

Cataract-causing α AG98R-crystallin mutant dissociates into monomers having chaperone activity

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Purpose: The G98R mutation in α A-crystallin is associated with autosomal dominant cataract in humans. We have reported that mutant G98R protein has substrate-dependent chaperone activity. Further studies on this G98R mutant protein revealed that mutant protein shows reduced oligomeric stability and accelerated subunit dissociation at a low protein concentration. The purpose of present study was to investigate the chaperone function of dissociated subunits of α AG98R-crystallin.

Methods: Substitution of glycine with arginine at position 98 in human α A-crystallin was accomplished by site-directed mutagenesis. The recombinant protein was expressed in *E. coli* cells and purified by chromatographic techniques. Purified α AG98R-crystallin was diluted to a concentration of 0.1 mg/ml in 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2) and incubated at 37 °C for 24 h. The monomeric subunits were isolated from the oligomers through 50 kDa cutoff filters. The monomers were analyzed by SDS-PAGE, mass spectrometry, and circular dichroism spectroscopy and characterized by multi-angle light-scattering methods. Chaperone activity was tested against four client proteins: citrate synthase, alcohol dehydrogenase, bovine β B2-crystallin and ovotransferrin.

Results: Gel filtration studies showed that α AG98R-crystallin oligomers dissociate readily into monomers. Subunits of α AG98R-crystallin, isolated either by size exclusion chromatography or filtration showed chaperone activity against heat-denatured alcohol dehydrogenase, citrate synthase, bovine β B2-crystallin, and chemically denatured ovotransferrin. SDS-PAGE analysis of the mutant protein incubated at 37 °C for 12 days showed autolysis, which was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS/MS) analysis of α AG98R-crystallin fragments recovered after SDS-PAGE.

Conclusions: The present study shows that the G98R mutation in α A-crystallin produces unstable oligomers that dissociate into active chaperone subunits. The chaperone activity of the dissociated subunits against four client proteins suggests that the α A-crystallin subunits are the minimal units of chaperone activity.

α -Crystallin belongs to the family of small heat shock proteins (sHSP) and is composed of two subunits, α A- and α B-, which form heteromers and homomers with varying number of subunits [1]. Size exclusion chromatography (SEC) analysis of a wide range of α A- and α B-crystallin oligomer concentrations, from as low as 5 μ g/ml to as high as 5 mg/ml, has shown that such oligomers, although dynamic, do not dissociate into monomers under physiologic conditions [2]. However, wild-type α A- and α B-crystallins have been found to dissociate into dimers and tetramers at <5 μ g/ml concentrations [3]. A detailed study on a truncated form of α B-crystallin, Q151X, has shown that this mutant dissociates into monomers at <0.1 mg/ml concentrations, whereas it aggregates at higher concentrations [4].

Like other sHSPs, α -crystallin prevents aggregation of denaturing proteins [5]. It is well documented that both α A- and α B-crystallin subunits show chaperone activity [5]. Many

of the mutant α A- and α B-crystallins studied thus far have shown altered structure, aggregation propensity and varying chaperone activity. Several mutations in the α -crystallin gene have been linked to cataract [6]. For example, an autosomal dominant G98R mutation in α A-crystallin, found in an Indian family, leads to pre-senile cataract in young adults [7]. Earlier studies with recombinant α AG98R-crystallin revealed that the mutation affects the protein's structure, stability and chaperone activity [8]. In a previous study we showed that α AG98R-crystallin exhibits substrate-dependent chaperone activity [9].

α A- and α B-crystallin oligomers as well as the dimers have been found to exhibit chaperone activity [10]. The evidence in support of this comes from studies where native oligomeric crystallins or truncated human α B-crystallin (α B 57–157), which exists as dimer, were used in chaperone assays [11]. Those data suggest that oligomer organization is not essential for the chaperone property of α -crystallin subunits. We have shown that short sequences of α A- and α B-crystallin, representing the chaperone sites of native proteins, possess chaperone activity [12,13]. Chaperone activity has also been found in truncated α B-crystallin capable of dissociating into

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monomers [4]. While we were characterizing α AG98R-crystallin protein, we observed that the α AG98R-crystallin oligomer readily dissociates into monomeric form. This gave us an opportunity to investigate whether chaperone function is an inherent property of full-length individual subunits of α A-crystallin. In the present study, chaperone function of dissociated α AG98R-crystallin subunits was investigated using citrate synthesis (CS), alcohol dehydrogenase (ADH), bovine β B2-crystallin, and ovotransferrin as client proteins. The results suggest that the dissociated subunits exhibit chaperone activity in a concentration-dependant manner.

Recent studies have shown that the majority of mutant crystallins form insoluble aggregates, nonspecifically interact with other proteins and precipitate, yet some mutants and truncated forms of α A- and α B-crystallins are less stable and form oligomers with increased polydispersity [4,14-18]. Additionally, one of the mutant α -crystallins, α BR120G-crystallin, was shown to undergo premature or accelerated degradation on storage [19]. In the present study we show that although the mutant α AG98R-crystallin aggregates at high concentrations, at low concentrations the protein dissociates into monomers and undergoes autolysis on storage.

METHODS

Preparation of α AG98R-crystallin mutant and wild-type α A-crystallin: Human α A-crystallin cDNA (obtained from J.M. Petrush, Washington University, St. Louis, MO) was cloned into pET-23d (+) vector (Novagen, Madison, WI). This cloned cDNA was used as a template to generate mutation in the α A-crystallin gene using a Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) with a set of primers as described earlier [9]. The G98R mutation was confirmed by automated DNA sequencing. Both mutant and wild-type proteins were expressed in *E.coli* BL21(DE3) pLysS cells (Invitrogen, Carlsbad, CA) and purified as described earlier [8]. Briefly, bacterial cell pellets were obtained from 1 l culture, suspended in 10 ml lysis buffer containing 50mM Tris-HCl (pH 8.0), 100 mM NaCl, and 2 mM EDTA, and treated with 50 μ l of protease inhibitor cocktail III, 1 mg lysozyme, and 10 mM DTT. The cell suspension was treated with 1 μ l of benzonase and incubated at 37 °C on a shaking platform for 30 min. The extract was centrifuged at 17,000 \times g for 1 h. The α AG98R-crystallin protein partitioned into insoluble fraction was washed and redissolved in 20mM Tris-HCl buffer (pH 7.2) containing 6 M urea and 1 mM EDTA. The urea-dissolved supernatant was filtered and loaded into a Q-Sepharose Fast Flow ion-exchange column equilibrated with Tris-EDTA buffer. The protein was eluted using a stepwise gradient of 1 M NaCl in 50 mM Tris-HCl (pH 7.2) containing 1 mM EDTA at a flow rate of 1 ml/min. The wild-type α A-crystallin protein partitioned into the soluble fraction was initially purified on a HiLoad 16/60 Superdex 200 gel filtration column equilibrated with 50 mM phosphate buffer containing 150 mM NaCl (pH

7.2). The peak containing the wild-type α A-crystallin was pooled, concentrated, treated with solid urea (6 M) and purified using anion exchange column, as described for the mutant protein. The purity of the proteins was examined by SDS-PAGE and the molecular mass was determined by mass spectrometry. The concentrations of the mutant and of the wild-type proteins were estimated using Bio-Rad protein assay reagent.

Light scattering studies: Protein samples were injected into a TSKG5000PW_{XL} (Tosoh Bioscience, Montgomeryville, PA) size-exclusion column equilibrated with 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2). The flow rate was 0.75 ml/min. The size-exclusion column was attached to a HPLC system connected with UV and refractive index detectors (Shimadzu, Columbia, MD) and coupled to static multi-angle laser light scattering (DAWN-EOS) and dynamic quasi-elastic light scattering detectors (Wyatt Technology, Santa Barbara, CA). The data were analyzed using ASTRA (5.3.4.14) software (Wyatt Technology).

Isolation of α AG98R-crystallin monomer protein: Purified α AG98R-crystallin mutant protein was diluted in 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2) to obtain a concentration of 0.1 mg/ml. This solution was incubated at 37 °C for 24 h and passed through 50 kDa cutoff membrane filters (Amicon, Millipore). Filtrate used in all experiments was analyzed by size exclusion chromatography, as described above.

Mass spectrometric analysis: Dissociated subunits of α AG98R-crystallin mutant proteins were desalted using PepClean C18 column before mass spectrometry (MS). The protein bound to the PepClean column was eluted in 40 μ l of 70% acetonitrile, dried on a SpeedVac, re-dissolved in 20 μ l of elution buffer, and subjected to MALDI TOF MS analysis.

Chaperone activity measurements: The chaperone activity of wild-type α A-crystallin and of the α AG98R-crystallin monomer protein was measured using substrates alcohol dehydrogenase (ADH; Biozyme, San Diego, CA), citrate synthase (CS; Sigma, St Louis, MO), β B2-crystallin, and ovotransferrin (Sigma, St. Louis, MO). 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. Assays were done in the absence or presence of wild-type or mutant proteins as a function of time. Aggregation of ADH (75 μ g) was induced by the addition of 100 mM EDTA in 50 mM phosphate buffer containing 150 mM NaCl (pH 7.3) at 37 °C. For CS aggregation assay, 75 μ g of CS in 1 ml of 40 mM HEPES-KOH buffer (pH 7.4) was heated to 43 °C. CS aggregation at 360 nm was measured up to 1 h. During ovotransferrin aggregation assay, 100 μ g of the protein in 1 ml of 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2) was kept at 37 °C and allowed to denature. To investigate the effect of α AG98R-crystallin on β B2-crystallin, 150 μ g of

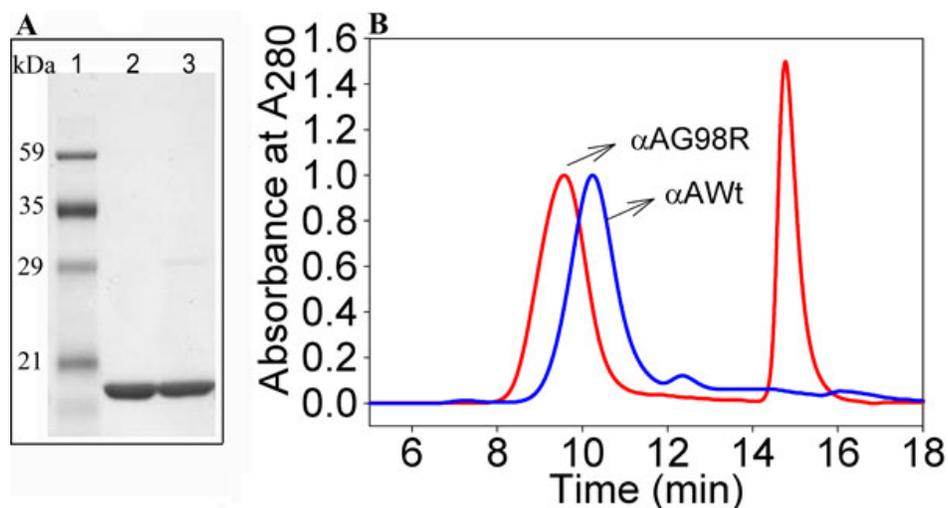


Figure 1. Electrophoretic and size exclusion chromatography profile of wild-type and α AG98R-crystallin. **A:** Coomassie Blue-stained SDS-PAGE of G98R mutant and wild-type α A-crystallin, showing >98% purity of the crystallins used in the study. Lane 1, protein markers; Lane 2, wild-type α A-crystallin; and Lane 3 α AG98R-crystallin. **B:** Size exclusion chromatography profile of α AG98R-crystallin and wild-type α A-crystallin. 100 μ g of protein at 1mg/ml concentration in phosphate buffer was injected into a TSK-G5000PWXL gel filtration column (7.6 mm \times 30 cm). Fractions of 0.75 ml were collected. The mutant G98R protein (red) shows two peaks, one at 9.5 min, corresponding to the oligomer, and another peak at 15 min, corresponding to monomer mass. Wild-type α -crystallin (blue) did not show two peaks.

β 2-crystallin isolated from bovine lens extract was used. The aggregation assay was performed at 37 °C, as described earlier [20].

Mutant protein stability studies: α AG98R-crystallin mutant protein (0.1 mg/ml) was incubated in 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2) under sterile conditions for 0, 2, 6, and 12 days at 37 °C. Aliquots of these samples were subjected to SDS-PAGE and MS analysis. In addition, 250 μ l of the sample was analyzed by size exclusion chromatography connected with MALS instrument. The data were analyzed as described above. Control experiments with wild-type α A-crystallin were run simultaneously with the mutant protein experiments.

RESULTS

Expression of wild-type α A- and α AG98R-crystallins and isolation and characterization of α AG98R-crystallin subunits: Recombinant proteins were expressed and isolated following the procedure described earlier [9]. Although the mutant protein expressed in *E.coli* cells forms inclusion bodies due to high in vivo protein concentration after urea solubilisation, purification and refolding at <2 mg/ml concentrations the recombinant protein remains soluble in assay buffers for several days to permit the characterization. On the basis of SDS-PAGE profile, both wild-type and mutant recombinant crystallins used in this study were >98% pure (Figure 1A). Size exclusion chromatography of mutant G98R protein at 1 mg/ml concentration gave an elution profile with an oligomer peak eluting at 9.5 min and a monomer peak eluting at 15 min, indicating the dissociation of oligomeric assembly (Figure 1B). In contrast, wild-type α A-crystallin

eluted as a single peak at 10 min, corresponding to the elution time of α A-crystallin oligomers (Figure 1B).

After observing the dissociation of mutant protein during size exclusion chromatography, the following experiment was performed to isolate a sufficient amount of monomer protein for further study. Purified α AG98R-crystallin protein was diluted to concentrations of 0.1 mg/ml, 0.2 mg/ml and 0.5 mg/ml in 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2). The diluted protein solutions were subjected to size exclusion chromatography. The elution profile showed an inverse relationship between the concentration of mutant protein and the degree of dissociation. Over 95% dissociation was observed when the protein concentration was 0.1 mg/ml (Figure 2A), whereas only partial dissociation occurred with higher α AG98R concentrations. Examination of the effect of pH on dissociation of α AG98R-crystallin mutant protein, at 1 mg/ml concentration, revealed about 70% dissociation of the mutant protein at pH 5.9. The precipitation of the mutant protein at higher concentrations did not allow us to fully evaluate the relationship between α AG98R-crystallin concentration and dissociation at pH 5.9 (data not shown). The dissociated subunits of α AG98R-crystallin were also analyzed by SDS-PAGE and western blot. The results showed that, in addition to a 20 kDa band corresponding to the molecular weight of α A-crystallin subunit, an additional band at ~11 kDa region was observed in immunoblots (Figure 2B). A corresponding protein band was not clearly visible, however, after staining with Coomassie Blue. When the immunoblot was analyzed using *ImageJ* software, the 11 kDa band was about 13% of the total. To investigate whether the 11 kDa protein band is a breakdown fragment of mutant

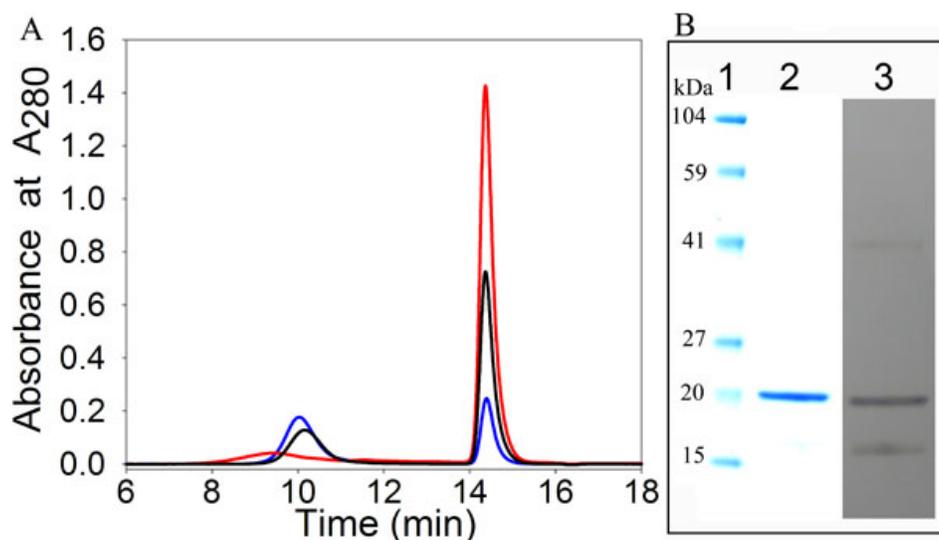


Figure 2. Size exclusion chromatography profile and immunoblot showing monomers of α AG98R-crystallin. **A:** Size-exclusion chromatography of three concentrations of α AG98R-crystallin. α AG98R-crystallin was injected to a TSK-5000 PW_{XL} column (7.6 mm \times 30 cm) in 3 concentrations and the elution profile was recorded by following the 280 nm absorbance. Blue, 0.5 mg/ml; Black, 0.2 mg/ml and Red, 0.1 mg/ml. **B:** SDS-PAGE and immunoblot of dissociated subunits of α AG98R-crystallin protein obtained during size exclusion chromatography analysis. Lane1, Marker proteins; lane 2, α AG98R-crystallin stained with Coomassie Blue; lane 3, western blot of lane 2 sample probed with anti- α A-crystallin.

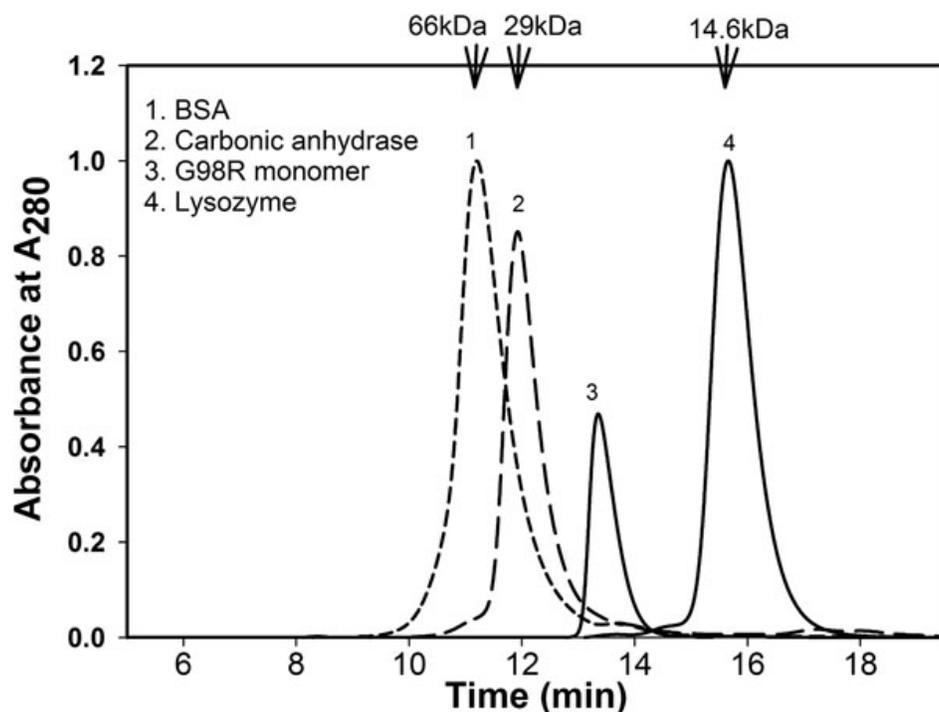


Figure 3. Relative elution profile of α AG98R-crystallin subunits during size exclusion chromatography on TSK-3000 PW_{XL} column (7.6 mm \times 30 cm). 1) BSA (66 kDa); 2) Carbonic anhydrase (29 kDa); 3) α AG98R-crystallin and 4) lysozyme (14.6 kDa).

protein or a contaminant co-purified with mutant protein, MALDI-TOF MS analysis of the 11 kDa band excised from the SDS-PAGE was performed. Trypsin digestion and MALDI-TOF MS analysis of the 11 kDa band showed peptides arising from both NH₂- and COOH-terminal regions of α A-crystallin protein and accounted for 47% of the native α A-crystallin sequence, confirming that the mutant protein was the source of the 11 kDa band. Filtration of the 0.1 mg/ml α AG98R solution through 50 kDa filter separated the oligomers from monomers. This was confirmed by size

exclusion chromatography of 50 kDa filtrate using TSK 3000 gel filtration column and using as standards BSA (66 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.6 kDa). As shown in Figure 3, the V_e/V_o value for α AG98R-crystallin collected as filtrate during 50 kDa filtration was 4.29, which corresponds to a molecular weight of 19,500 Da, estimated using the elution profile of standard proteins – serum albumin, carbonic anhydrase and lysozyme.

To determine whether structural changes are present in dissociated α AG98R-crystallin subunits as compared to the

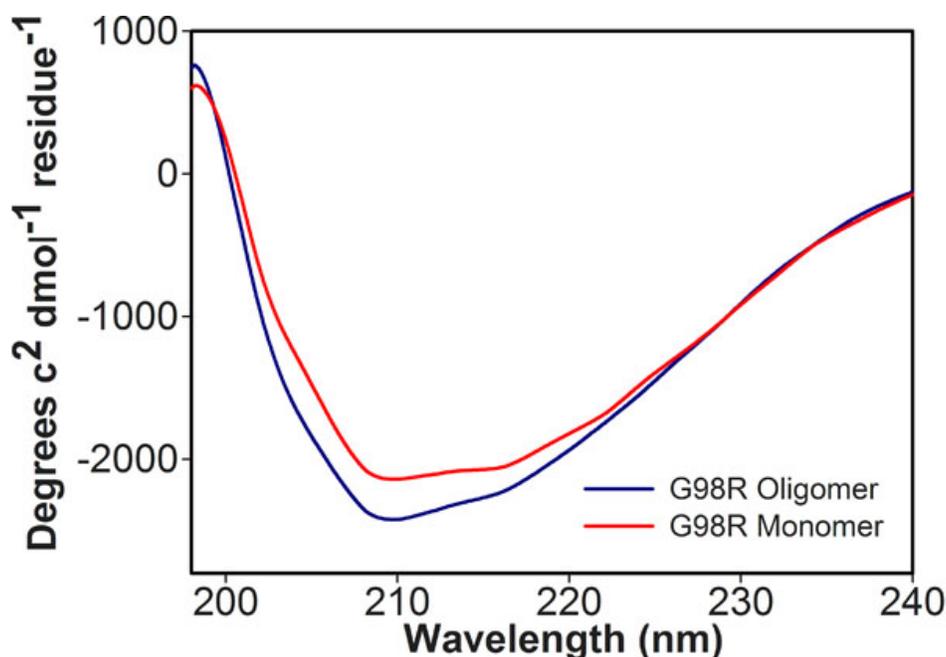


Figure 4. Far-UV Circular dichroism (CD) spectra of α AG98R-crystallin monomeric protein. CD spectra were recorded using 0.1 mg/ml protein in a 0.2 cm cell path length cuvette at 25 °C. The spectra shown represent an average of six scans.

wild-type protein, the subunits of the mutant protein collected by filtration were subjected to far-UV CD spectroscopy in Jasco J 815 spectropolarimeter. When the protein sample (0.1 mg/ml) was analyzed in a 0.2-cm cuvette, the spectrum of the monomer sample showed increased negative ellipticity at 208 nm, indicating that the mutant protein has increased α -helical content similar to that of oligomeric α AG98R-crystallin (Figure 4). The secondary structural elements of the monomer protein (α -helix 3.8%, β -sheet 41.3%, β -turn 22.1%, and random coil 32.8%) did not significantly differ from the structural elements of the oligomeric form of α AG98R-crystallin analyzed at the same time (α -helix 3.7%, β -sheet 42.4%, β -turn 22.5%, and random coil 32.5%). However, the secondary structural contents of the mutant protein in both monomeric and oligomeric forms slightly differed from that of wild-type α A-crystallin (α -helix 2.1%, β -sheet 41%, β -turn 22.8%, and random coil 34.1%) [9].

Chaperone-like activity of monomeric protein: To investigate whether oligomerization is essential for chaperone activity of α A-crystallin, we performed chaperone assays using monomers of α AG98R-crystallin and the four different client proteins. Aggregation of CS, bovine β B₂-crystallin, ADH, and ovotransferrin was measured in the presence and absence of α AG98R-crystallin monomers. Monomers of α AG98R-crystallin displayed chaperone activity against EDTA-induced ADH aggregation (Figure 5A). However, the dissociated subunits showed 25% less protection than the oligomeric wild-type protein (Figure 5A). CS aggregation assay showed that the monomers of mutant α AG98R-crystallin have chaperone activity at 43 °C (Figure 5C) and the activity was comparable to that of wild-type oligomer.

α AG98R-crystallin monomers also suppressed the aggregation of denaturing ovotransferrin (Figure 5D) and β B₂-crystallin (Figure 5B). However, against denaturing β B₂-crystallin and ovotransferrin, the mutant monomers showed 25% and 50%, respectively, lower activity than the wild-type oligomer. In all of the chaperone assays, the activity of α AG98R-crystallin monomers was concentration dependent. Doubling the amount of mutant protein in assays completely suppressed the aggregation of all 4 client proteins.

Impaired stability of α AG98R-crystallin mutant protein: To examine whether stability of the protein in its monomer form is affected by the α AG98R-crystallin mutation, the recombinant G98R protein (100 μ g) in 1 ml of 50 mM phosphate buffer containing 150 mM NaCl (pH 7.2) was incubated under sterile conditions in presence of protease inhibitor cocktail at 37 °C for 0, 2, 6, and 12 days. The aggregates formed were removed by centrifugation and the supernatant analyzed by size exclusion chromatography and MALS system. Prior to incubation, α AG98R-crystallin eluted as two peaks: one at the expected oligomer elution region and the other corresponded to the monomer size at 14.5 min (Figure 6A, green line). The non-incubated sample showed an average oligomeric mass of 2,250 kDa. After 2 days of incubation, the oligomeric peak was significantly reduced to a 420 kDa peak (Figure 6A inset). The 280 nm profile showed a decreased oligomeric peak and an increased monomeric peak, indicating that the dissociation of the oligomer into subunits occurred during 2 days of incubation. The samples incubated for 6 days and 12 days showed complete loss of oligomeric peak, probably owing to the precipitation of some of the oligomers, since α AG98R-crystallin is known to

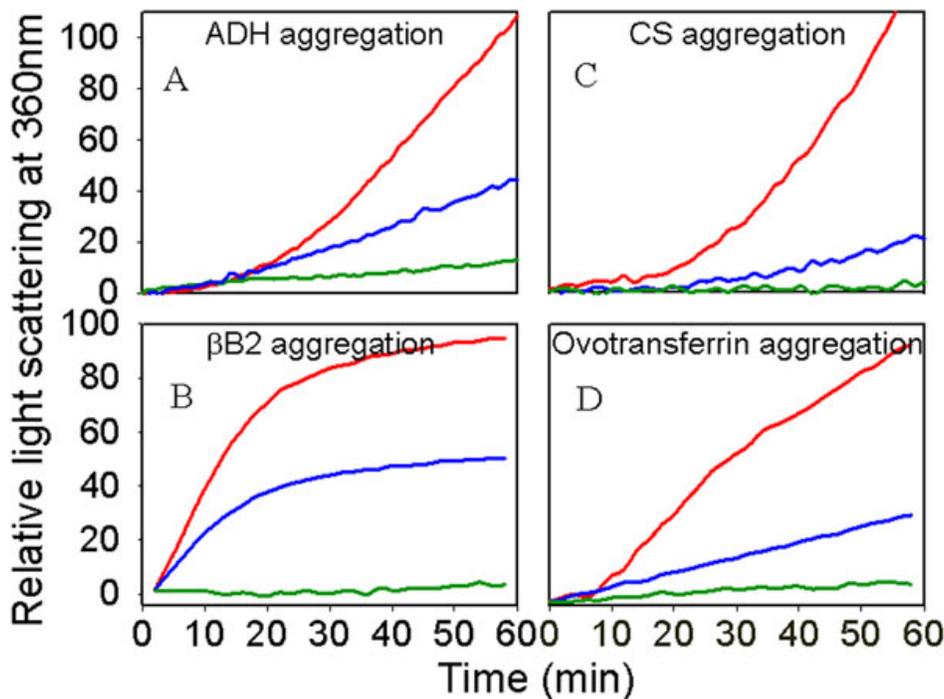


Figure 5. Chaperone activity of α AG98R-crystallin monomers measured with different substrates. The assays were performed as described under methods. Nine μ g of the mutant and 10 μ g of the wild-type proteins were used in assays. **A:** Thermal aggregation of ADH in the absence or presence of wild-type or α AG98R-crystallin monomer at 37 °C. In each experiment 75 μ g of ADH was used. (Red, ADH; blue, + α AG98R-crystallin; green, + wt α A-crystallin). **B:** Thermal aggregation of β B2-crystallin in the presence of α AG98R-crystallin monomers. In each experiment 150 μ g of β B2-crystallin was used. (Red, β B2; blue, + α AG98R-crystallin; green, + wt α A-crystallin). **C:** Thermal aggregation of CS in the presence and absence of α AG98R-crystallin monomers. In each experiment 75 μ g of CS was used. (Red, CS; blue, + α AG98R-crystallin; green, + wt α A-crystallin). **D:** Thermal aggregation of ovotransferrin in presence or absence of α AG98R-crystallin monomers. In each experiment 100 μ g of ovotransferrin was used. (Red, ovotransferrin; blue, + α AG98R-crystallin; green, + wt α A-crystallin).

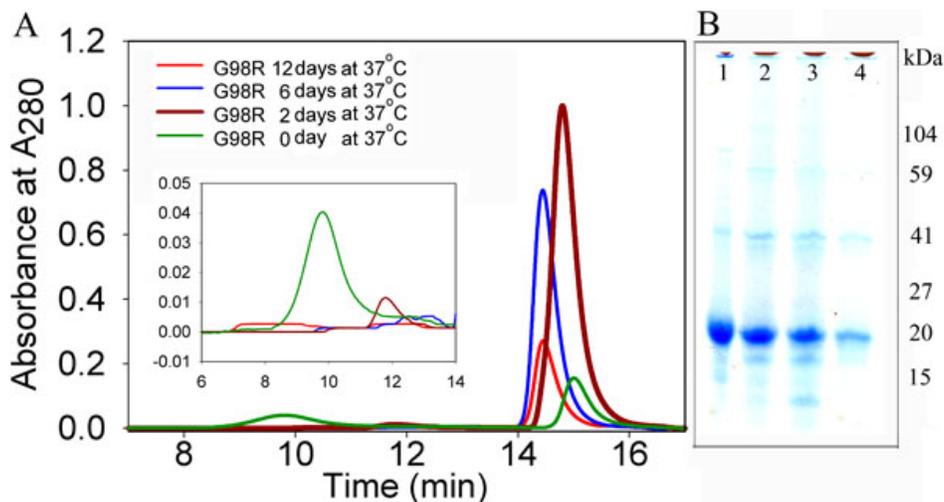


Figure 6. Stability of α AG98R-crystallin. **A:** α AG98R Mutant protein was removed from -80 °C (stored for a year) and size exclusion chromatography was performed at room temperature after 0 – 12 days of incubation of the protein at 0.1 mg/ml at 37 °C. Day 0, Green; day 2, brown; day 6, blue and day 12, red. Inset picture shows enlarged view of the elution profile at the oligomer region, indicating loss of oligomer peak. **B:** SDS-PAGE of samples incubated for 0, 2, 6 and 12 days. (Lane 1, 0; lane 2, 2 days; lane 3, 6 days; lane 4, 12 days).

precipitate on prolonged incubation [9]. The slight difference among the samples in the elution profile around 14.5 min is likely due to the interaction of breakdown peptides with the monomeric form of the protein. Samples incubated 0 to 12 days were also analyzed by SDS-PAGE without centrifugation to remove the precipitates (Figure 6B). The electrophoretic profile showed a breakdown of α AG98R-

crystallin into several <20 kDa size protein bands. There was also some aggregate formation (specifically dimer) in the samples incubated for 2 and 6 days. In the protein sample incubated for 12 days, most of the protein had undergone degradation. The degradation of the protein was confirmed by MALDI TOF MS/MS analysis (data not shown).

DISCUSSION

A novel α A-crystallin mutation, G98R, is known to cause early-onset cataract [7]. We [9] and others [8] have reported that the α AG98R-crystallin mutant protein forms larger oligomers of a polydisperse nature and exhibits lower thermal stability than the wild-type α A-crystallin. At concentrations below 1 mg/ml, the mutant protein begins to dissociate into a monomeric form. The monomers of α AG98R-crystallin can be separated from the oligomers by size exclusion chromatography (Figure 2A) or by filtration using molecular weight cutoff filters. Surprisingly, the oligomers of intermediate size (between 1000 kDa and 20 kDa) were undetectable during gel chromatography (Figure 2A). We found that the dissociation of α AG98R-crystallin oligomer was maximal around pH 5.9. Under similar experimental conditions, the wild-type α A-crystallin did not dissociate into monomers. The findings of our present study confirm earlier observation [2] that wild-type α A- does not dissociate into monomers under physiologic conditions but demonstrate that mutant form does dissociate into monomers at low concentrations. It is known that the truncated α A- and α B-crystallins form oligomers of various sizes [2,4,21,22]. NH₂-terminally truncated α A⁵⁶⁻¹⁷³ and α B⁶⁰⁻¹⁷⁵ crystallins form dimers, trimers, and tetramers [2]. In addition, the α B⁶⁶⁻¹⁷⁵ shows concentration-dependent changes in oligomer size. At 0.07 mg/ml concentration, α B⁶⁶⁻¹⁷⁵ elutes as mostly dimers and trimers, whereas at ~3.3 mg/ml concentration, the protein elutes as high-molecular weight oligomers [2]. The myopathy-causing truncated α B-crystallin, α BQ151X, elutes from a Superose 6 column with characteristics similar to those of a monomeric state [4]. Earlier it was reported that native α -crystallin at low concentrations (0.1 mg/ml) dissociates into monomers [3] but this was contradictory to another study [2] that showed no dissociation of oligomers. However, dissociation of α -crystallin into monomers has also been observed during nano-electrospray MS studies [23] which could be characterized as non-physiologic conditions. The dissociation of sHSP 27 into <70 kDa oligomers following phosphorylation [24] or into dimers after R148G mutation [25] and to monomers after R127W and S135F mutation [26] has been reported. Previous studies have also suggested that sHSPs dimers are likely the building blocks of oligomers [27,28]. To our knowledge there are no reports of a full-length α A-crystallin mutant that dissociates into monomers under physiologic conditions.

The G98R mutation resides in the protein at a strand region equivalent to the β 5-strand in sHSP 16.5 and α A-crystallin [29]. This substitution introduces a positively charged bulky residue at this subunit interaction region likely affecting the subunit interactions that form the stable oligomers. It is unlikely that the relative increase in pI is responsible for the dissociation of mutant protein since it was reported earlier that α AS173K, with similar change in pI did not dissociate into monomers [30]. Mutation of G98 to a

noncharged, less bulkier residue Cys was found to not result in a significant alteration in the oligomer size [29]. Our observation of less α AG98R-crystallin dissociation at higher protein concentrations is likely related to a “molecular crowding effect” in which the subunits tend to interact with one another and remain as oligomers. Molecular crowding is known to affect protein-protein interactions and, at high concentrations, proteins tend to aggregate more [31]. Therefore, although α AG98R-crystallin dissociates into monomeric form at low concentration in vitro, it is unlikely that α AG98R-crystallin exists in the lens as a monomer in vivo because of high protein concentration and the propensity to form large aggregates.

α -Crystallin chaperone activity in the lens has been investigated in both wild-type and mutant α A- and α B-crystallins. Chaperone activity is dependent on the rate of subunit exchange, a characteristic of sHSPs [32]. Studies with Hsp27 and T4 lysozyme showed that dissociation of oligomer is required for client protein binding [33]. Dimers have been identified as the preferred chaperone units in sHSPs [11,33,34]. Several reports suggest that subunit dissociation is required for better chaperone activity [26,32-34]. However, in another study, α -crystallin chaperone activity did not change after crosslinking to prevent the dissociation [35]. Our studies with α AG98R-crystallin show that the monomeric form of mutant protein displays chaperone activity toward aggregating of ADH, bovine β B2-crystallin, CS, and ovotransferrin. Among these 4 client proteins, ADH, CS, and bovine β B2-crystallin were denatured by heat, whereas ovotransferrin was denatured by reduction. The results show that the mutant is active against client proteins denatured by heat or chemical methods. The monomers of α AG98R-crystallin showed varying amounts of chaperone activity during aggregation assays with CS, β B2-crystallin, and ovotransferrin as client proteins. While there was nearly a 50% protection of β B2-crystallin with monomers of α AG98R-crystallin when used in 5:1 ratio (w/w), the same ratio between ovotransferrin and α AG98R-crystallin was found to give nearly 100% protection from aggregation (Figure 5B,D). Such a wide range in chaperone activity in both mutant and wild-type α A-crystallin has been observed in other studies [8,9,14,18,21]. The reasons for such substrate-dependent chaperone activity are yet to be elucidated. It is plausible that the mutation affects one of the chaperone binding sites involved in interactions with a particular client protein, whereas the interactions with another client protein and chaperone are not altered because a different binding site is involved. Earlier we found that ⁸⁹VLGDVIEVHGK⁹⁹ [36] is one of the substrate binding sites in α A-crystallin and that the G98R mutation is part of this region. The scientific literature on α -crystallin chaperone activity point to multiple regions of the protein as having a role in chaperone activity. To our knowledge the present study is the first demonstration

of chaperone activity in an isolated full-length α A-crystallin monomer.

The monomers of α AG98R-crystallin mutant protein showed lower chaperone activity than wild-type α A-crystallin. A factor may be that the complexes of ADH and α AG98R-crystallin monomers are less stable than the complexes formed with wild-type α A-crystallin and lead to the formation of light scattering aggregates with time. SDS-PAGE analysis of the aggregates supports this view (data not shown). While this study demonstrates chaperone activity in monomers of α AG98R-crystallin, it does not clarify the necessity of subunit dissociation for chaperone activity. Our study does, however, provide enough support for the argument that a subunit in α A-crystallin is a functional unit and dimers are not the active minimum chaperone units in α A-crystallin. Recent studies with mutant forms of HSPB1 (HSP27) found that monomerization of the protein leads to hyperactivity [26]. However, unlike α AG98R-crystallin, HSPB1 mutants only partially dissociated into monomers.

Studies have shown that some mutations affect the size and stability of α A- and α B-crystallin oligomers, but other mutations do not [2,8,9,14,16,22]. The present study reveals that α AG98R-crystallin undergoes autolysis on storage, as has been reported with α BR120G-crystallin [19]. It is possible that trace amount of co-purified protease may cause the degradation of recombinant proteins. However, it is unlikely that the degradation we observed was due to protease contamination because the wild-type α A-crystallin expressed, purified and stored under similar conditions did not show degradation. Degradation of aggregated lipase, γ -crystallin and scFv protein (single-chain variable fragment antibody) has been reported [37], and the autolysis was attributed to the action of surface serine residues in the aggregated proteins. It is yet to be determined whether aggregation-induced proteolysis and serine residues are also involved in α AG98R-crystallin autolysis.

In conclusion, the present study shows that the monomers of α AG98R-crystallin have chaperone activity and that G98R mutation in α A-crystallin is sufficient to cause the dissociation of the oligomers.

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