αA-Crystallin–Derived Mini-Chaperone Modulates Stability and Function of Cataract Causing αAG98R-Crystallin

Murugesan Raju¹, Puttur Santhoshkumar¹, K. Krishna Sharma¹,²

¹Department of Ophthalmology, University of Missouri–Columbia School of Medicine, Columbia, Missouri, United States of America, ²Department of Biochemistry, University of Missouri–Columbia School of Medicine, Columbia, Missouri, United States of America

Abstract

Background: A substitution mutation in human αA-crystallin (αAG98R) is associated with autosomal dominant cataract. The recombinant mutant αAG98R protein exhibits altered structure, substrate-dependent chaperone activity, impaired oligomer stability and aggregation on prolonged incubation at 37°C. Our previous studies have shown that αA-crystallin–derived mini-chaperone (DFVIFLDVKHFSPEDLTVK) functions like a molecular chaperone by suppressing the aggregation of denaturing proteins. The present study was undertaken to determine the effect of αA-crystallin–derived mini-chaperone on the stability and chaperone activity of αAG98R-crystallin.

Methodology/Principal Findings: Recombinant αAG98R was incubated in presence and absence of mini-chaperone and analyzed by chromatographic and spectrometric methods. Transmission electron microscope was used to examine the effect of mini-chaperone on the aggregation propensity of mutant protein. Mini-chaperone containing photoactive benzoylphenylalanine was used to confirm the interaction of mini-chaperone with αAG98R. The rescuing of chaperone activity in mutantα-crystallin (αAG98R) by mini-chaperone was confirmed by chaperone assays. We found that the addition of the mini-chaperone during incubation of αAG98R protected the mutant crystallin from forming larger aggregates that precipitate with time. The mini-chaperone-stabilized αAG98R displayed chaperone activity comparable to that of wild-type αA-crystallin. The complexes formed between mini-αA–αAG98R complex and ADH were more stable than the complexes formed between αAG98R and ADH. Western-blotting and mass spectrometry confirmed the binding of mini-chaperone to mutant crystallin.

Conclusion/Significance: These results demonstrate that mini-chaperone stabilizes the mutant αA-crystallin and modulates the chaperone activity of αAG98R. These findings aid in our understanding of how to design peptide chaperones that can be used to stabilize mutant αA-crystallins and preserve the chaperone function.

Introduction

Alpha A-crystallin, a major structural protein of the vertebrate eye lens [1], belongs to the small heat shock protein (Hsp) family [2–4]. Like other members of this family, αA-crystallin exhibits chaperone-like activity [5–9]. The chaperone function of αA-crystallin prevents aggregation of unfolding proteins and is essential for maintaining transparency of the lens [6,7]. In humans, the αA-crystallin gene is located on chromosome 21 and encodes a polypeptide of 173 residues [10]. Several point mutations have been reported in human αA-crystallin and these mutations cause structural changes in the protein and impair its chaperone activity. The loss of chaperone activity is considered one of the causes for the development of cataract [7,11–13]. Congenital cataract is associated with R12C [14], R21L [15], R49C [16], R54C [17], and R116C [18] mutations, which occur at the conserved arginine residues. Pre-senile cataract is associated with a novel G90R mutation in αA-crystallin [19]. In the G90R mutation, a bulky basic amino acid, arginine, replaces the neutral glycine. Earlier studies of the recombinant G90R mutant protein revealed altered structure, substrate-dependent chaperone activity and impaired oligomer stability compared to wild-type recombinant αA-crystallin [20–22].

Generally, mutant proteins are prone to misfolding in the endoplasmic reticulum (ER) and subsequent aggregation. Paradoxically, some mutant proteins seem to fold efficiently in the ER but are subsequently misfolded at their target sites due to modification in their microenvironment. Functionally impaired mutant proteins or protein aggregates are generally rapidly degraded by the intracellular quality-control system [23] but...
some escape the quality-control mechanisms. Misfolded proteins are a hallmark of several pathological conditions including cataract. Several lines of evidence suggest that small molecular chaperones would be potential therapeutic molecules for diseases associated with misfolded proteins. Collectively called pharmacological chaperones, such molecules include native ligands, substrate analogues and small peptides [24,25], which bind to mutant proteins and stabilize the mutant proteins to the extent that they function normally in vivo as well as in vitro.

We identified the major chaperone site in αA-crystallin and demonstrated that a 19 amino acid peptide (αA70-88, KFVIFLDVKHFSPEDLTVK), representing the chaperone site in the protein, functions like a molecular chaperone [26]. We have designated such a peptide as a “αA-mini-chaperone.” The amino acid sequence of this mini-chaperone is a highly conserved region among several small Hsp70s [27] and structure analysis shows that αA-mini-chaperone region aligns to the β3 and β4 region in the αA-crystallin. Our studies revealed that the αA-mini-chaperone is effective in suppressing aggregation of H2O2-induced β-crystallin [20] and denaturing substrate proteins ADH, citrate synthase, insulin and α-lactalbumin [26,29,30]. The mini-chaperone also inhibits amyloid fibril formation and its toxicity [31]. Because both β-sheet structure and hydrophobicity are necessary for maximal activity of the mini-chaperone, we concluded that direct interaction between the chaperone peptide and client protein is responsible for chaperone-like activity.

In this study, we examined the effect of αA-crystallin–derived mini-chaperone on the stability and function of the mutant αA-crystallin G98R. We show that mini-chaperone stabilizes the unstable mutant protein. Compared to the mutant protein, the mini-chaperone–stabilized αAG98R has a better capacity to chaperone denaturating protein. Our studies demonstrate specific interaction between the mini-chaperone and the mutant αA-crystallin. Using synthetic mini-chaperone harboring a benzoyl phenylalanine (Bpa) residue in place of a Phe we found that the mini-chaperone interacts at least at 1:1 ratio with mutant αAG98R subunits and the stabilized protein has the chaperone activity comparable to that of the WT-αA-crystallin.

Materials and Methods

Proteins and peptides

Recombinant wild-type αA-crystallin and αAG98R mutants were expressed and purified as described earlier [21]. In brief, the full-length human αA-crystallin cDNA cloned into pET-23d (+) vector (Novagen, Madison, WI) was used as a template to generate the G98R mutation. Both mutant and wild-type proteins were expressed in E. coli BL21(DE3)pLysS cells (Invitrogen, Carlsbad, CA) and purified by column chromatography. The purity of the proteins was checked by SDS-PAGE and the molecular mass was determined by mass spectrometry. The concentration of the mutant and wild-type protein was estimated using Bio-Rad protein assay reagent. Mini-chaperone peptide (DFVIFLDVKHFSPEDLTVK), also called αA-mini-chaperone, and Pro-substituted mini-chaperone (DFVPFSLDVKHFSPEDLTVK) were supplied by GenScript Corp. (Piscataway, NJ). Biotin-DFVIFLDVKH(Bpa)SPEDLTVK was supplied by Aapptec (Louisville, KY). The peptides used in the study were >95% pure as determined by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Alcohol dehydrogenase (ADH) was obtained from Biozyme, (San Diego, CA). All other chemicals were of the highest grade commercially available.

Aggregation and multi-angle light scattering studies

αAG98R or wild-type αA-crystallin (75 μg) were incubated in the presence and absence of αA-crystallin–derived mini-chaperone (10 μg) for 1 hr in 100 μl PO4 buffer at 43°C, the temperature at which αAG98R readily aggregates [21]. Samples were injected on to a TSK G5000PWXL (Tosoh Bioscience, Montgomeryville, PA) size-exclusion column equilibrated with 50 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.2). The flow rate was set to 0.75 ml/min. The column was attached to a HPLC system connected with UV and refractive index detectors and coupled to a static multi-angle laser light scattering (DAWN-EOS) and dynamic quasi-elastic light scattering detectors (Wyatt Technology, Santa Barbara, CA). The molar mass (Mr), hydrodynamic radius (Rh) and polydispersity index (PDI) were determined using ASTRA (5.3.2) software developed by Wyatt Technology.

Fluorescence spectroscopy

For measurement of intrinsic Trp fluorescence, protein samples (200 μg) were diluted in 1 ml of PO4 buffer (50 mM, pH 7.2, containing 150 mM NaCl) in the absence and the presence of mini-chaperone (10 μg). The sample was excited at 295 nm (slit width 5 nm) and the emission was recorded at 300–400 nm range (slit width 5 nm). The relative surface hydrophobicity of wild-type αA-crystallin and αAG98R proteins was measured using bis-ANS. Bis-ANS (1 mM solution), 10 μl, was added to 0.2 mg protein in 1 ml buffer (50 mM phosphate buffer containing 150 mM NaCl, pH 7.2) in the absence and in the presence of mini-chaperone (10 μg). The samples were excited at 385 nm and the emission spectra were recorded between 400–600 nm using a Jasco spectrofluorimeter FP-750.

Effect of αA-crystallin-derived mini-chaperone on chaperone activity measurements

The chaperone-like activity of wild-type αA-crystallin and αAG98R proteins was determined in the presence and absence of mini-chaperone using denaturing ADH as the aggregating substrate. Aggregation assay was performed in 1 ml 50 mM phosphate buffer containing 150 mM NaCl and 100 mM EDTA at 43°C. The chaperone activity of Biotin-DFVIFLDVKH(Bpa)SPEDLTVK as well as a Pro-substituted mini-chaperone (DFVPFSLDVKHFSPEDLTVK) was also measured by ADH aggregation assay. The extent of aggregation was estimated by monitoring the light scattering at 360 nm using a Shimadzu UV-VIS spectrophotometer equipped with a temperature-controlled multi-cell transporter.

Circular dichroism measurements

Circular dichroism (CD) spectropolarimeter, J815 (Jasco, Easton, MD), equipped with a temperature control system, was employed to record CD spectra. Far-UV CD measurements were carried out over the wavelength range of 190 to 250 nm with bandwidth 0.5 nm, scan speed 10 nm/min using 0.1-cm path length cuvettes. Protein samples were prepared in 10 mM sodium phosphate buffer (pH 7.2). Spectra are the average of five scans. Buffer signal was subtracted prior to reporting the data. Thermal denaturation data were collected from 25°C to 45°C, with protein concentration of 100 μg/400 μl. Thermal denaturation experiments were performed with a heating rate of 1°C/min, and CD signals at 218 nm were used to determine transition midpoints. Near-UV CD spectra were recorded using a protein sample of 1.5 mg/ml in the buffer used for far-UV studies.
Confirmation of αA-crystallin–derived mini-chaperone binding to αAG98R protein

The interaction between αA-crystallin–derived mini-chaperone and αAG98R-crystallin was studied using benzoyl phenylalanine-substituted αA-mini-chaperone. Phenylalanine corresponding to Phe-80 in wild-type αA-crystallin was substituted in αA-mini-chaperone with benzoyl-phenylalanine (Bpa). αA-Mini-chaperone also contained a biotin moiety at the N-terminus, creating a biotinyl αA-mini-chaperone-Bpa peptide. Biotinyl-αA-mini-chaperone-Bpa peptide (200 μg) was incubated with 200 μg of αAG98R crystallin in buffer, pH 7.2, at 37°C for 1 hr. Subsequently, the sample was filtered in a Microcon 10 kDa cut of filter (Millipore, Bedford, MA) to remove free peptides. The sample was photolyzed for 30 min, at 4°C, using a UV lamp (365 nm) held at a distance of 7 cm from the sample. The photolyzed sample was desalted using C18 zip tip spin columns (Thermo Fisher Scientific, Rockford, IL), as per the manufacturer’s protocol, and the bound peptides were eluted in 70% acetonitrile. The binding of αA-mini-chaperone to αAG98R was confirmed by MALDI-TOF/TOF mass spectrometry. The UV-photolyzed sample was subjected to SDS-PAGE and western blot analysis using antibody against αA-crystallin.

Electron microscopy

To examine the aggregation of G98R mutant protein (100 μg) in the absence and presence of αA-crystallin–derived mini-chaperone (10 μg), the purified protein was incubated at 37°C or 40°C in 7.2 pH phosphate buffer and the samples were analyzed by transmission electron microscopy (TEM). Aliquots of 5 μl were withdrawn at different time intervals (0 min, 10 min, 30 min) and placed on carbon-coated, 200 mesh copper grids and left for 1 min. The excess solution was wicked away with a filter paper. The proteins on the grid were stained with 5 μl of freshly prepared 5% uranyl acetate solution for 10 min. This solution was then wicked off, and the grid was air-dried and then examined using a JEOL 1400 TEM (120 kV). The images were captured on a digital camera with 20,000 magnification and imaging software from Gatan Digital Micrograph (Gatan, Inc., Warrendale, PA). The protein samples incubated at 37°C in presence and absence of mini-chaperone for 8 hrs and processed as above was also examined by TEM.

Results

Recombinant crystallin proteins were expressed and isolated according to the procedure described earlier [21]. SDS-PAGE analysis confirmed that both wild-type and mutant forms of recombinant αA-crystallins were as pure (>98%) as the proteins used in earlier studies [21,22]. Size-exclusion chromatographic profile of the mutant protein gave an elution profile with an oligomer peak and a peak of dissociated subunits, indicating that the mutant protein has an unstable oligomeric assembly, as described earlier [22]. On the other hand, the wild-type αA-crystallin eluted from the same column as a single peak with the expected elution time for αA-crystallin oligomer. Incubation of αAG98R at 37°C led to gradual aggregation over a period of time, whereas incubation at 43°C resulted in rapid aggregation of the mutant protein, as we reported earlier [21].

αA-Crystallin–derived mini-chaperone increases the recovery of soluble αAG98R

Following purification of αAAG98R, we examined the ability of αA-mini-chaperone to stabilize the mutant protein. We know from previous studies that αA-mini-chaperone suppresses the aggregation and precipitation of denaturing proteins [26,29,30]. The purified αAAG98R (75 μg), which aggregates and precipitates on incubation at 37–45°C [21] was incubated in the presence and absence of αA-mini-chaperone (10 μg) at 43°C for 1 hr. The samples were centrifuged to remove any precipitate formed during incubation, and the supernatant was analyzed by TSKG5000 PWxL gel filtration column connected to a multi-angle laser light scattering (DAWN-EOS) and dynamic quasi-elastic light scattering detectors. The elution profile showed two peaks (Figure 1B). The first peak corresponded to the oligomeric form of αAAG98R, whereas the second peak represented dissociated subunits of the mutant protein. In the absence of mini-chaperone, only 7.1 μg (9.5%) of the mutant protein was recovered, whereas 59.2 μg (79%) of the mutant protein was recovered when the incubation was carried out with αA-mini-chaperone. The monomeric peak also decreased in the presence of αA-mini-chaperone. The binding of αA-mini-chaperone to αAAG98R was confirmed by HPLC analysis of the protein peak eluting between 8.5–11 min from the TSKG5000PWxL column (the data are shown in Figure S1). Both αAAG98R and αA-mini-chaperone were present in the protein peak, indicating that the peptide chaperone was in complex with αAAG98R during gel filtration analysis. The average molar mass of αAAG98R oligomer (non-aggregated) recovered in the absence of αA-crystallin–derived mini-chaperone was 2.3 × 10^6, whereas in the presence of the mini-chaperone, the average molar mass of the stabilized αAAG98R was 2.8 × 10^6, indicating that the αA-mini-chaperone prevented the dissociation of αAAG98R protein and that the slightly increased molar mass might be due to binding of αA-mini-chaperone (Figure 1B). The hydrodynamic radius (Rh) of the stabilized αAAG98R increased from 15.3 nm to 16.4 nm, consistent with increase in molar mass. Under similar experimental conditions, wild-type αA-crystallin oligomeric size and molar mass did not change upon incubation at 43°C (Figure 1A) and the mini-chaperone did not interact with wild-type αA-crystallin. This was confirmed by HPLC analysis of wild-type protein oligomer incubated with αA-mini-chaperone and isolated by gel filtration (Figure S1).

Stabilization of recombinant αAAG98R by αA-crystallin–derived mini-chaperone

To investigate the thermal behavior of mutant αAAG98R protein and the effect of αA-mini-chaperone on αAAG98R stability, we incubated the mutant protein (750 μg) at 43°C in the presence and absence of mini-chaperone, in a 1:7:1 (mol/mol) ratio. Light scattering was monitored at 360 nm for 90 min using a spectrophotometer. As shown in Figure 2, αAAG98R begins to form light scattering aggregates in 40 min. It is well known that under similar conditions, the wild-type αA-crystallin does not form light scattering aggregates. The chaperone peptide DVFVLFDVHKHFSPLEDTVK, is known to suppress aggregation of proteins denatured by heat [26], chemicals [30] and oxidation [28], completely suppressed αAAG98R aggregation (Figure 2). Because αAAG98R is a structurally perturbed protein [20,21], we hypothesize that the mini-chaperone interacted with mutant αAAG98R and prevented aggregation and light scattering. Under similar conditions, incubation of αAAG98R with a Pro substituted mini-chaperone (DVFVLFDVHKHFSPLEDTVK), which has no chaperone activity (Figure S2), failed to suppress precipitation of the mutant protein (data not shown). The aggregation and precipitation of αAAG98R also occurred at 37°C but at a slower rate. It took ~8 hrs to see light scattering by αAAG98R at 37°C and addition of αA-mini-chaperone completely suppresses light scattering (data not shown). In a separate experiment, when different amounts (1–30 μM) of mini-αA-crystallin was used with 10 μM of αAAG98R.


\[ \text{Figure 1. Molar mass distribution of } \alpha\text{-AG98R and wild-type } \alpha\text{-crystallin in the absence and presence of } \alpha\text{-crystallin-derived mini-chaperone.} \]

\[ A, \text{ } \alpha\text{-crystallin wild-type (75 } \mu \text{g, solid line, filled circle); } \alpha\text{-crystallin wild-type (75 } \mu \text{g in presence of } 10 \mu \text{g } \alpha\text{-mini-chaperone (broken line, open circle). Samples were incubated at } 43^\circ \text{C for } 30 \text{ min in PO4 buffer, pH 7.2, and were injected into a TSK-}
\]

\[ \text{Figure 2. Thermal behavior of the wild-type } \alpha\text{-crystallin and } \alpha\text{AG98R incubated with and without mini-chaperone. The protein samples (750 } \mu \text{g were incubated in 1 ml PO4 buffer, pH 7.2 at } 43^\circ \text{C and light scattering was continually monitored at 360 nm for 90 min in a spectrophotometer. The results show that mini-chaperone prevents the light scattering by aggregates formed by } \alpha\text{AG98R incubated at } 43^\circ \text{C. The figure is representative of 3 independent experiments.} \]

\[ \text{doi:10.1371/journal.pone.0044077.g002} \]

\[ \alpha\text{AG98R incubated in the presence of } \alpha\text{-mini-chaperone, in a mutant-to-peptide chaperone ratio of 1: 0.9 (mol/mol), did not form clusters or amorphous aggregates of oligomers (Figure 3 F). Additionally, the size of } \alpha\text{AG98R oligomer incubated with } \alpha\text{-mini-chaperone was slightly smaller than the mutant incubated alone for 10 min at } 40^\circ \text{C. A similar pattern of aggregation and suppression of aggregation with mini-chaperone was also observed by TEM when the mutant protein was incubated at } 37^\circ \text{C for 8 hrs (compare B and C in Figure 3).} \]

\[ \text{Structural changes in } \alpha\text{AG98R in the presence of } \alpha\text{-crystallin-derived mini-chaperone} \]

\[ \text{The thermal behavior of } \alpha\text{AG98R mutant in the presence and absence of } \alpha\text{-mini-chaperone was investigated at both near-UV and far-UV range, using a CD spectrometer equipped with a temperature controller. The temperatures of wild-type } \alpha\text{-crystallin and } \alpha\text{AG98R mutant samples, from } 25^\circ \text{C to } 45^\circ \text{C, were raised slowly and negative ellipticity was recorded. The far-UV CD-spectra showed that wild-type } \alpha\text{-crystallin is very stable until } 40^\circ \text{C, and at temperature above } 40^\circ \text{C, the negative ellipticity at 218 nm increased with increasing sample temperature, indicating structural changes in the protein (Figure 4A). The mutant } \alpha\text{-crystallin began to show a significant increase in ellipticity above } 27^\circ \text{C, and at temperatures above } 40^\circ \text{C, the increases in ellipticity were moderated. Incubation } \alpha\text{AG98R with } \alpha\text{-crystallin mini-chaperone stabilized the protein, as the negative ellipticity at 218 nm remained stable up until } 35^\circ \text{C. Above } 35^\circ \text{C, there was a gradual increase in 218 nm ellipticity, suggesting structural changes in the mutant protein occur at these temperatures even in presence of mini-chaperone. The near-UV CD spectrum of } \alpha\text{AG98R-mini-chaperone was similar to that of wild-type protein in 272-260 nm region, whereas the spectrum in the 300-272 nm region showed minor changes suggestive of interactions between } \alpha\text{AG98R and the mini-chaperone (Figure 4B) but the minimal nature of the interaction may be indicative of the interactions occurring away from the aromatic residues. This is supported by the absence of the peptide effect on intrinsic tryptophan fluorescence of } \alpha\text{AG98R (Figure S3).} \]

\[ \text{doi:10.1371/journal.pone.0044077.g002} \]
Chaperone activity of αAG98R is stabilized after interaction with αA-crystallin-derived mini-chaperone

Earlier we reported that, compared to wild-type αA-crystallin, αAG98R mutant protein showed chaperone activity against denaturing ADH at 37°C during the early phase of the assay [21]. However, αAG98R chaperone activity diminished after 30 min of reaction at 45°C and precipitation of proteins was observed [21]. SDS-PAGE analysis of the precipitate revealed that αAG98R protein co-precipitated along with substrate ADH. We postulated that the precipitation was due to the unstable nature of ADH-αAG98R complex, and investigated whether αA-mini-chaperone would stabilize the complex. Similar to our earlier observation [21], αAG98R suppressed ADH aggregation during the early part of the 2 hr assay but the assay mixture started to scatter light after 60 min (Figure 5A). However, the addition of αA-crystallin-derived mini-chaperone to the reaction mixture of ADH-αAG98R significantly reduced the aggregation of denaturing protein (Figure 5A). It should be noted that the addition of αA-mini-chaperone did not solubilize the aggregates already formed. The suppression of αAG98R aggregation beyond the point of the addition of αA-mini-chaperone could be either due to the effect of αA-mini-chaperone itself or due to the stabilization of the ADH-αAG98R complex by the αA-mini-chaperone. To examine the latter possibility, αAG98R protein was incubated with αA-mini-chaperone at 37°C for 30 min, and the αAG98R-αA-mini-chaperone complex was isolated by gel filtration using a TSK G5000PWxl column and the chaperone activity of the complex was determined employing ADH aggregation assay. The αAG98R treated with αA-mini-chaperone exhibited better chaperone activity than the untreated αAG98R (Figure 5B). Further, the chaperone activity of αA-mini-chaperone-stabilized mutant protein was comparable to that of wild-type αA-crystallin.

Confirmation of αA-crystallin–derived mini-chaperone binding to αAG98R

We took advantage of photoaffinity labeling with Bpa, which was incorporated into the αA-mini-chaperone at one of the Phe sites, to elucidate the αA-mini-chaperone interaction with αAG98R. The biotin at the N-terminal of the peptide chaperone allowed the detection of the αA-mini-chaperone-AG98R complex. Since biotin was attached at the N-terminus and the Bpa group was away from the critical Phe (corresponding to Phe 71 in αA-crystallin), the αA-mini-chaperone retained chaperone activity after these modifications. The photoaffinity labeling of αAG98R was performed using biotin-labeled Bpa-mini-αA and αAG98R. Excess Bpa-mini-chaperone was removed by filtration prior to photolysis. The photolyzed protein was analyzed by SDS-PAGE and western blot using avidin-horseradish peroxidase conjugate against biotin and mass spectrometry. Western blot of UV-irradiated mixture of αAG98R and Bpa-mini-αA separated by SDS-PAGE showed the presence of αAG98R-Bpa-mini-αA cross-linked protein band (Figures 6). The molecular weight of the biotin-containing protein band suggests that one peptide was incorporated into one subunit of αAG98R during photolysis. Image analysis of the stained gel showed that mini-chaperone–αAG98R had photo-crosslinked about 10% of αAG98R. MALDI TOF/TOF mass spectrometric profile of the photolyzed sample also showed that about 10% of αAG98R was bound with one biotin-Bpa-peptide (Figure 7B), whereas the unphotolyzed sample did not show binding of biotinyl-Bpa-mini-chaperone (Figure 7A).

Discussion

The αAG98R mutation in αA-crystallin is associated with early-onset cataract [19]. We and others have shown that αAG98R protein has altered structure, stability and chaperone activity [20–
A binding interface exposed to interact with another oligomer, only a few subunits in an oligomer may have aggregation. Although each subunit in the oligomer has mutation recognizing another oligomer and this process could lead to the formation of aggregates of 2–10 oligomers. Further studies are required to identify all of the exposed residues as a consequence of the G98R mutation. Stabilization of Mutant αA-Crystallin

Figure 4. A, Far-UV CD analysis during thermal denaturation of αAG98R in the presence and absence of αA-mini-chaperone. Proteins, 100 μg, were prepared in 400 μl of 10 mM phosphate buffer, pH 7.2, and the sample temperatures were slowly raised from 25 to 48°C in 2°C steps, equilibrated for 5 min at each temperature prior to far-UV CD measurement. Molar ellipticity changes at 218 nm were plotted to determine relative stability of samples. B, Near-UV CD spectra of αAG98R and αAG98R+αA-mini-chaperone. The spectra were recorded using a protein sample of 1.5 mg/ml. The profile shown is the average of 5 scans. The far-UV CD results show that the αA-mini-chaperone has the stabilizing effect on αAG98R.

doi:10.1371/journal.pone.0044077.g004

In vitro incubation of mutant protein at 37°C leads to the formation of soluble aggregates which, with time, coalesce and precipitate [21,22]. Almost all of the mutant protein precipitates if incubation continues for several hours at 37°C or 40°C. This behavior is typical of many mutant forms of lens crystallins, such as αD mutants L5S, V75S and I90F [32], and αB mutants F27R [33] and D140N [34]. Aggregation and precipitation are hallmarks of cataract-causing crystallin mutations. TEM studies show that αAG98R oligomers interact with one another to form clusters of 2 to 3 oligomers or linear structures composed of 3 to 8 oligomers in 10 min of incubation at 40°C (Figure 3). At 37°C, it takes 6–8 hrs to form such aggregates, whereas at the slightly higher temperature of 40°C, aggregation begins as early as 10 min (compare Figures 3B and 3D). With time, the aggregates coalesce to form irregular aggregates having several oligomers, as shown in Figure 3E. We do not yet know which residues on the surface of the oligomers are involved in oligomer dimerization or initial aggregation. Although each subunit in the oligomer has mutation and altered structure, only a few subunits in an oligomer may have a binding interface exposed to interact with another oligomer, since all the subunits are not equally positioned due to the irregular polydisperse nature of αAG98R oligomers [21]. Such a limitation of interaction sites would initially result in a linear arrangement or the formation of dimers and trimers of the oligomer rather than the formation of an oligomer fully decorated with additional oligomers to form a cluster of several oligomers. With time, however, the aggregates of 2–10 oligomers would interact with one another to form amorphous aggregates, as shown in images of the 30 min sample at 40°C (Figure 3E). Because the mutant protein has an altered structure and increased hydrophobicity [20–22], we hypothesize that the G98R mutation exposes specific hydrophobic regions and these interact with other oligomers to form aggregates. Further studies are required to identify all of the exposed residues as a consequence of the G98R mutation. Alternately, it is possible that the increased chaperone property of the subunits in the mutant oligomer is responsible for recognizing another oligomer and this process could lead to the formation of aggregates of 2–10 oligomers. In support of this, it
was shown earlier that zAG98R variant [21] and cataract-causing mutant of zA-crystallin R116C has enhanced affinity toward client proteins [35].

Earlier we discovered that a peptide representing the chaperone site of zA-crystallin is sufficient to suppress the aggregation of denaturing proteins. We showed that the peptide chaperone stabilizes the partially unfolded proteins [26–30] and prevents fibril formation by β-amyloid [31]. Since zAG98R has an altered structure, we investigated whether the zA-crystallin–derived mini-chaperone would prevent the mutant protein from precipitation during incubation. When zA-crystallin–derived mini-chaperone was incubated with zAG98R, we found that the zAG98R was stabilized and remained in solution (Figure 2) and the stabilized zAG98R can be isolated by chromatography (Figure 1 B). This observation was confirmed by TEM study (Figure 3), which showed that aggregation of zAG98R was prevented by the zA-mini-chaperone. We believe that the mini-chaperone interacts with the exposed hydrophobic regions of the mutant proteins and prevents these sites from binding to another oligomer to form aggregates that precipitate during the incubation at 37°C or 40°C. Further studies are required to confirm this since interaction of mini-chaperone with zAG98R did not result in significant change in hydrophobic probe Bis-ANS binding (Figure S3 A).

The addition of the mini-chaperone to the zAG98R sample prior to incubation at 43°C and chromatography by gel filtration increased by 8-fold the recovery of zAG98R in the soluble form (Figure 1B). However, an inactive form of mini-chaperone (DFVPFLDVKHFSPEPDLTK) did not prevent the precipitation of zAG98R, suggesting the chaperone activity of the peptide was responsible for maintaining the mutant protein in soluble form. The interaction of the zA-crystallin–derived mini-chaperone with zAG98R was confirmed by reversed-phase HPLC analysis of the zAG98R peak recovered following incubation of active mini-chaperone and mutant protein (Figure S1). Under similar experimental conditions, the wild-type zA-crystallin showed negligible interaction with mini-chaperone (Figure S1), suggesting that the conformational change in zAG98R perhaps acted as a chaperone sensor.

Peptides substituted with the photoactive amino acid Bpa have been used to confirm the interaction between ligand and receptor [36–38]. We substituted one of the three phenylalanines in zA-mini-chaperone with Bpa and biotinylated the N-terminal amino group to obtain biotin-DFIVFLDVKH(benzoylphenylalanine)-SPEDLTK. The chaperone peptide was active in suppressing the aggregation of heat-denatured ADH. When the biotinyl-Bpa-mini-chaperone–zAG98R incubation mixture was photolyzed and subjected to SDS-PAGE and western blot analysis, covalent association of zA-mini-chaperone with zAG98R subunits was observed (Figure 6A). The binding of biotinyl-Bpa-chaperone to zAG98R was also confirmed by MS analysis. The mass of the complex, 22592.3 m/z (Figure 6B) is equal to 1:1 binding of zA-mini-chaperone and zAG98R subunit. We found that only about 10% of the Bpa-peptide was incorporated into zAG98R subunit. Bpa photocross linking efficiency is dependent on the duration of UV exposure, the affinity of the ligands and the orientation of the Bpa residue [37]. The low insertion of Bpa in our hands may in part be due to shorter photolysis time. We did not extend the photolysis time to minimize any UV-induced structural change in the protein that may influence the interaction of peptide with zAG98R. Further, it is unlikely that all subunits in the zAG98R oligomer interact with the mini-chaperone equally because of uneven exposure of hydrophobic regions to the surface in mutant crystallin.

Modulation of wild-type z-crystallin chaperone activity by small molecules such as ATP [39], glutathione [40], arginine and aminoguanidine [41,42] has been previously reported. In those studies the modulator was used in 10- to 30-fold higher concentrations than the z-crystallin [39–42] and the conformational changes in z-crystallin in the presence of the modulator was considered to be responsible for the activity enhancement. Our study shows that 2-fold higher concentration of mini-chaperone is sufficient to stabilize the mutant zAG98R-crystallin in solution and the mini-chaperone stabilized crystallin has chaperone activity comparable to that of WT-zA-crystallin (Fig. 5B). We reported earlier that diithiothreitol (DTT) treatment of z-crystallin in the water-insoluble fraction of lens proteins restores some of the lost chaperone activity [43]. Oxidation of methionine in z-crystallin leads to loss of chaperone activity and this can be reversed by treatment with methionine sulfoxide reductase [44]. However, none of the studies carried out thus far attempted to rescue the chaperone activity in mutant forms of zA- or zB-crystallins. We have previously shown that zA-crystallin–derived mini-chaperone can suppress the aggregation of several proteins [26–30] and prevent fibril formation by β-amyloid [31]. This study is the first report on the stabilization of a mutant zA-crystallin by a mini-chaperone derived from zA-crystallin. The rescuing of chaperone activity in zAG98R by zA-mini-chaperone can be compared to the interaction of a C-terminal peptide of p53 with inactive mutant forms of the same protein and restoration of its activity [45,46]. The specific interaction between the zA-mini-chaperone and zAG98R subunit demonstrates the ability of the zA-mini-chaperone to act as a chaperone toward structurally perturbed
\(\alpha\)AG98R, akin to the mini-chaperone suppressing the aggregation of denaturing proteins [26,30,31].

In summary, we have shown that the \(\alpha\)A-crystallin–derived mini-chaperone can suppress the aggregation of mutant parent protein. The increased stability of the mutant protein, coupled with only marginal increase in Rh in the presence of chaperone peptide suggests that the \(\alpha\)A-mini-chaperone has the potential to become a therapeutic agent to stabilize the cataract-causing mutant forms of \(\alpha\)A-crystallin. We propose that \(\alpha\)A-crystallin–derived mini-chaperones or synthetic chaperones with mini-chaperone electrostatic surface would have the capacity to control the aggregation of crystallin-client protein complexes or conformationally challenged proteins. Further, peptide chaperones may serve as universal chaperones for controlling diseases involving protein aggregation.

**Supporting Information**

**Figure S1** Elution profile of mini-\(\alpha\)A, \(\alpha\)AG98R-mini\(\alpha\)A complex and \(\alpha\)A-WT treated with \(\alpha\)A-mini-chaperone from a C8 column. 100 µg of \(\alpha\)AG98R or WT-\(\alpha\)A-crystallin and 10 µg of peptides were used in the study. Samples were passed through TSK5000pw column was used to separate \(\alpha\)A-crystallin peak from the unbound peptides. The protein from the \(\alpha\)A-crystallin peak was subsequently analyzed in a Vydac 208TP column (250 mm × 4.6 mm) fitted to a Shimadzu HPLC system. Acetonitrile gradient (0–80%) over a period of 40 min was used to resolve the components. Eluent A was 0.1% trifluoroacetic acid in...
water and eluent B was acetonitrile. Detector was set at 220 nm and the flow rate 1 ml/min. A. Analysis of αA-mini-chaperone-AG98R and αA-mini-chaperone. B. Analysis of αA-minichaperone and WT-αA-crystallin. The HPLC analysis of the fractions at α-crystallin elution region from gel filtration column shows the binding of αA-mini-chaperone to mutant protein but not to wild-type αA-crystallin. The figure is representative of 3 independent experiments.

(TIF)

Figure S2 Chaperone assay in presence of either αA-mini-chaperone or αA-mini-chaperone with proline substitution. The EDTA-induced aggregation of ADH assay was performed at 37°C as described under methods. In each experiment 250 µg of ADH was used. Curve 1, ADH alone; Curve 2, ADH+αA-mini-chaperone (pro) 50 µg; Curve 3, ADH+αA-min-chaperone, 50 µg. The results show that Pro-substitution abolishes the chaperone activity of mini-chaperone. The figure is representative of two independent experiments.

(TIF)

Figure S3 Fluorescence studies of αA99R in presence or absence of αA-mini-chaperone. A, bis-ANS (1,1'-bi(4-anilino)naphthalene-5,5'-disulfonic acid) interaction with mutant protein before and after addition of αA-mini-chaperone was recorded as described under methods. The spectra shows minimal change in fluorescence after the peptide interaction with αA99R. B, Intrinsic fluorescence spectra of αA99R before and after addition αA-mini-chaperone. The data, representative of two independent experiments, shows minimum change in the bis-ANS binding or intrinsic tryptophan fluorescence in mutant protein following treatment with αA-mini-chaperone.

(TIF)

Acknowledgments

We thank Sharon Morey for help with preparation of the manuscript and Beverly DaGue for performing mass spectrometry analysis.

Author Contributions

Conceived and designed the experiments: KKS MR PS. Performed the experiments: MR PS. Analyzed the data: KKS MR PS. Contributed reagents/materials/analysis tools: KKS. Wrote the paper: KKS MR PS.

References

1. Bloemendal H (1977) The vertebrate eye lens. Science 197: 127–138.
2. Ingolia TD, Craig EA (1982) Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc Natl Acad Sci USA 79: 2360–2364.
3. Merck KB, Groenroj EP, Voorster CE, de Haard-Hoekman WA, Horvitz J, et al. (1993) Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein. A family of proteins. J Biol Chem 268: 1046–1052.
4. Klementz B, Frohli E, Steiger RH, Schaefer R, Aoyama A (1991) Alpha B-crystallin is a small heat shock protein. Proc Natl Acad Sci USA 88: 3632–3636.
5. Horvitz J (1992) Alpha crystallin can function as a molecular chaperone. Proc Natl Acad Sci USA 89: 1049–1053.
6. Rao PV, Huang QL, Horvitz J, Zigler JS, Jr. (1995) Evidence that alpha-crystallin prevents non-specific protein aggregation in the intact eye lens. Biochim Biophys Acta 1245: 439–447.
7. Horvitz J (2000) The function of alpha-crystallin in vision. Semin Cell Dev Biol 11: 53–60.
8. Rajaraman K, Raman B, Ramakrishna T, Rao CM (2001) Interaction of human recombinant alpha-A and alpha-B-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation. FEBS Lett 497: 118–123.
9. Reddy GB, Reddy PY, Suryarayana P (2001) alpha-A- and alpha-B-crystallins protect glucose-6-phosphate dehydrogenase against UV irradiation-induced inactivation. Biochem Biophys Res Commun 282: 712–716.
10. Hawkins JW, Van Keuren ML, Plagionsky J, Law ML, Patterson D, et al. (1987) Confirmation of assignment of the human alpha 1-crystallin gene (CRYA1) to chromosome 21 with regional localization to q22.3. Human Genetics 76: 375–380.
11. Graw J, Kratochvilova J, Summer KH (1984) Genetical and biochemical studies of a dominant cataract mutant in mice. Exp Eye Res 39: 37–45.
12. Graw J (2009) Genetics of crystallins: cataract and beyond. Exp Eye Res 89: 173–189.
13. Sharma KK, Santoshkumar P (2009) Lens aging: effects of crystallins. Biochim Biophys Acta 1790: 1095–1108.
14. Hanlon L, Yao W, Eberg H, Jager KW, Baggesen K, et al. (2007) Genetic heterogeneity in microcornea-cataract: five novel mutations in CRYAA, CRYGD, and GJA8. Invest Ophthalmol Vis Sci 48: 3937–3944.
15. Graw J, Kopp N, Illig T, Lorenz B (2006) Congenital cataract and macular hypoplasia in humans associated with a de novo mutation in CRYAA. Mol Gen Genet 17: 7–15.
16. Mackay DS, Andley UP, Shiels A (2003) Cell death triggered by a novel neurokinin-1 tachykinin receptor. Eur J Biochem 270: 4161–4169.
17. Xia CH, Liu H, Chang B, Cheung C, Cheung D, et al. (2006) Arginine 54 and Tyrosine 118 residues of alpha A-crystallin are crucial for lens formation and transparency. Invest Ophthalmol Vis Sci 47: 1069–1075.
18. Lit M, Kramer P, LaMorticella DM, Murphy W, Lovrien EW, et al. (1998) Autosomal dominant congenital cataract associated with a missense mutation in the human alpha crystallin gene CRYAA. Hum Mol Genet 7: 471–474.
19. Santhoshkumar P, Sharma KK (2004) Inhibition of amyloid fibrillogenesis and toxicity by a peptide chaperone. Mol Cell Biochem 267: 147–155.
20. Moreau KL, King J (2009) Hydrophobic core mutations associated with cataract development in mice destabilize human gammaD-crystallin. J Biol Chem 284: 33285–33295.
21. Horvitz J, Bova M, Huang QL, Ding L, Yaron O, et al. (1998) Mutation of alpha B-crystallin: effects on cataract-like activity. Int J Biol Macromol 22: 263–269.
22. Liu Y, Zhang X, Luo L, Wu M, Zeng R, et al. (2006) A novel alphaB-crystallin mutation associated with autosomal dominant congenital lamellar cataract. Invest Ophthalmol Vis Sci 47: 1069–1073.
23. Kotecha HA, McHasourah HS (2006) Mechanism of a hereditary cataract phenotype. Mutations in alpha A-crystallin activate substrate binding. J Biol Chem 281: 14273–14279.
24. Dorman G (1998) Estimation of the binding site of drugs by means of new types of photoactive ligands. Acta Pharm Hung 68: 95–105.
25. Dorman G, Prestwich GD (1994) Benzophenone photophores in biochemistry. J Biochem 281: 14273–14279.
26. Girault S, Sagan S, Bologh B, Lavielle S, Chassaing G (1996) The use of photoactive ligands. Acta Pharm Hung 68: 95–105.
27. Raju M, Santhoshkumar P, Sharma KK (2011) Cataract-causing alpha(AG98R)-crystallin mutant dissociates into monomers having chaperone activity. Molecular Vision 17: 7–15.
28. Sreelakshmi Y, Sharma KK (2000) Chaperone-like activity of a synthetic peptide toward oxidized gamma-crystallin. J Peptide Res 56: 157–164.
29. Bhattacheraya J, Sharma KK (2003) Conformational specificity of mini-alphaA-crystallin as a molecular chaperone. J Peptide Res 57: 428–434.
30. Sreelakshmi Y, Sharma KK (2000) Interaction of alpha-lactalbumin with mini-alphaA-crystallin. J Pept Res 10: 125–130.
31. Santoshkumar P, Sharma KK (2004) Identification of amyloid fibrillogenesis and toxicity by a peptide chaperone. Mol Cell Biochem 267: 147–155.
32. Moreau KL, King J (2009) Hydrophobic core mutations associated with cataract development in mice destabilize human gammaD-crystallin. J Biol Chem 284: 33285–33295.
33. Del Rio V, Lagage M, Bichet DG, Bouvier M (2004) Pharmacological chaperones: potential treatment for conformational diseases. Trends Endocrinol Metab 15: 222–228.
34. Cohen FE, Kelly JW (2003) Therapeutic approaches to protein-misfolding diseases. Nature 426: 905–909.
40. Pal J, Bera S, Ghosh SK (1998) The effect of glutathione upon chaperone activity of alpha-crystallin is probably mediated through target modulation. Ophthal Res 30: 271–279.
41. Srinivas V, Raman B, Rao KS, Ramakrishna T, Rao Ch M (2003) Structural perturbation and enhancement of the chaperone-like activity of alpha-crystallin by arginine hydrochloride. Protein Sci 12: 1262–1270.
42. Srinivas V, Raman B, Rao KS, Ramakrishna T, Rao Ch M (2005) Arginine hydrochloride enhances the dynamics of subunit assembly and the chaperone-like activity of alpha-crystallin. Molecular Vision 11: 249–253.
43. Sharma KK, Ortwerth BJ (1995) Effect of cross-linking on the chaperone-like function of alpha crystallin. Exp Eye Res 61: 413–421.
44. Kantorow M, Hawse JR, Cowell TL, Benhamed S, Pizarro GO, et al. (2004) Methionine sulfoxide reductase A is important for lens cell viability and resistance to oxidative stress. Proc Natl Acad Sci USA 101: 9654–9659.
45. Friedler A, Hansson LO, Veprintsev DB, Freund SM, Rippin TM, et al. (2002) A peptide that binds and stabilizes p53 core domain: chaperone strategy for rescue of oncogenic mutants. Proc Natl Acad Sci USA 99: 937–942.
46. Selivanova G, Iotsova V, Okan I, Frische M, Strom M, et al. (1997) Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. Nature Med 3: 632–638.