM in a total reaction volume of 200 μ l. Following incubation, the reaction mixture was briefly spun down, and 175 μ l of the supernatant was injected on a TSKG5000PW_{XL} column. The α A–crystallin peak eluted from SEC was analyzed for α A–crystallin bound ADH, molar mass and hydrodynamic radius using the ASTRA 6.1 software package. All values were averaged from three independent analysis for statistical significance.

2.7. Membrane Binding Assays

Bovine lens fiber cell membranes were isolated as per the procedure described by Russell et al. (Russell et al., 1981). α A–WT and α A–R21Q were labeled with Alexa Fluor 350 as per manufacturer's instructions. Varied concentrations of Alexa Fluor–labeled α A–WT and α A–R21Q were incubated for 8 and 24 hours with 500 μ g of bovine lens membranes in binding buffer (PBS + 5 mM MgCl₂ pH 7.4) at 37 °C. Following incubation, the reaction mixture was centrifuged at 15,000 rpm, and the supernatant containing the unbound protein was aspirated. The pellet was washed twice in PBS pH 7.4 and spun down as described earlier. The resultant pellet was resuspended in PBS pH 7.4 and analyzed for bound protein by monitoring the emission maxima for the dye in a Spectramax i3 fluorescence plate reader. All experiments were repeated thrice for statistical significance.

2.8. Urea Unfolding Studies

Protein aliquots (100 µg) were incubated in a range of urea concentrations from 0–8 M at 0.5 M intervals. The mixtures were made to a final volume of one ml and incubated for 16 hours at 37 °C. Fluorescence spectra for the incubated samples were recorded from 300–400 nm using 295 nm as the excitation wavelength in a Jasco FP750 spectrofluorometer. The extent of unfolding is estimated using the I_{337}/I_{350} ratio, which is the ratio of the emission maxima of tryptophan in a folded protein to that in an unfolded protein.

2.9. Limited Proteolysis Using Trypsin

αA–WT, αA–R21Q (500 µg) were individually mixed with sequencing-grade modified trypsin (Promega Corporation, Madison, WI) at a ratio of 1:200 w/w in PBS pH 7.4, to a final reaction volume of 500 µl. From the parental mixture, 50 µg aliquots were incubated at 37 °C for time intervals ranging from 0–90 minutes. Reconstituted hetero-oligomers of α–crystallin — αL and αR were also subjected to limited proteolysis to assess the effect of αB–WT on the susceptibility of oligomeric α–crystallin to the protease. For every time point, the reaction was terminated using formic acid at a final concentration of 0.1%. Aliquots of samples (10 µl) were run on a 15% polyacrylamide gel and stained with Acqua stain (Bulldog Bio, Portsmouth, NH).

2.10. Cellular Apoptosis Assays

Authenticated ARPE-19 cells were obtained from the ATCC. Cells were seeded in 96-well plates in Dulbecco's Modified Eagle's Medium (DMEM) + 2% FBS, and maintained at 37 °C in an incubator maintained at 5% CO₂ flow, until they were 60–70% confluent. The cells were then serum starved for 2 hours at 37 °C, after which they were treated with 5 µg/ml and 10 µg/ml of either αA–WT, αA–R21Q and reconstituted αL and αR respectively. Cellular apoptosis was induced using 150 µM H₂O₂ in serum-free DMEM. Following incubation for 16 hours, the degree of apoptosis was determined using the EarlyTox cell integrity kit (Molecular Devices, Sunnyvale, CA) according to manufacturer's instructions. The degree of apoptosis was estimated by measuring the fluorescence intensity at 541 nm. Empirically, cells with average fluorescence intensities of ~1500 units at 541 nm were considered as apoptotic. The generated statistical data are an average of four readings for statistical significance.

3. Results

3.1. The R21Q Point Mutation Does Not Affect αA-Crystallin oligomerization

Recombinant human αA–WT and αA–R21Q were purified according to established protocols. Both the proteins were soluble and were ~95% pure as judged by SDS-PAGE analysis (Figure 1).

Proteins were subjected to MALS measurements to determine the mass distribution of αA–crystallin homo- and hetero-oligomers (Figures 2A and B). On incubation at 37 °C for three hours αA–WT homo-oligomers exhibited an average molar mass of 4.70×10^5 g/mol, with oligomeric masses ranging from $3.97 - 8.10 \times 10^5$ g/mol. αA–R21Q showed an average molar mass of 5.01×10^5 g/mol, ~6 % larger than αA–WT with two distinct mass

distributions (Figure 2B) within the eluted peak. α B-WT on incubation exhibited an average molar mass of 5.59×10^5 g/mol, with most oligomer species siding within the molar mass range of $4.60 - 6.40 \times 10^5$ g/mol.

α A-R21Q segregated as an autosomal dominant mutation in affected individuals (Laurie et al., 2013), as only one copy of the mutant gene was sufficient to initiate cataractogenesis. To assess whether α A-R21Q influenced the oligomerization of the α -crystallin hetero-oligomer, we incubated α A-WT, α A-R21Q and α B-WT for 3 hours at 37 °C in the pre-determined lenticular molar ratio of 3:1 (α L), and 1.5:1.5:1 (α R) to allow subunit exchange, as determined previously (Bova et al., 1997; Bova et al., 2000). The average molar masses for α L and α R were 5.98×10^5 g/mol and 5.83×10^5 g/mol respectively. Mass distribution analyses of α L and α R oligomers indicated that α L oligomers were more polydisperse than α R (Figure 2B, Table 1). Taken together, it can be summarized that the R21Q mutation enhances the polydispersity of α A-crystallin, and that α B-WT stabilizes the oligomeric assembly of the α A-R21Q oligomer on subunit exchange.

3.2. The R21Q point mutation does not significantly affect the α A-crystallin structure

To estimate the effect of the point mutation on the secondary and tertiary structure of α A-crystallin, circular dichroism measurements were taken for both α A-WT and α A-R21Q. No significant changes were observed in both near- and far-UV spectra for α A-R21Q in comparison to α A-WT (Figure 3A and B). It is possible that the R21Q point mutation exerts subtle effects on the protein structure which are not conferred by CD studies.

3.3. The α A-R21Q Mutation Increases the Surface Hydrophobicity of α A-Crystallin

Monomeric α A-crystallin subunit has one tryptophan residue, in the N-terminal at position 9. It is therefore valuable to assess the effect of a point mutation in the N-terminal region on the local microenvironment of the tryptophan residue and to gather clues toward the higher ordered assembly of the mutant α A-crystallin. As shown in Figure 4A, α A-R21Q exhibits increased tryptophan fluorescence in comparison to α A-WT. Like other members of the sHSP family, α A-crystallins prevent insolubilization of aggregation-prone proteins using hydrophobic “patches” on their surface (Sharma et al., 1998). These hydrophobic patches interact with the hydrophobic residues exposed by the unfolding intermediate, stabilizing them in the process. 4,4'-Dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (Bis-ANS) is a hydrophobic probe essentially non-fluorescent in an aqueous environment and fluoresces on binding hydrophobic residues on proteins (Das and Surewicz, 1995). Bis-ANS binding studies, therefore, give an overall estimate of the surface hydrophobicity of the protein. To determine whether the point mutation affects the surface hydrophobicity of α A-R21Q, the mutant α A-crystallin was incubated in the presence of bis-ANS at room temperature for 30 minutes, after which its fluorescence was measured. As demonstrated in Figure 4B, α A-R21Q showed ~75% higher fluorescence in comparison to α A-WT when incubated with bis-ANS. It can thus be concluded that the R21Q mutation increases the surface hydrophobicity of α A-crystallin.

3.4. α A–R21Q exhibits Enhanced Chaperone-like Activity

Since α A-crystallin contributes to lens transparency by preventing non-specific protein aggregation, we tested the ability of α A–R21Q crystallin to prevent induced protein aggregation *in vitro*. Chaperone-like activity assays were carried out with α -crystallin homo-oligomers (α A–WT and α A–R21Q) and reconstituted hetero-oligomers (α L, α M, and α R) using alcohol dehydrogenase (ADH) and bovine pancreatic insulin as unfolding client protein substrates. As shown in Figure 5A and C, α A–R21Q homo-oligomers exhibit enhanced chaperone-like activity against nonspecific aggregation of insulin and ADH. Compared to α A–WT, α A–R21Q exhibited 3.4-fold higher chaperone-like activity against insulin and 1.7-fold higher chaperone-like activity against ADH.

Native α -crystallin isolated from lenses exists in a hetero-oligomeric state composed of both α A– and α B–crystallin subunits. To test whether α B–WT affected the chaperone-like activity of α A–R21Q, reconstituted α L, α M (α A–R21Q: α B–WT mixed in molar ratios of 3:1) and α R hetero-oligomers were co-incubated with unfolding ADH and insulin. Compared to α L hetero-oligomers, α M hetero-oligomers showed 2.2-fold higher chaperone-like activity during insulin aggregation assays (Figure 5B) and 2.4-fold higher chaperone-like activity in ADH aggregation analysis (Figure 5D). α R hetero-oligomers, reconstituted to mimic the heterozygous nature of the mutation *in vivo*, exhibited 2.9-fold higher chaperone-like activity against aggregating ADH, and 3.2-fold higher against aggregation of insulin. The R21Q mutation, therefore, increased the chaperone-like activity of α A-crystallin in its homo-oligomeric state, as well as its hetero-oligomeric state, with α B–WT. A quantitative comparison of the chaperone-like activities of α A–WT and α A–R21Q, with or without α B–WT is summarized in Table 2.

3.5. The R21Q point mutation increases the affinity of α A–crystallin towards ADH

Since recombinant α A–R21Q exhibited an increase in its surface hydrophobicity along with a concomitant increase in its chaperone-like activity, we tested the effect of the point mutation on the affinity of α A–crystallin towards aggregating ADH. Seventy five μ g of α A–crystallin was empirically determined to be sufficient to completely suppress EDTA induced aggregation of 250 μ g ADH in a reaction volume of 200 μ l (data not shown). Since both α A–WT and α A–R21Q stock solutions were stored in the cold, the proteins were allowed to equilibrate at room temperature for three hours to avoid altered binding patterns that could arise due to temperature fluctuations. α A–crystallins were co-incubated with ADH in the presence of EDTA for different time intervals, and each reaction mixture was subjected to SEC to determine the amount of ADH that was associated with α A–crystallin. SEC analysis was limited to the elution time range of 7.2–10.7 minutes to accommodate the polydisperse nature of the α A–crystallin–ADH complex. Figure 6 summarizes the SEC elution profiles of both α A–WT (A) and α A–R21Q (B) incubated with ADH in the presence of EDTA. Incubation of α A–WT with ADH for increasing time intervals resulted in successive emergence of broad peak shoulders that correspond to the ADH– α A–WT complex. Approximately 36 μ g ADH was calculated to be associated with α A–WT following incubation at 37 °C for 60 minutes. In contrast, 35 μ g of ADH was calculated to have associated with the α A–R21Q peak on incubation for 30 minutes at 37 °C and 49.7 μ g of ADH was found to be associated with α A–R21Q after incubation for 60 minutes at 37 °C.

Since the reaction mixtures were identical in composition, it can be interpreted that the observed increase in association of ADH with α A-R21Q was due to the increased affinity of the mutant protein towards ADH. The R21Q mutation, therefore, appears to enhance the affinity of α A-crystallin towards unfolding protein substrates. In comparison to α A-WT—ADH complexes, the α A-R21Q—ADH complex peak was less polydisperse (Figure 6A and B), suggesting that R21 also played a role in substrate recognition, as well as the reorganization of the α A-crystallin—ADH oligomeric complex.

3.6.Recombinant α A-R21Q Shows Enhanced Binding with Lens Fiber Cell Membranes

Mutations in α A-crystallin are known to affect the association of α A-crystallins with lens fiber cell membranes (Cobb and Petrash, 2000, 2002). To determine the extent of this effect, both α A-WT and α A-R21Q were incubated with bovine lens fiber cell membranes for 8 and 24 hours at 37 °C. After 8 hours, Alexa fluor 350 labeled α A-R21Q showed a two-fold increase in its association with lens fiber cell membranes in comparison with Alexa fluor 350 labeled α A-WT (Table 4). However, a 24 hour incubation of α A-R21Q with bovine lens membranes showed nearly a 6.3-fold increase in its association compared to α A-WT (Figure 7). The comparison of the binding patterns of the two proteins used in different concentrations is summarized in Table 4. It is plausible that R21Q mutation associated increase in the surface hydrophobicity may have a role in increased binding of the mutant α A-crystallin to lens fiber cell membranes.

3.7. The R21Q mutation alters the stability of α A-Crystallin in urea

The polydisperse nature of α A-crystallin has hindered the high-resolution structural analysis of the protein. Circular dichroism studies with both α A-WT and α A-R21Q did not reveal significant changes in the structure of the mutant protein. However, bis-ANS binding studies showed an increased surface hydrophobicity profile for α A-R21Q in comparison to α A-WT, indicating a subtle change that could not be detected during CD-analysis. To further assess the impact of R21Q mutation, both α A-WT and α A-R21Q homo-oligomers were tested for their stability in different concentrations of urea (Figure 8). α A-WT exhibited stability in urea concentrations up to 1.5 M, following which it underwent an unfolding transition between urea concentrations ranging between 2–3M, and completely unfolded at urea concentrations around 4 M. The $C_{1/2}$ for α A-WT was calculated to be 2.64 M from the unfolding curve. In contrast, α A-R21Q exhibited an unfolding transition in urea concentrations as low as 0.5 M, with its $C_{1/2}$ estimated as 1.64 M. The subtle structural alteration influenced by the point mutation, therefore, altered the stability of the α A-crystallin oligomer in urea, causing it to unfold in lower denaturing conditions.

3.8. The R21Q mutation-induced structural change influences the stability of α A-crystallin against limited proteolysis

To further validate the results of the urea unfolding studies, α A-WT and α A-R21Q were subjected to limited proteolysis using trypsin. The limited proteolysis approach was selected based on the hypothesis that the point mutation results in conformational changes in the α A-crystallin oligomer, leading to exposure of tryptic sites that are otherwise masked in α A-WT oligomer. As shown in Figure 9A, α A-R21Q demonstrates nearly complete loss of the 20 kDa band after 60 minutes of incubation with trypsin in contrast to α A-WT, where a 20

kDa band is visible corresponding to the 90-minute timepoint following incubation. The point mutation induced structural change exposes potential cleavage sites in the α A-R21Q oligomer, promoting faster cleavage by trypsin in comparison to α A-WT.

Since lenticular α -crystallin exists as a hetero-oligomer of α A and α B subunits, we also subjected reconstituted hetero-oligomers of α -crystallin, α L (α A-WT: α B-WT = 3:1) and α R (α A-WT: α A-R21Q: α B-WT = 1.5:1.5:1) to limited proteolysis. Both hetero-oligomers exhibited identical tryptic digestion profiles for the different time points tested (Figure 9B). This observation suggests a possible role of both α A- and α B-crystallin on stabilizing heterooligomers by masking tryptic sites otherwise exposed in the α A-R21Q.

3.9. Transduced α A-R21Q prevents oxidative damage-induced apoptosis in ARPE-19 cells

Multiple reports support the anti-apoptotic properties of both α A- and α B-crystallins in response to various stimuli (Christopher et al., 2014; Hamann et al., 2013; Liu et al., 2004; Pasupuleti et al., 2010; Xi et al., 2003). Pasupuleti *et al.* suggested a direct correlation between the anti-apoptotic property of α A-crystallin and its chaperone-like activity (Pasupuleti et al., 2010). Additionally, studies by Biswas *et al.* revealed that a mutant α A-crystallin, α AR21A, exhibited better chaperone-like activity than α A-WT (Biswas et al., 2006). Since both R21A and R21Q mutations target the same arginine residue, we tested the effect of transduced α A-R21Q in ARPE-19 cells in response to H_2O_2 -mediated oxidative stress.

More than 80% of ARPE-19 cells undergo apoptosis in response to H_2O_2 in the absence of α A-crystallins (Figure 10A, Row 2). Cell viability was retained at $41.4 \pm 2\%$ with $5 \mu\text{g/ml}$ of α A-WT (Figure 10A, Row 3 and Figure 10B). α A-R21Q efficiently prevented H_2O_2 induced apoptosis in ARPE-19 cells, with a cell viability of $93 \pm 3\%$ when $5 \mu\text{g/ml}$ protein was used to transduce the cells (Figure 10A Row 4 and Figure 10B). The enhanced chaperone-like activity of α A-R21Q thus corresponds to its increased ability to prevent cell death under conditions of oxidative stress.

Limited proteolysis assays suggest a stabilizing role for both α A-WT and α B-WT on α A-R21Q. The heterozygous oligomer α R, however, shows an increased chaperone-like activity against insulin and ADH. To further assess the effect of hetero-oligomerization of α A-R21Q on its cytoprotective ability, we transduced reconstituted α L (α A-WT: α B-WT = 3:1) and α R (α A-WT: α A-R21Q: α B-WT = 1.5:1.5:1) into ARPE-19 cells. In the absence of H_2O_2 , both α L and α R transduced cells exhibited an average viability of 93%. On exposure to $150 \mu\text{M}$ H_2O_2 , ARPE-19 cells treated with $5 \mu\text{g/ml}$ of α L exhibited a cellular viability of $84 \pm 4\%$ (Figure 10B, Group 4). ARPE-19 cells transduced with α R hetero-oligomers were also more resilient to H_2O_2 mediated oxidative stress, and the viability was maintained at $90 \pm 2\%$ after $5 \mu\text{g/ml}$ of α R treatment (Figure 10B, Group 5). The presence of α B-WT within the reconstituted heterooligomers therefore contributes to their increased resistance toward oxidative stress induced apoptosis in ARPE-19 cells.

4. Discussion

The N-terminal of small heat shock proteins (sHSP) has been the subject of several studies, yet there is ambiguity about its contribution to protein structure and function (Basha et al., 2006; Carver et al., 2017; Kundu et al., 2007). The domain has been shown to affect sHSP oligomerization as well as influence their interaction with unfolding protein intermediates (Basha et al., 2006). Isolated N-terminal domains of both α A- and α B-crystallins have been shown to localize in the urea-soluble fraction during protein purification, owing to their high hydrophobicity (Asomugha et al., 2011). Earlier studies on *C. elegans* HSP 16-2 (Leroux et al., 1997) and the crystal structure of *M. jannaschii* HSP 16.5 (Kim et al., 1998) positioned the N-terminal of the protein in the interior of the sHSP oligomer. However, crystallographic data and NMR spectra corresponding to the N-terminal domain are not well resolved (Basha et al., 2012), and hydrogen-deuterium exchange studies on the protein show a high degree of deuterium labeling (Aquilina et al., 2003), indicating that the N-terminal region is very much mobile (Basha et al., 2012; Derham and Harding, 1999; Smith et al., 1996). Deletion mutants of the N-terminal domain region exhibit altered oligomeric structures and subunit exchange dynamics (Kundu et al., 2007). The mobility of the N-terminal may, therefore, contribute towards inter- and intrasubunit interactions responsible for the α A-crystallin oligomerization, in a manner similar to α B-crystallin (Jehle et al., 2011). There are four conserved arginine residues in the N-terminal of α A-crystallin (Derham and Harding, 1999). Arginine residues are critical for the stability of the protein, owing to their highly charged guanidinium group, which can interact with electronegative atoms via salt bridges. Bioinformatics analyses have shown that arginine residues can also interact with backbone carbonyl oxygen atoms, providing additional stability to the protein (Borders et al., 1994). Therefore, the loss of arginine residues at critical positions can compromise protein stability and affect protein function by altering these crucial interactions.

Three point mutations have thus far been shown to target arginine 21 of α A-crystallin — α A-R21L, α A-R21W, and α A-R21Q. The arginine 21 residue is a part of a conserved motif ²⁰SRLFDQFFG²⁸ in α -crystallins and other sHSP family members (Pasta et al., 2003). Informatics analyses reveal a considerable similarity between α A-crystallin residues 20–24 and residues 7–11 in Hsp16.9 (Kim et al., 1998). Hsp16.9 residues 7-SNVFD-11 interact with amino acid residues 109 and 110 in the α -crystallin domain, which is thought to contribute towards stabilization of sHSP oligomer and its chaperone-like activity (van Montfort et al., 2001). Deletion of residues 20–28 in α A-crystallin was found to result in mutants with enhanced subunit dynamics, altered tertiary structure, increased surface hydrophobicity and enhanced chaperone-like activity (Pasta et al., 2003). The deletion mutant also showed lesser stability in equilibrium unfolding experiments. Our work on recombinant α A-R21Q shows that the properties of the mutant protein mimic those of the α A(20–28) deletion mutant with increased surface hydrophobicity, enhanced chaperone-like activity, increased affinity towards unfolding substrate proteins and lens fiber cell membranes, increased susceptibility to urea mediated unfolding and limited proteolysis with trypsin. The point mutation exerts subtle effects on protein structure, as near and far UV CD spectra did not reveal any significant differences in the secondary and tertiary structure signatures (Figure 3A and B). This observation could possibly result from other residues in the motif

that help to maintain optimal side chain packing in the α A-crystallin oligomer. The reduced stability of α A-R21Q to urea could be interpreted to be the result of the loss of “compactness” of the α A-R21Q oligomer due to the loss of stabilizing interactions otherwise mediated and stabilized through R21. This assumption is partially supported from polydispersity analyses as the α A-R21Q homo-oligomers were more polydisperse compared to α A-WT and α R hetero-oligomers. Further studies are therefore needed to elucidate the role of R21 in regulating inter- and intra-subunit interactions of α A-crystallin.

Arginine 21 has also been the subject of attention in studies of age-related cataracts resulting from post-translational modifications on α A-crystallins. Advanced Glycosylation Endproduct (AGE)-modified α A-crystallins at R21 and R103 have been shown to exhibit enhanced chaperone-like activity *in vitro* (Biswas et al., 2006; Nagaraj et al., 2003). AGE modification on α A-crystallins has been proposed as an evolutionary adaption to compensate for the decreased chaperone-like activity of the protein (Nagaraj et al., 2003). The conserved arginine residues play a vital role in maintaining appropriate subunit-subunit and chaperone-substrate interactions.

The loss of a charged residue increases the surface hydrophobicity of α A-R21Q such that it could bind potential targets with a much higher affinity than α A-WT, a likely explanation for its higher chaperone-like activity and increased binding to membranes. This observation was further supported by the results of the ADH binding assays, which showed an increased association of unfolding ADH with α A-R21Q (Figure 6 and Table 3). Additionally, a higher polydispersity of α L hetero-oligomers and the observed higher IC₅₀ values in comparison to α A-WT suggests a stabilizing effect of α B-WT (Table 2). Reconstituted α R hetero-oligomers exhibited lower polydispersity, their IC₅₀ values were much lower than those of α M and α L, suggesting that the presence of α B-WT did not affect the functional aspect of α A-R21Q. Considering that the lens has a defined supply of α B-crystallin due to lack of protein turnover, there arises a possibility that most α B-WT in the lens could be used up early in life in stabilization of α A-R21Q. This could be detrimental during lens development, as both α -crystallin subunits have been shown to have multiple cytoplasmic and cytoskeletal targets (Andley, 2007, 2008) and to influence many cellular processes (Xi et al., 2003) during development.

In vitro membrane binding studies involving an α A-crystallin mutant R116C revealed an 8-fold increase in the membrane binding capacity of the mutant protein compared to wild-type α A-crystallin (Cobb and Petrash, 2000). The altered membrane-binding ability of mutant α A-crystallins was further validated *in vivo* using a knock-in mouse model (Andley, 2009), where another mutant α A-crystallin, R49C, showed higher localization with lens fiber cell membranes and altered cell morphology. Both α A-R116C and α A-R49C also showed an increase in their surface hydrophobicities in comparison to α A-WT. Our studies on Alexa Fluor 350 labeled α A-R21Q show an extensive association of the protein with lens fiber cell membranes on prolonged incubation in comparison to Alexa fluor 350 labeled α A-WT. Taken together with observations made by others, it appears that the exposed hydrophobic regions in α A-R21Q may be driving the association of mutant protein to the membrane surface. Further studies are needed to determine the components of lipid bilayer responsible for interaction with mutant protein. The relative orientation of the fiber cells with each other

and a high refractive index of their proteinaceous content assure efficient focusing of light on the retina for the perception of vision. Abnormal interactions with fiber cell membranes can alter membrane morphology, thereby interfering with the refractive properties of the lens and its transparency.

Studies involving a recombinant mutant of R21, α A-R21A, have correlated its chaperone-like activity with its ability to prevent apoptosis of mammalian cells under stress. *In vitro*, α A-R21A exhibited enhanced chaperone-like activity and an enhanced surface hydrophobicity profile as compared to that of wild-type α A-crystallin (Biswas et al., 2006). α A-R21A transfected CHO and HeLa cells on treatment with staurosporine and etoposide showed lower levels of activated pro-apoptotic molecules, such as Bax and Bim, diminished release of cytochrome C from mitochondria and subsequent activation of executioner caspases (Pasupuleti et al., 2010). Our cell culture studies with R21Q uphold this observation, as ARPE-19 cells transduced with the mutant protein exhibited increased survival on exposure to H_2O_2 . In addition, ARPE-19 cells transduced with reconstituted α R (α A-WT: α A-R21Q: α B-WT = 1.5:1.5:1) and or α L (α A-WT: α B-WT = 3:1) hetero-oligomers exhibited better resistance toward H_2O_2 induced apoptosis. Since the report by Laurie *et al.* suggested the autosomal dominant nature of the mutation, further studies are needed to understand whether increased cytoprotective nature of α R hetero-oligomer plays a role in culminating in lamellar cataract phenotype. The observed enhanced chaperone-like activity and cytoprotective ability, either directly or indirectly, appears to relate to R21 localized within the N-terminal of α A-crystallin.

Lens development includes a controlled apoptosis phase, which involves degradation of cellular organelles, with the retention of the membrane architecture and a simultaneous increase in the production of the crystallin proteins (Andley, 2008). The denucleation phase is prevalent within the cortical fibers and shows directionality towards the lens nucleus. Any interference in the programmed apoptosis cascade can result in loss of the optimal transparency and refractive properties of the lens. The R21Q mutation in α A-crystallin resulted in a congenital lamellar type cataract with variable severity. The observed phenotype along with the findings of our studies raises the possibility of α A-R21Q mediated interference in normal lens development, especially during denucleation of the cortical lens fiber cells. It would be interesting to examine the repercussions of a gain-of-function mutant in terms of its interactions with other lenticular crystallin proteins as well to ascertain the expansive role of R21 in α A-crystallin function.

In summary, we report that the α A-R21Q mutant of α A-crystallin is a gain of function mutant that displays an enhanced affinity towards unfolding proteins and lens fiber cell membranes, and promotes cell survival on transduction in ARPE-19 cells. The enhanced activity could be a result of the loss of conventional intermolecular and intramolecular interactions otherwise mediated by R21. The study, therefore, emphasizes the role of R21 in maintaining optimal α A-crystallin structure and function.

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Abbreviations

αA-WT	wild-type α A-crystallin
αA-R21Q	α A-crystallin mutant R21Q
sHSP	small heat shock protein
ACD	alpha crystallin domain
SEC	Size Exclusion Chromatography
Bis-ANS	4,4'-Dianilino-1,1'-Binaphthyl-5,5'-Disulfonic Acid
ADH	alcohol dehydrogenase
PBS	phosphate buffered saline
IC₅₀	half maximal inhibitory concentration
C_{1/2}	half maximal denaturant concentration.

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Highlights

Mutant α A-R21Q exhibits an increase in its surface hydrophobicity.

α A-R21Q displays better chaperone-like activity and increased lens membrane binding.

Transduced α A-R21Q prevents apoptosis of ARPE-19 cells under oxidative stress.

α A-R21Q gain of function may be compromising lens transparency by increased binding of unfolding crystallins accumulating in lens.

Figure 1. SDS-PAGE analysis of the purified recombinant crystallins

Lane 1) Molecular weight markers, Lane 2) α A-WT, Lane 3) α A-R21Q, Lane 4) α B-WT.

Figure 2. Representative SEC elution and polydispersity profiles of α A-WT, α B-WT and α A-R21Q homo- and hetero-oligomers

Proteins (100 μ g) were injected on a TSKG5000_{PWXL} size-exclusion column following incubation, and differential refractive index (dRI) and A_{280} were monitored for screening elution times. A. SEC and Molar Mass measurements of α -crystallin homo- and hetero-oligomers. B. Polydispersity profile analysis of elution peaks in A. as determined by ASTRA 6.1.

Figure 3. Near-UV (A) and Far-UV (B) circular dichroism spectra of $\alpha\text{A-WT}$ and $\alpha\text{A-R21Q}$
Near-UV spectra were recorded using 1 mg/ml protein in PBS pH 7.4, and Far -UV spectra were recorded with 0.2 mg/ml protein in 5 mM phosphate buffer, pH 7.4. Representative spectra are an average of five scans recorded at 25 °C following correction for buffer blank.

Figure 4. Fluorescence spectrum measurements of α A-WT and α A-R21Q

A. Intrinsic tryptophan spectra of α A-crystallins (0.2 mg/ml in PBS pH 7.4) were recorded from 300–450 nm using 295 nm as the excitation wavelength. B. Surface hydrophobicity measurements reflecting Bis-ANS binding to α A-crystallins (0.2 mg/ml in PBS pH 7.4) were recorded from 400–600 nm with 395 nm as the excitation wavelength. Spectra are an average of three recordings.

Figure 5. Chaperone-like activity assays of αA-WT and αA-R21Q homo-oligomers and hetero-oligomers with αB-WT

A. αA-R21Q and αA-WT activity against EDTA-induced aggregation of ADH. B. Chaperone-like activity of αL, αM and αR hetero-oligomers against ADH aggregation. C. αA-R21Q and αA-WT chaperone-like activity against TCEP induced insulin aggregation. D. Chaperone-like activity of αL, αM and αR hetero-oligomers against insulin aggregation. Chaperone-like activity assays with hetero-oligomers were performed by pre-incubating the αA-WT, αB-WT and/or αA-R21Q in fixed molar ratios at 37 °C for 3 hours IC₅₀ values, composition and the relative chaperone-like activities for crystallin homo- and hetero-oligomers are summarized in Table 2.

Figure 6. SEC elution profiles of ADH-bound α A-crystallin

Relative binding affinities of α A-WT (A) and α A-R21Q (B) toward unfolding ADH were assessed by incubating 75 μ g of α A-crystallin with 250 μ g ADH in the presence of EDTA for 0 (solid line), 15 (long dash), 30 (short dash) and 60 (dotted line) minutes at 37 °C. The reaction mixture (175 μ l) was injected on a TSKG5000PW_{XL} column for SEC analysis. The range of elution time chosen to determine the amount of ADH associated with α A-crystallin is marked with red dashes. Details of the analysis are summarized in Table 3.

Figure 7. Binding of α A- and α A-R21Q to bovine lens fiber cell membranes

Alexa Fluorlabeled crystallins at varied concentrations were incubated with 500 μ g bovine lens membrane in binding buffer (PBS + 5 mM MgCl_2) for 8 and 24 hours at 37 °C. The data shown is the average of three independent measurements and is summarized in Table 4.

Figure 8. Equilibrium unfolding studies of αA - and αA -R21Q

αA -WT (0.1 mg/ml) and αA -R21Q aliquots were incubated in urea concentrations ranging from 0.5–8 M for 16 hours at 37 °C. Samples were later scanned for emission maxima of their intrinsic tryptophan fluorescence as a function of urea concentration.

Figure 9. SDS-PAGE analysis of α A-crystallin homo-oligomers (A) and hetero-oligomers (B) after limited proteolysis using trypsin

Fifty μ l aliquots of 1 mg/ml α A-WT, α A-R21Q, α L, and α R were incubated with trypsin (1:200 protease to protein w/w) for defined reaction times. The reaction was terminated with the addition of formic acid to a final concentration of 0.1%. The extent of proteolysis was evaluated on a 15% polyacrylamide gel by gel electrophoresis.

Figure 10. α A-crystallin transduced in ARPE-19 cells influence their viability when exposed to H_2O_2 induced oxidative stress

A. Cell viability is greatly reduced on exposure to H_2O_2 , which corresponds to a marked increase in the intensity of the green signal (Column 1, Row 2). H_2O_2 induced apoptosis is reduced in the case of ARPE-19 cells treated with α A-WT and α A-R21Q (Rows 3 and 4), and is more profound in the case of α A-R21Q (Row 4) treated cells. B. Statistical representation of the H_2O_2 induced apoptosis assays. Group 1, ARPE-19; Group 2, ARPE-19 + 5 μ g/ml α A-WT; Group 3, ARPE-19 + 5 μ g/ml α A-R21Q; Group 4, ARPE-19 + 5 μ g/ml α L; Group 5, ARPE-19 + 5 μ g/ml α R. α -Crystallin hetero-oligomers were reconstituted as described earlier prior to transduction in ARPE-19 cells. The represented

data is an average of four reads for statistical significance. ** p-value <0.05 and *** p-value <0.0005, as determined by ANOVA test for analysis of variance.

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Table 1Quaternary structural parameters of α A-WT, α B-WT and α A-R21Q homo- and hetero-oligomers.

Protein	Rh at peak apex (nm)	Average Molar Mass ($\times 10^5$ g/mol)	Molar Mass Range ($\times 10^5$ g/mol)	Population Group (PG)	Mass Range ($\times 10^5$ g/mol)	Cumulative Percent (%)
α A-WT	7.07 ± 0.05	4.70 ± 0.02	$3.97 - 8.10$	1	$3.97 - 4.35$	28.6
				2	$4.37 - 5.62$	63.4
				3	$5.66 - 8.03$	6.3
α B-WT	7.39 ± 0.10	5.59 ± 0.02	$4.65 - 8.15$	1	$4.60 - 5.70$	64.0
				2	$5.70 - 6.40$	22.5
				3	$6.50 - 8.10$	10.5
α A-R21Q	7.36 ± 0.07	5.01 ± 0.10	$3.98 - 7.99$	1	$3.98 - 4.92$	50.7
				2	$4.98 - 5.75$	26.1
				3	$5.80 - 6.57$	9.7
				4	$6.63 - 7.92$	5.9
α L [*]	7.83 ± 0.15	5.98 ± 0.05	$4.02 - 8.50$	1	$4.02 - 5.18$	39.8
				2	$5.22 - 6.17$	35.4
				3	$6.21 - 6.94$	9.4
				4	$6.97 - 8.50$	7.6
α R [†]	7.81 ± 0.13	5.83 ± 0.02	$4.40 - 8.30$	1	$4.40 - 6.20$	78.1
				2	$6.20 - 8.30$	17.8

* α L : α -crystallin oligomer where α A-WT: α B-WT = 3:1.

† α R : α -crystallin oligomer where α A-WT: α A-R21Q: α B-WT = 1.5:1.5:1.

Table 2

The chaperone-like activity of wild-type α A-crystallin and α A-R21Q homo- and hetero-oligomers against Insulin and ADH.

Protein	Substrate	IC ₅₀ (μg)	Fold increase in the chaperone-like activity of α A-R21Q homo-oligomers comparison to α A-WT controls
α A-WT	Insulin	48	3.4
α A-R21Q	Insulin	14	
α A-WT	ADH	10.8	1.7
α A-R21Q	ADH	6.4	
Protein	Substrate	IC ₅₀ (μg)	Fold increase in the chaperone-like activity of α A-R21Q hetero-oligomers comparison to α L control
α L [*]	Insulin	75	—
α M ^{**}	Insulin	34.5	2.20
α R [†]	Insulin	23.4	3.19
α L	ADH	29	—
α M	ADH	12	2.95
α R	ADH	9.8	2.42

* α L : α -crystallin oligomer where α A-WT: α B-WT = 3:1.

** α M : α -crystallin oligomer where α A-R21Q: α B-WT = 3:1.

† α R : α -crystallin oligomer where α A-WT: α A-R21Q: α B-WT = 1.5:1.5:1.

Table 3

Qualitative analysis of ADH— α A—crystallin complex peaks eluted during SEC.

Crystallin	ADH	Timepoint (min)	ADH (μ g) associated with α A—crystallin peak	Molar Mass at peak apex ($\times 10^5$ g/mol)	Average Rh (nm)
α A-WT	250 μ g	0	0	6.83	9.1
		15	15.05	15.3	13.8
		30	25.58	31.8	18.5
		60	36.76	—	24.5
α A-R21Q	250 μ g	0	0	7.35	9.4
		15	17.40	11.30	11.6
		30	35.04	17.94	13.6
		60	49.72	27.18	15.4

Seventy five μ g of α A—crystallin was incubated with ADH in the presence of EDTA for different time intervals, after which the reaction mixture was subjected to SEC fractionation. Analysis of the peak corresponding to α A—crystallin and complex was performed using ASTRA 6.1 software suite. Additional experimental details are included under methods.

Table 4

Comparison of membrane binding patterns of Alexa Fluor 350-labeled α A-WT and α A-R21Q, showing increased binding affinity of the mutant protein towards bovine lens fiber cell membranes.

Time point (hours)	Membrane (μ g)	Protein	[α A-crystallin] incubated (μ g)	[α A-crystallin] bound (ng)	α A-crystallin(ng)
					membrane (μ g)
8	500	α A-WT	10	996 $\pm$ 168	1.99
			25	1259 $\pm$ 10	2.52
			50	1863 $\pm$ 68	3.73
			100	2816 $\pm$ 265	5.63
		α A-R21Q	10	699 $\pm$ 94	1.4
			25	1887 $\pm$ 518	3.77
			50	2198 $\pm$ 175	4.4
			100	4683 $\pm$ 211	9.37
24	500	α A-WT	50	3642 $\pm$ 44	7.3
			75	3715 $\pm$ 477	7.43
			100	4196 $\pm$ 236	8.39
		α A-R21Q	50	16592 $\pm$ 3601	33.18
			75	21199 $\pm$ 555	42.4
			100	26363 $\pm$ 1347	52.73

The experimental details are described in Methods. Measurements are an average of three independent readings.