Molecular Vision 2018; 24:297-304 <http://www.molvis.org/molvis/v24/297>
Received 8 December 2017 | Accepted 9 April 2018 | Published 11 April 2018

αA-crystallin-derived minichaperone stabilizes αAG98R-crystallin by affecting its zeta potential

Ashutosh S. Phadte,[1,2] Puttur Santhoshkumar,[1] K. Krishna Sharma[1,2]

[1]Department of Ophthalmology, University of Missouri–Columbia School of Medicine, Columbia, MO; [2]Department of Biochemistry, University of Missouri–Columbia School of Medicine, Columbia, MO

Purpose: The G98R mutant of αA-crystallin is associated with the development of presenile cataracts. In vitro, the recombinant mutant protein exhibits altered structural and functional characteristics, along with the propensity to aggregate by itself and precipitate. Previously, we have reported that the N-terminal aspartate substituted form of the antiaggregation peptide, D^{71}FVIFLDVKHFSPEDLTVK^{88} (αA-minichaperone or mini-αA) prevented aggregation of αAG98R. However, the mechanism of stabilization of αAG98R from aggregation is not fully understood. The purpose of this study was to determine whether the surface charge (zeta (ζ) potential) of αAG98R in the presence of the peptide chaperone contributed to the stabilization of mutant protein, and to identify the sites of interaction between αAG98R and the peptide chaperone.

Methods: Wild-type αA-crystallin (αAWT) and recombinant mutant αAG98R were purified from *Escherichia coli* BL21(DE3)pLysS cells. The ζ potential values of αA-crystallins in the presence or absence of αA-minichaperone and purified protein–peptide complexes were estimated in a ζ potential analyzer. Potential regions within αAG98R that bind the αA-minichaperone were investigated by incubating the protein with a photoactivable minichaperone variant, followed by mass spectrometric analysis.

Results: Binding of the αA-minichaperone to aggregation-prone αAG98R was accompanied by an increase in the ζ potential from −15.19±0.870 mV corresponding to αAG98R alone to −28.64±1.640 mV for the purified complex. Mass spectrometric analysis identified ^{1}MDVTIQHPWFK^{11}, ^{13}TLGPFYPSR^{21}, ^{55}TVLDSGISEVR^{65}, and ^{113}EFHRR^{117} as the αA-minichaperone-binding regions in αAG98R. The results suggest the involvement of the N-terminal region and the α-crystallin domain in the peptide-mediated stabilization of αAG98R.

Conclusions: The αA-crystallin–derived minichaperone stabilizes αAG98R by compensating its lost surface charge. Methods for increasing the ζ potential of aggregating proteins can be a potential approach for therapy to protein aggregation diseases.

The eye lens relies on the ordered arrangement of its fiber cells and their constituent proteins to focus light on the retina for visual perception [1]. The highly conserved lens α-crystallins account for nearly 40% of the total lens proteins. α-Crystallins belong to the small heat shock protein (sHSP) family and are composed of αA and αB subunits [1-3]. Both αA- and αB- subunits have been reported to exhibit chaperone-like activity as homo-oligomers by themselves and as hetero-oligomers with each other [4,5]. The chaperone-like activity of α-crystallin is essential for lens transparency. Several α-crystallin mutations affect the protein structure and chaperone activity and result in cataracts [6-14]. The G98R mutation in αA-crystallin is associated with presenile cataract, with affected individuals developing cataract in adolescence [10]. The glycine-to-arginine mutation adds one positive charge and increases the isoelectric point (pI) of the protein from 5.52 and 5.69. We hypothesize that this change in charge contributes the nonspecific aggregation shown by the mutant protein. In vitro studies have shown that recombinant αAG98R protein exhibited an altered structure, decreased stability, lower chaperone activity, and an increased propensity for self-aggregation [15,16]. The mutation is believed to promote the early development of cataract because of depletion of αB-crystallin chaperone-like activity, in addition to the presence of misfolded mutant αAG98R-crystallin in the lens [15].

Thus far, our structure-function studies of αA- and αB-crystallins have identified key regions that contribute to the chaperone-like activity of the protein [17-19]. We discovered that the ^{70}KFVIFLDVKHFSPEDLTVK^{88} region of the αA-crystallin sequence can function like a chaperone by suppressing aggregation of unfolding proteins and protecting cells from apoptosis [19-21]. The αA70–88 peptide was modified by substituting an aspartate residue at the N-terminus to enhance the solubility and the chaperone-like activity [9]. We called this peptide, D^{71}FVIFLDVKHFSPEDLTVK^{88}, an “αA-minichaperone” or “mini-αA” due to its ability to

Correspondence to: K. Krishna Sharma, Department of Ophthalmology, EC213, University of Missouri School of Medicine, One Hospital Dr., Columbia, MO 65212; Phone: (573) 882-8478; FAX: (573) 884-4100; email: sharmak@health.missouri.edu

mimic the function of full-length αA-crystallin. We have previously showed the efficacy of αA-minichaperone in preventing aggregation of αAG98R, with the rescue of its loss of chaperone-like activity in vitro [22]. Using biotin-labeled αA-minichaperone, gel filtration, and mass spectrometry, we showed that the chaperone peptide binds to αAG98R-crystallin. However, the role of the surface charge in αAG98R aggregation and the ability of αA-minichaperone to restore αAG98R stability have not been investigated. Furthermore, it is not known whether the sites targeted by the αA-minichaperone promote this stabilization. To investigate how the αA-minichaperone restores the stability of αAG98R, we investigated the changes in the zeta (ζ) potential of αAG98R before and after interaction with the peptide chaperone.

All proteins in solution are charged owing to the spatial arrangement of their constituent charged amino acids, which also contribute to the protein structure and function. The net surface charges on a protein dictate its stability in solution and are responsible for its interactions with other proteins in vivo and in vitro. For chaperone proteins, the surface charges enable them to physically interact with aggregation-prone protein intermediates and stabilize them. The surface charges of protein in solution exhibit a charge density around it, and the resulting electrostatic potential differences establish its ζ potential [23]. In a protein solution, the magnitude of the ζ potential results in forces of repulsion between adjacent molecules and promotes its homogeneity. When the ζ potential is close to zero, the weak repulsive forces are compensated by attractive forces, which lead to protein aggregation and precipitation.

For the experiments, the ζ potential was determined by calculating the electrophoretic mobility (μ_{ep}) of the proteins in the presence of an external electric field using phase analysis light scattering (PALS) [24]. PALS calculates μ_{ep} by estimating the change in the phase of the light beam that passes through the sample cell in comparison to the reference beam. μ_{ep} is then related to the ζ potential using the following equations:

$$\phi = \langle A \rangle \int \bar{q}(\mu_{ep}\bar{E})dt$$

where

$$\bar{q} = \frac{4\pi n}{\lambda}\sin\frac{\theta}{2}$$

and

$$\mu_{ep} = \frac{\varepsilon_i \varepsilon_0 \zeta}{\eta}$$

ϕ is the phase shift of the scattered light, $\langle A \rangle$ is the average scattered amplitude $\bar{q}$ is the scattering vector, $\bar{E}$ is the electric field, n is the refractive index of the medium, λ is the wavelength, θ is the scattering angle, ε_i is the permittivity of the surrounding liquid, and ε_0 is the permittivity of the vacuum, ζ is the zeta potential, and η is the viscosity of the solution.

In this study, we show that the binding of αA-minichaperone has a direct effect on the ζ potential of αAG98R. αA-minichaperone–bound αAG98R showed a higher ζ potential than the αA-minichaperone or αAG98R by itself. To understand the nature of this stabilization, we determined the αA-minichaperone binding site(s) in αAG98R using a biotinylated version of αA-minichaperone containing a p-benzoyl-phenylalanine (Bpa) residue at Phe80 to cross-link the chaperone peptide with the protein. The cross-linking experiments identified four regions in αAG98R-crystallin as αA-minichaperone binding sites. We conclude that αA-minichaperone–mediated stabilization of αAG98R occurs through interactions of the peptide with these regions, which is reflected in the increased ζ potential of the protein–peptide complex.

METHODS

Recombinant wild-type αA-crystallin (αAWT) and αAG98R-crystallin were expressed in *Escherichia coli* BL21(DE3) pLysS cells (Invitrogen, Carlsbad, CA) and purified using column chromatography, as described previously [16]. The purity of the protein was checked with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and the molecular mass was determined with mass spectrometry (MS). The minichaperone peptide D^{71}FVIFLDVKHF-SPEDLTVK^{88} was supplied by GenScript (Piscataway, NJ). Biotinyl-D^{71}FVIFLDVKH(Bpa)SPEDLTVK^{88} was obtained from Apptec (Louisville, KY). The peptides used in the study were >95% pure as determined with high-performance liquid chromatography (HPLC) and mass spectrometry. All buffers for experimental measurements were made from American Chemical Society–grade reagents.

Preparation of αA-minichaperone–αAG98R complexes for zeta potential measurements: Recombinant αA-crystallin, 1 mg, was mixed with 200 µg of αA-minichaperone, and the reaction mixture was made to a final volume of 350 µl using 20 mM sodium phosphate and 10 mM NaCl pH 7.4 (20/10 buffer). The sample was then incubated at 37 °C for 2 h or at 42 °C for 1 h. Following incubation, the reaction mixture

was loaded on a Sephadex-G50 column (50 cm × 1.5 cm; Sigma Aldrich, St. Louis, MO). The protein peak eluting from the Sephadex-G50 column corresponding to the elution time of αA-crystallin was collected and used for further analysis. Our previous study confirmed that this peak contains the complex formed between αAG98R and mini-αA [22]. The ζ potential measurements were recorded in a Nanobrook Omni (Brookhaven Instruments, Holtsville, NY) in PALS mode using a Uzgiris type electrode assembly [24] to compensate for bulk motion effects. Each replicate value is an average of five reads, with 30 cycles in each read, per the recommendation of the equipment manufacturer.

Chaperone-like activity assay: The interaction between αA-minichaperones and unfolding alcohol dehydrogenase (ADH) was studied using native and photoactive version of mini-αA-crystallin (biotinyl-mini–αA-Bpa). Chaperone-like activity assays were performed using ADH as a substrate following the procedure described previously [22]. The efficacy of mini-αA-crystallin and biotinyl-mini–αA-Bpa to prevent ADH aggregation was monitored as relative absorbance at 360 nm at 37 °C in a Shimadzu ultraviolet-visible (UV-VIS) spectrophotometer equipped with a temperature-controlled multicell transporter.

Photocrosslinking, trypsin digestion, and mass spectrometry: Biotinyl-mini-αA-Bpa, 200 µg, was incubated with 200 µg of αAG98R-crystallin in phosphate buffer, pH 7.2, at 37 °C for 1 h in the absence of light. Subsequently, the sample was filtered using a 10 kDa cutoff centrifugal filter (Millipore, Bedford, MA) to remove any unbound peptide. The concentrate was then photolyzed for 30 min, at 4 °C, using an ultraviolet lamp at 365 nm (Spectroline ENF-280C, 8 W, 60 Hz, 0.20 A) held at a distance of 7 cm from the sample as described previously [22]. The photolyzed sample was desalted using C18 ZipTip spin columns (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's protocol, and the bound protein was eluted in 70% acetonitrile. The sample was subjected to SDS–PAGE using 4–20% gradient gel and stained with Coomassie blue. The covalently crosslinked biotinyl-mini-αA-αAG98R complex band was excised from SDS–PAGE and subjected to in-gel digestion with trypsin, as described in [22]. Tryptic peptides were extracted from the gel and analyzed with Nano-LC-QTOF MS (Agilent 6520A). Analysis of MS plus MS/MS data was performed with a "find compounds by auto MS/MS" program in the Agilent Mass Hunter software (version B.04.00) suite. The compound list obtained by "auto MS/MS analysis" was further examined visually, and the MS/MS spectra were annotated using the fragment ion nomenclature described previously by Roepstorff and Fohlman [25] and Johnson et al. [26]. The peptides mass searches were performed against the NCBInr Human protein database. Evidence for aspartic acid dehydration was included in the search [27]. The search parameters included variable mini-αA-Bpa peptide modifications of glycine, isoleucine, lysine, leucine, methionine, proline, arginine, and valine, to which Bpa is expected to insert [28-30]. Other fixed modifications were not selected for the mass search. MS analysis performed on Bpa-untreated and UV-photolyzed αAG98R and αAWT were used as controls.

RESULTS AND DISCUSSION

The ζ potential is an inherent property of biomolecules that can be measured when they are suspended in a liquid owing of the nature of the arrangement of charged groups on their surface. For proteins, the ζ potential is determined by charged amino acids exposed to the solvent, which determines solution stability as well as prevents nonspecific aggregation among adjacent molecules due to charge repulsion. Nonspecific interactions, post-translational modifications, mutations, etc., affect the surface charge of the proteins [30]. This leads to decreased protein stability, increased aggregation, and precipitation. A consequential effect of mutations and modifications of proteins is alteration of the surface charges that contribute to the ζ potential of the protein [31]. The ζ potential measured for proteins above their isoelectric point is negative. In this study, ζ potential measurements were used to evaluate the superficial electrical charges on wild-type and mutant αA-crystallin oligomers. αAG98R and wild-type αA-crystallin (αAWT) were dialyzed against 20/10 buffer before the ζ potential measurements were obtained. αAWT exhibited an average ζ potential of –16.84±0.640 mV, whereas αAG98R showed an average ζ potential of–15.37±1.190 mV. The αA-minichaperone dissolved in 20/10 buffer showed a ζ potential of –19.64±2.160 mV. The average ζ potential values for all samples are summarized in Table 1. The calculated isoelectric points (pI) of αAWT- and αAG98R-crystallin are 5.52 and 5.69, respectively. The pI of αA-minichaperone is 4.49. There is no correlation between the theoretical pI values and the ζ potential of proteins as the ζ potential depends on the net surface charge on the protein. The αAG98R mutant showed a lower ζ potential value than αAWT, an effect that could be due to the altered structure of the mutant protein. Previous studies of αAG98R involving circular dichroism, bis-ANS binding, and light scattering found that αAG98R exhibited an altered structure and decreased stability, which promoted its aggregation and precipitation over time [15,16]. The precipitation occurs because the attractive forces begin to overpower like-charge repulsion between adjacent αA-crystallin oligomers in solution. In the case of αAG98R,

Table 1. Comparison of the zeta potential of wild-type and mutant αAG98R-crystallins treated with mini–αA-chaperone.

Sample	Incubation time (minutes)	Incubation temperature (° C)	Zeta (ζ) potential (mV)	Number of replicates (n)
αAWT, 1 mg	0	25	−16.84±0.64	4
αAG98R, 1 mg	0	25	−15.19±0.87	5
Mini–αA, 200 µg	0	25	−19.64±2.16	3
1 mg αAG98R + 200 µg mini–αA	0	25	−28.64±1.68	2
1 mg αAG98R + 200µg mini–αA	120	37	−28.99±1.20	2
1 mg αAG98R + 200µg mini–αA	60	42	−27.36±1.63	2
1 mg αAWT + 200µg mini–αA	0	25	−17.29±0.59	2
1 mg αAWT + 200µg mini–αA	120	37	−17.15±0.81	2
1 mg αAWT + 200µg mini–αA	60	42	−17.83±1.39	2

Each replicate was scanned 5 times during each zeta potential measurement.

the mutation-induced changes in the tertiary structure oligomer assembly may be affecting the surface charge distribution, resulting in an altered ζ potential and an increased propensity of the protein to aggregate and precipitate with time, as observed in electron microscopy [22].

The αA-crystallin–derived minichaperone (mini-αA) has been shown to bind and stabilize αAG98R [22]. In this study, we used the ζ potential as a tool for monitoring changes in surface charges of aggregation-prone αAG98R and mini-αA-bound αAG98R. The mutant αAG98R, 1 mg, was incubated with 200 µg mini-αA at 37 °C for 120 min and at 42 °C for 60 min. Following incubation, the mixture was loaded on a Sephadex-G50 size exclusion column to separate the peptide–protein complex from the unbound peptide. The eluted complex peak was subjected to HPLC analysis to confirm the presence of the protein, and the peptide in the SEC eluted peak (data not shown). The ζ potential of the mini-αA–αAG98R complex recovered after 2 h of incubation at 37 °C and Sephadex G50 column chromatography was –28.99±1.200 mV. This value was similar to the ζ value of –28.64±1.680 mV in the sample not subjected to incubation suggesting a rapid interaction between the chaperone peptide and αAG98R. The incubation of αAG98R and αA-minichaperone at 42 °C for 1 h resulted in a ζ value of –27.36±1.630 mV.

Taken together, the higher ζ values of the αA-minichaperone–αAG98R complex indicate that the peptide stabilizes the aggregation-prone protein by compensating its loss of charge. When the αA-minichaperone–treated αAWT was passed through Sephadex G50 column, and the eluted αA-crystallin peak was subjected to ζ potential measurement, a ζ value of –17.15±0.830 mV was observed for a sample processed after 2 h of incubation at 37 °C and a ζ value of –17.83±1.380 mV for samples processed after 1 h of incubation at 42 °C. Previous studies have shown the aggregation and precipitation of αAG98R following incubation at 37 °C and 42 °C for 1–2 h. Therefore, it is likely that the higher ζ potential of the αA-minichaperone–αAG98R complex is responsible for keeping the complex in solution. The increased ζ potential helps overcome the effects of forces that contribute to aggregation, helps maintain a uniform dispersion, and prevents nonspecific aggregation. The present experiments show that ζ potential measurements are a valuable tool for monitoring changes in protein stability. Furthermore, it also hints at the possibility that modulation of surface charge could be a possible mechanism for maintaining protein stability in solution.

To identify whether the peptide targeted specific sites within αAG98R to promote the observed stability, a photoactive version of the αA-minichaperone was employed. The photoactive peptide, biotinyl-mini–αA-Bpa incorporated a benzoyl phenylalanine (Bpa) residue instead of phenylalanine at position 80 corresponding to human αA-crystallin sequence. Bpa was chosen over other crosslinking strategies to avoid attaching a photophore to chemically reactive groups within the peptide sequence of the αA-minichaperone [27]. The reactive group in Bpa is its carbonyl group, which on absorbing a photon at about 350 nm, a wavelength not detrimental to proteins [28], promotes an electron to an antibonding π* orbital, enabling it to react with C–H bonds [27,28]. Native mini-αA and biotinyl-mini-αA-Bpa were subjected to chaperone-like activity assays to determine whether the amino acid substitution at F80 to Bpa affected the efficacy of the peptide to prevent protein aggregation. As

shown in Figure 1, the αA-minichaperone and biotinyl-mini-αA-Bpa showed similar efficacy in preventing EDTA-induced aggregation of ADH. The results of the activity assays, therefore, suggest that the Bpa incorporation did not interfere in the chaperone function of αA-minichaperone. As an experimental control, we also tested the effect of UV irradiation on wild-type αA-crystallin (αAWT). Wild-type αA-crystallin was used as a control to circumvent possible complications from irradiation of self-aggregation-prone αAG98R. Based on the intrinsic tryptophan fluorescence measurement, hydrophobic probe 1, 1'-bi(4-anilino) naphthalene-5,5′-disulfonic acid binding, near UV-circular dichroism (CD) spectra and chaperone activity assays, UV irradiation at 365 nm had minimal effect on the structure and function of wild-type αA-crystallin or purified bovine α-crystallin (data not shown). The exposure to UV light in the current experimental setting, therefore, is conducive to promote crosslinking but is insufficient to exert deleterious effects on the structure and function of αA-crystallin.

Incubation of photoactive biotinylated αA-minichaperone with αAG98R and subsequent ultraviolet light treatment resulted in covalent binding of αA-minichaperone to αAG98R, as observed with SDS–PAGE and MS analysis [22]. The binding of αA-minichaperone to αAG98R imparts a ζ potential that is conducive to the stability of the complex. Therefore, we initiated a study to identify the sequences in αAG98R involved in the binding of photoactive αA-minichaperone. The reaction protocol was designed such that the occurrence of non-specific crosslinks was minimal, as Bpa mediated crosslinking heavily depends on the time of UV exposure. The crosslinking reactions were performed at an optimal wavelength of 365 nm to prevent detrimental damage to the protein [28]. Crosslinked products have been shown to undergo a range of modifications during photolysis or sample ionization in MS. For example, products obtained by reaction at proline can rearrange and lose the adduct while forming dehydroproline. Water may be lost from products obtained by reaction at glycine [27]. As a countermeasure, search parameters included the

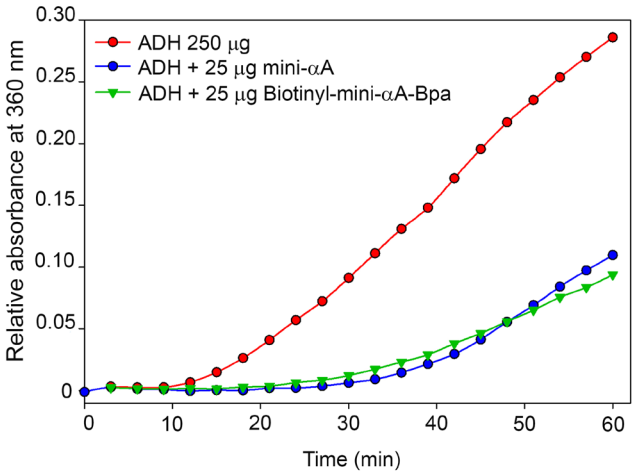

Figure 1. Chaperone-like activity of αA-minichaperone and biotinyl-mini-αA-Bpa using ADH. Experimental details are included in the Methods section.

all variable biotinyl-mini-αA-Bpa peptide modifications involving glycine, isoleucine, lysine, leucine, methionine, proline, arginine, and valine amino acid residues. Additional searches were also performed to include aspartic acid dehydration, cysteine carbamidomethylation, and methionine oxidation as variable modifications. Despite these defined parameters, if the peptide deviated from the included variable modifications, we would have missed it in the MS/MS search. The photocrosslinked complex of biotinyl-mini-αA-Bpa and αAG98R was digested with trypsin and subjected to MS analysis. Searches with the MS and MS/MS data revealed four peptide signals that contained the crosslinks. The features of the multiply charged cross-linked peptide are shown in Table 2. The inter-crosslinks between a portion of the photoactive αA-minichaperone and αAG98R region in these crosslinked peptides was confirmed with MS/MS. Tandem mass spectrum of a quadruply charged crosslinked peptide (m/z: 609.06) showed crosslinking between L57 of αAG98R ^{55}TVLDSGISEVR^{65} and [Bpa] of biotinyl αA-minichaperone fragment, ^{10}H[Bpa]SPEDLTVK^{19} (Figure 2). Examination of the MS/MS spectra also indicated dehydration of aspartic acid

Table 2. Identification of αA–mini–chaperone interaction sites in αAG98R crystallin.

Crosslinked precursor ion mass	Theoretical mass	Relative mass error	Mini–αA sequence	αAG98R sequence
404.804 $^{5+}$	2018.9963	6 ppm	H[Bpa]SPEDLTVK	^{113}EFH**R**R^{117}
579.043 $^{4+}$	2312.1477	2 ppm	H[Bpa]SPEDLTVK	^{13}TL**G**PFYPSR^{21}
536.267 $^{5+}$	2676.3046	2 ppm	H[Bpa]SPEDLTVK	^{1}**M**DVTIQHPWFK^{11}
609.063 $^{4+}$	2432.252	3 ppm	H[Bpa]SPEDLTVK	^{55}TV**L**DSGISEVR^{65}

. The amino acid in αAG98R involved in the crosslink is shown in bold.

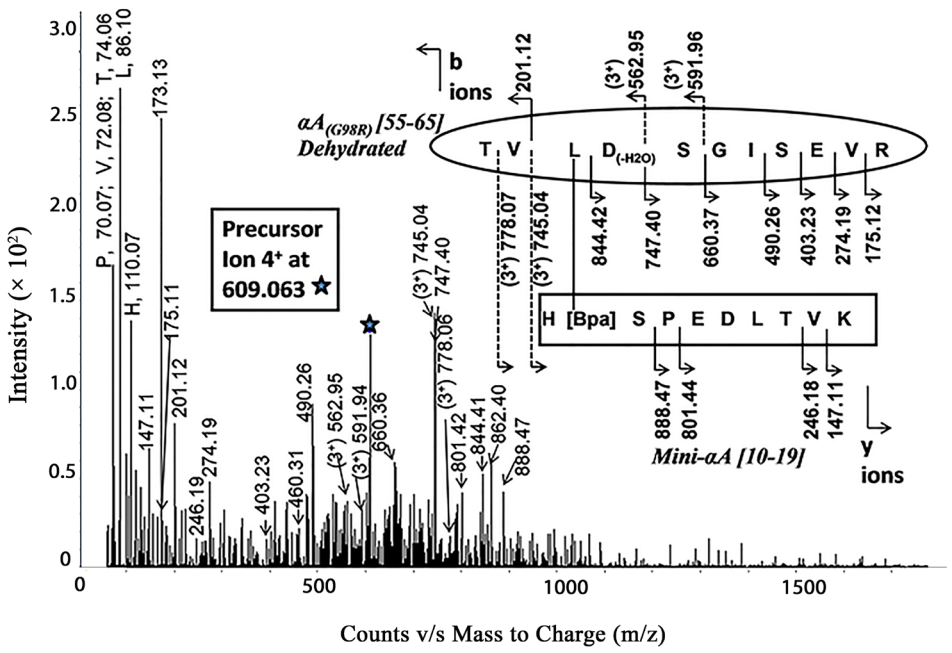

Figure 2. Fragmentation mass spectrum of the identified precursor ion (m/z 609.063 4^+) obtained using MS/MS. The masses of the identified ions are indicated. The crosslink between the two peptides is also shown in the inset.

of αAG98R peptide involved in the crosslink. The other sites of interaction between Bpa-containing αA-minichaperone and αAG98R were ^{1}MDVTIQHPWFK^{11}, ^{13}TLGPFYPSR^{21}, and ^{113}EFHRR^{117} regions (Figure 3).

MS analysis of the mini-αA–αAG98R complex previously [22] showed two prominent peaks and a small peak, which could signify the binding of three αA-minichaperone peptides to a subunit of αAG98R. Taking into consideration that the current analysis identified four different mini-αA binding sites within αAG98R, it is likely that the presumed 1–3:1 binding stoichiometry of mini-αA to αAG98R could result from the processing of the complex for MS analysis, owing to the polydispersity of αA-crystallin. The analysis of tryptic peptides from several subunits of αAG98R with αA-minichaperone bound at different sites when summarized in one sequence appears as four regions of binding in one subunits as shown in Figure 3. Of the four identified sites, one of the peptides, ^{113}EFHRR^{117}, is charged whereas the other three sites (^{1}MDVTIQHPWFK^{11}, ^{13}TLGPFYPSR^{21}, and ^{55}TVLDSGISEVR^{65}) are hydrophobic. The αAG98R sequences ^{55}TVLDSGISEVR^{65} and ^{113}EFHRR^{117}are part of first and fifth beta (β) strands in characteristic β sandwich of αA-crystallin [32-34]. Circular dichroism studies have shown that mini-αA adopts a β-sheet conformation in solution as well [19]. Therefore, it is likely that the mini-αA interacts with the two accessible β strand regions of αAG98R during complex formation and stabilization. It is tempting to say that the residues M1, G15, L57, and R116 in minichaperone

```
        10         20         30         40
MDVTIQHPWF KRTLGPFYPS RLFDQFFGEG LFEYDLLPFL
        50         60         70         80
SSTISPYYRQ SLFRTVLDSG ISEVRSDRDK FVIFLDVKHF
        90        100        110        120
SPEDLTVKVQ DDFVEIHrKH NERQDDHGYI SREFHRRYRL
       130        140        150        160
PSNVDQSALS CSLSADGMLT FCGPKIQTGL DATHAERAIP
       170
VSREEKPTSA PSS
```

Figure 3. Localization of αA-minichaperone–binding regions in αAG98R-crystallin. The underlined sequences in red represent regions of αAG98R that interact with the photoactive peptide chaperone from liquid chromatography–mass spectrometry (LC-MS) data, further confirmed with tandem mass spectrometry (MS/MS). The amino acids involved in crosslinking are labeled in blue. The mutant residue (r) is shown in the lowercase.

stabilized αAG98R form part of a single groove, similar to the groove found in HSP16.9 lined by residues V4, F10, W48, and F110 [33]. The binding and crosslinking of the peptide chaperone at different orientations on this groove might be responsible for the identification of M1, G15, L57, and R116 residues as biotinyl-mini-αA-Bpa crosslinked amino acids during mass spectrometric analysis. However, the observation that some of the subunits have more than one chaperone peptide bound to them, based on the mass spectrometric analysis reported previously [22], suggests that the minichaperone stabilizes, at least some of the oligomers through binding at an additional site away from the groove although further studies are needed to confirm this hypothesis. Because it is known that α-crystallin subunits have the propensity to exist in multiple conformations in an oligomer [34,35], it is possible that the minichaperone binds to different subunits present in a polydisperse form of αAG98R at different regions such that analysis of the oligomer–minichaperone complexes result in the identification of more than one site as a binding site. Structural determination of peptide chaperone-αAG98R complex with cryoelectron microscopy (cryo-EM) in the future may provide better insight into the molecular interactions involved in the stabilization of unstable αAG98R by αA-minichaperone. Previous studies have highlighted the involvement of the N-terminal of αA-crystallin in influencing the oligomer structure and function of the protein in vivo and in vitro. The present binding studies also validate an interaction between N-terminal residues 1–11 and mini-αA-crystallin. This observation also suggests destabilization of the N-terminal region of in αAG98R because the minichaperone generally recognized unfolding proteins.

In conclusion, we showed that αA–derived minichaperone stabilizes the cataract-causing unstable αAG98R by interacting with the mutant protein at multiple sites. By doing so, the ζ potential of the mutant protein is altered, and so are the repulsive forces between the oligomers that prevent the protein from aggregation and precipitation. The findings indicate that the ζ potential can be used to determine the protein stabilization effect of peptides of interest.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant R01EY023219 and Beryl J. Ortwerth Professorship to K. Krishna Sharma. The authors thank Sharon Morey for help in the preparation of the manuscript, Dr. Murugesan Raju for preparing the chaperone peptide-crystallin crosslinks and Beverly DaGue of the MU Proteomic Core Facility for mass spectrometric analysis of the samples.

REFERENCES

1. Jaffe NS, Horwitz J. Lens and Cataract: Gower Medical Pub.; Distributed in the USA and Canada by Raven Press; 1992.
2. Andley UP. Crystallins in the eye: Function and pathology. Prog Retin Eye Res 2007; 26:78-98. [PMID: 17166758].
3. Ingolia TD, Craig EA. Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc Natl Acad Sci USA 1982; 79:2360-4. [PMID: 6285380].
4. Horwitz J. Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci USA 1992; 89:10449-53. [PMID: 1438232].
5. Horwitz J. The function of alpha-crystallin in vision. Semin Cell Dev Biol 2000; 11:53-60. [PMID: 10736264].
6. Devi RR, Yao W, Vijayalakshmi P, Sergeev YV, Sundaresan P, Hejtmancik JF. Crystallin gene mutations in Indian families with inherited pediatric cataract. Mol Vis 2008; 14:1157-70. [PMID: 18587492].
7. Graw J, Klopp N, Illig T, Preising MN, Lorenz B. Congenital cataract and macular hypoplasia in humans associated with a de novo mutation in CRYAA and compound heterozygous mutations in P. Graefes Arch Clin Exp Ophthalmol 2006; 244:912-9. [PMID: 16453125].
8. Xia CH, Liu H, Chang B, Cheng C, Cheung D, Wang M, Huang Q, Horwitz J, Gong X. Arginine 54 and Tyrosine 118 residues of {alpha}A-crystallin are crucial for lens formation and transparency. Invest Ophthalmol Vis Sci 2006; 47:3004-10. [PMID: 16799046].
9. Kore R, Hedges RA, Oonthonpan L, Santhoshkumar P, Sharma KK, Abraham EC. Quaternary structural parameters of the congenital cataract causing mutants of alphaA-crystallin. Mol Cell Biochem 2012; 362:93-102. [PMID: 22045060].
10. Santhiya ST, Soker T, Klopp N, Illig T, Prakash MV, Selvaraj B, Gopinath PM, Graw J. Identification of a novel, putative cataract-causing allele in CRYAA (G98R) in an Indian family. Mol Vis 2006; 12:768-73. [PMID: 16862070].
11. Laurie KJ, Dave A, Straga T, Souzeau E, Chataway T, Sykes MJ, Casey T, Teo T, Pater J, Craig JE, Sharma S, Burdon KP. Identification of a novel oligomerization disrupting mutation in CRYAlphaA associated with congenital cataract in a South Australian family. Hum Mutat 2013; 34:435-8. [PMID: 23255486].
12. Javadiyan S, Craig JE, Souzeau E, Sharma S, Lower KM, Pater J, Casey T, Hodson T, Burdon KP. Recurrent mutation in the crystallin alpha A gene associated with inherited paediatric cataract. BMC Res Notes 2016; 9:83-[PMID: 26867756].
13. Panda AK, Nandi SK, Chakraborty A, Nagaraj RH, Biswas A. Differential role of arginine mutations on the structure and functions of alpha-crystallin. Biochim Biophys Acta 2016; 1860:1 Pt B199-210. [PMID: 26080000].
14. Mackay DS, Andley UP, Shiels A. Cell death triggered by a novel mutation in the alphaA-crystallin gene underlies autosomal dominant cataract linked to chromosome 21q. Eur J Hum Genet 2003; 11:784-93. [PMID: 14512969].

15. Singh D, Raman B, Ramakrishna T, Rao Ch M. The cataract-causing mutation G98R in human alphaA-crystallin leads to folding defects and loss of chaperone activity. Mol Vis 2006; 12:1372-9. [PMID: 17149363].

16. Murugesan R, Santhoshkumar P, Sharma KK. Cataract-causing alphaAG98R mutant shows substrate-dependent chaperone activity. Mol Vis 2007; 13:2301-9. [PMID: 18199971].

17. Sharma KK, Kaur H, Kumar GS, Kester K. Interaction of 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid with alpha-crystallin. J Biol Chem 1998; 273:8965-70. [PMID: 9535881].

18. Sharma KK, Kaur H, Kester K. Functional elements in molecular chaperone alpha-crystallin: identification of binding sites in alpha B-crystallin. Biochem Biophys Res Commun 1997; 239:217-22. [PMID: 9345298].

19. Sharma KK, Kumar RS, Kumar GS, Quinn PT. Synthesis and characterization of a peptide identified as a functional element in alphaA-crystallin. J Biol Chem 2000; 275:3767-71. [PMID: 10660525].

20. Sreekumar PG, Chothe P, Sharma KK, Baid R, Kompella U, Spee C, Kannan N, Manh C, Ryan SJ, Ganapathy V, Kannan R, Hinton DR. Antiapoptotic properties of alpha-crystallin-derived peptide chaperones and characterization of their uptake transporters in human RPE cells. Invest Ophthalmol Vis Sci 2013; 54:2787-98. [PMID: 23532520].

21. Raju M, Santhoshkumar P, Xie L, Sharma KK. Addition of alphaA-crystallin sequence 164–173 to a mini-chaperone DFVIFLDVKHFSPEDLT alters the conformation but not the chaperone-like activity. Biochemistry 2014; 53:2615-23. [PMID: 24697516].

22. Raju M, Santhoshkumar P, Sharma KK. alphaA-Crystallin-derived mini-chaperone modulates stability and function of cataract causing alphaAG98R-crystallin. PLoS One 2012; 7:e44077-[PMID: 22970163].

23. Salgýn SS. U.; Bahadir S. Zeta Potentials and Isoelectric Points of Biomolecules: The Effects of Ion Types and Ionic Strengths. Int J Electrochem Sci 2012; 7:121404-14. .

24. Tscharnuter WW. Mobility measurements by phase analysis. Appl Opt 2001; 40:3995-4003. [PMID: 18360433].

25. Roepstorff P, Fohlman J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom 1984; 11:601-[PMID: 6525415].

26. Johnson RS, Biemann K. Computer program (SEQPEP) to aid in the interpretation of high-energy collision tandem mass spectra of peptides. Biomed Environ Mass Spectrom 1989; 18:945-57. [PMID: 2620156].

27. Dorman G, Prestwich GD. Benzophenone Photophores in Biochemistry. Biochemistry 1994; 33:5661-73. [PMID: 8180191].

28. Chin JW, Schultz PG. In vivo photocrosslinking with unnatural amino Acid mutagenesis. ChemBioChem 2002; 3:1135-7. [PMID: 12404640].

29. Wilson CJ, Husain SS, Stimson ER, Dangott LJ, Miller KW, Maggio JE. p-(4-Hydroxybenzoyl)phenylalanine: A Photoreactive Amino Acid Analog Amenable to Radioiodination for Elucidation of Peptide−Protein Interaction Application to Substance P Receptor. Biochemistry 1997; 36:4542-51. [PMID: 9109663].

30. Pinkse MWH, Rijkers DTS, Dostmann WR, Heck AJR. Mode of Action of cGMP-dependent Protein Kinase-specific Inhibitors Probed by Photoaffinity Cross-linking Mass Spectrometry. J Biol Chem 2009; 284:16354-68. [PMID: 19369251].

31. Strickler SS, Gribenko AV, Gribenko AV, Keiffer TR, Tomlinson J, Reihle T, Loladze VV, Makhatadze GI. Protein stability and surface electrostatics: a charged relationship. Biochemistry 2006; 45:2761-6. [PMID: 16503630].

32. Kim KK, Kim R, Kim SH. Crystal structure of a small heat-shock protein. Nature 1998; 394:595-9. [PMID: 9707123].

33. van Montfort RL, Basha E, Friedrich KL, Slingsby C, Vierling E. Crystal structure and assembly of a eukaryotic small heat shock protein. Nat Struct Biol 2001; 8:1025-30. [PMID: 11702068].

34. Rajagopal P, Tse E, Borst AJ, Delbecq SP, Shi L, Southworth DR, Klevit RE. A conserved histidine modulates HSPB5 structure to trigger chaperone activity in response to stress-related acidosis. eLife 2015; •••:4-[PMID: 25962097].

35. Lampi KJ, Fox CB, David LL. Changes in solvent accessibility of wild-type and deamidated betaB2-crystallin following complex formation with alphaA-crystallin. Exp Eye Res 2012; 104:48-58. [PMID: 22982024].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 11 April 2018. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.