Identification of Subunit-Subunit Interaction Sites in \(\alpha\)-WT Crystallin and Mutant \(\alpha\)-A-G98R Crystallin Using Isotope-Labeled Cross-Linker and Mass Spectrometry

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Abstract

Cataract is characterized by progressive protein aggregation and loss of vision. \(\alpha\)-Crystallins are the major proteins in the lens responsible for maintaining transparency. They exist in the lens as highly polydisperse oligomers with variable numbers of subunits, and mutations in \(\alpha\)-crystallin are associated with some forms of cataract in humans. Because the stability of proteins is dependent on optimal subunit interactions, the structural transformations and aggregation of mutant proteins that underlie cataract formation can be understood best by identifying the residue-specific inter- and intra-subunit interactions. Chemical crosslinking combined with mass spectrometry is increasingly used to provide structural insights into inter- and intra-protein interactions. We used isotope-labeled cross-linker in combination with LC-MS/MS to determine the subunit–subunit interaction sites in cataract-causing mutant \(\alpha\)-A-G98R crystallin. Peptides cross-linked by isotope-labeled (heavy and light forms) cross-linkers appear as doublets in mass spectra, thus facilitating the identification of cross-linker-containing peptides. In this study, we cross-linked wild-type (\(\alpha\)-WT) and mutant (\(\alpha\)-A-G98R) crystallins using the homobifunctional amine-reactive, isotope-labeled (\(d_3\) and \(d_4\)) cross-linker–BS\(^3\)G (bis[sulfosuccinimidyl]glutamate). Tryptic in-solution digest of cross-linked complexes generates a wide array of peptide mixtures. Cross-linked peptides were enriched using strong cation exchange (SCX) chromatography followed by both MS and MS/MS to identify the cross-linked sites. We identified a distinct intermolecular interaction site between K88 — K99 in the \(\beta\) strand of the mutant \(\alpha\)-A-G98R crystallin that is not found in wild-type \(\alpha\)-crystallin. This interaction could explain the conformational instability and aggregation nature of the mutant protein that results from incorrect folding and assembly.

Introduction

Crystallins (\(\alpha\), \(\beta\) and \(\gamma\)) are the major water-soluble proteins of the lens and are responsible for its transparency. The structural interactions of crystallins and \(\alpha\)-crystallin chaperone activity are critical to lens transparency. \(\alpha\)-Crystallins (800–1000 kDa) constitute 40% of the lens crystallins and are made up of two subunits: \(\alpha\)-A- and \(\alpha\)-B-crystallins (20 kDa each). Chaperone activity of \(\alpha\)-crystallin prevents the aggregation of lens proteins [1–3]. Loss of protein stability and \(\alpha\)-crystallin chaperone activity causes changes in the lens architecture, leading to protein aggregation, increasing lens opacity and, ultimately, cataract development. The formation of high-molecular weight aggregates is the hallmark of cataract development, in both hereditary and age-related cataract. Hereditary, or congenital, cataract results from mutations in crystallin genes. Several \(\alpha\)-A-crystallin mutants, such as R116C, R21L, R12C, R54C and R49C, are known to cause congenital cataract [4–6]. G98R mutation in the \(\alpha\)-A-crystallin subunit is associated with a dominant, progressive total cataract that starts in the teenage years [7]. The mutation of G to R introduces charge and bulkiness to the \(\alpha\)-crystallin domain of the protein to which the mutation is localized and thus disrupts the immunoglobulin fold. The mutation results in misfolded and destabilized protein, with altered secondary and tertiary structure, increased oligomeric size and a propensity to aggregate [8–11].

Crystallins belong to the family of small heat shock proteins, a monomer of which is characterized by a central \(\alpha\)-crystallin domain of ~90 amino acids, flanked by an N-terminal arm and a C-terminal extension [11,12]. \(\alpha\)-Crystallins assemble as polydisperse hetero-oligomers comprising a variable number of \(\alpha\)-A and \(\alpha\)-B subunits, with a constant exchange of subunits. The structural assembly of \(\alpha\)-crystallin and therefore its chaperone function are largely regulated by appropriate interactions between the neighboring subunits [12], but identifying the subunit interaction regions by structural studies has been hampered by the large mass and polydisperse nature of the protein. The available structures of crystallins are often comprised of truncated proteins or portions removed to increase the solubility or crystallization property [13]. Still largely a mystery is the molecular basis for stability of the wild-type protein and instability and aggregation of mutant proteins in congenital cataract and the interfacial interactions...
that contribute to the specific characteristics of the wild-type and mutant protein.

Chemical cross-linking combined with mass spectrometry offers a promising tool for studying the structural aspects of the proteins and multiprotein complexes [14–16]. The technique is used to provide a topological map of multi-protein complexes and interactions sites between protein subunits as well as protein-peptide complexes. The linkage sites identified in a protein are used in structural modeling to elucidate protein conformational folds [15,17]. Isotope-labeled cross-linkers, available as a mixture of “heavy” and “light” forms, are particularly attractive in these studies. Both forms cross-link lysine’s in close proximity within and between proteins. After digestion of cross-linked species, the peptides modified by “heavy” and “light” forms carry a unique isotopic signature in mass spectra, facilitating identification of the cross-linked peptides among a large excess of non-cross-linked peptides [18,19].

Cross-linkers are available with varied reactivities and spacer length. The most common cross-linkers are amine-reactive N-hydroxysuccinimide (NHS) esters. In this study, we used an isotope-labeled (d0 and d4) homobifunctional NHS ester, bis(sulfosuccinimidyl) glutarate (BS2G) cross-linker, in combination with mass spectrometry to define and differentiate subunit interaction sites in native and a mutant crystallin. NHS esters form a stable amide or imide bond with the primary amines in lysine and in the N-termini of proteins, in the process releasing the NHS or sulfo-NHS group [20]. The deuterated (d4) and non-deuterated (d0) forms of the cross-linker were used in a 1:1 ratio, and the cross-linked peptides appear as distinct doublet with a 4.025 amu mass difference in the mass spectra [21]. Using this

![Figure 1. Amine-reactive NHS ester cross-linker BS2G- (Bis[ sulfo succinimidyl] glutarate) in its light and heavy form. Four hydrogen atoms in light (d0) form are replaced by four deuterium atoms in heavy (d4) form. The corresponding mass shifts associated with d0 and d4 cross-linked peptides are shown. doi:10.1371/journal.pone.0065610.g001](image)

![Figure 2. Identification of the interaction sites in protein complexes by isotope-labeled cross-linking and mass spectrometry. A— Strategy for cross-linking. The interacting partners are treated with equimolar amounts of light and heavy cross-linkers. Following labeling the samples are digested, enriched and analyzed by LC-MS. Inter cross-linked peptides were identified by GPMAW software and confirmed by MS/MS analysis. B— The peptide types generated after digestion of the cross-linked proteins. doi:10.1371/journal.pone.0065610.g002](image)
approach we have identified a unique cross-linked site in the mutant \( \alpha A-G98R \)-crystallin. This site is not found in wild-type protein. The location of the cross-linked site explains the conformational difference and therefore the abnormal interactions that might be responsible for aggregation of the mutant protein.

**Materials and Methods**

The cross-linking reagent Bis[Sulfosuccinimidyl] glutarate (BS\( ^2 \)Gd\( _0/d_4 \)) was from Proteochem, Inc (Denver, CO). Protease (Bovine) Trypsin (sequencing grade) was from G Biosciences (St Louis, MO). All other chemicals were of the highest grade commercially available.

**\( \alpha A-WT \) and \( \alpha A-G98R \) crystallins**

Human recombinant \( \alpha A-WT \) and \( \alpha A-G98R \) crystallins were expressed and purified as described earlier [22]. Briefly, both recombinant proteins were expressed in \( E. coli \) BL21(DE3) pLysS cells (Invitrogen, Carlsbad, CA). \( E. coli \) extracts were lyzed and centrifuged. Supernatants were processed for \( \alpha A-WT \) crystallin purification, and insoluble pellets were processed for \( \alpha A-G98R \) crystallin purification. The purification was achieved by gel-filtration (Superdex G-200) and anion-exchange chromatography (Q-Sepharose Fast Flow ion-exchange column). SDS-PAGE and mass spectrometry were used to determine the purity and molecular mass of the purified proteins.

**Cross-linking of proteins**

\( \alpha A-G98R \)-Crystallin and \( \alpha A-WT \) crystallin (35 \( \mu \)M) in 50 mM phosphate buffer (pH 7.4), 150 mM NaCl (final volume 250 \( \mu \)l), were incubated separately at 37°C for 30 min prior to the addition of homobifunctional cross-linker BS\( ^2 \)Gd\( _0/d_4 \) (bis[sulfosuccinimidyldyl]glutarate). The deuterated and non-deuterated forms of the cross-linker were prepared as 1 M stock solution in DMSO. Prior to the addition to proteins, a 1:1 ratio of deuterated and non-deuterated form was prepared. Preliminary studies were performed using 1:10, 1:20, 1:50 and 1:100 (protein to cross-linker ratio) to determine the optimal conditions that gives good cross-linking yield. To avoid nonspecific and excessive cross-linking, a 1:20 molar ratio was chosen and found to be optimal under the experimental conditions. A 20-fold molar excess of cross-linker mixture was added to \( \alpha A-WT \) and \( \alpha A-G98R \) protein separately. The samples were kept in ice for 1 hr. The cross-linking reaction was terminated by adding Tris (50 mM final concentration). After incubation at room temperature for 10 min, the samples were passed through 10 kDa centrifugal filters (Centricon) to remove unreacted cross-linker. Aliquots of the reaction mixture were run on 4–20% SDS-PAGE.

**In-solution digestion of cross-linked proteins**

In-solution digestion was carried out with a modified procedure described earlier [23]. In order to make the proteolytic sites more accessible to trypsin, the cross-linked protein samples (\( \alpha A-WT \) and \( \alpha A-G98R \)) were solubilized in 50 mM Tris buffer containing 8 M urea and 4 mM DTT for 2 h and then 12.5-fold diluted (final urea and DTT concentration of 0.64 M and 0.32 mM respectively) by adding trypsin digestion buffer (0.2 M ammonium bicarbonate, pH 7.9). Trypsin (1.75 \( \mu \)g (sequencing grade Trypsin, G. Biosciences) was added to 175 \( \mu \)g of cross-linked proteins. The trypsin-treated mixtures were incubated overnight at 37°C.

**Enrichment of cross-linked peptides**

The trypic digests of cross-linked \( \alpha A-WT \) and \( \alpha A-G98R \) protein were offline fractionated using SCX-Stage tips (Thermo Scientific), following the manufacturer’s protocol. Peptides were eluted from the SCX column using a stepwise gradient of ammonium acetate. Eluted fractions were pooled and used for LC-MS analysis.

**Mass spectrometry analysis**

SCX-enriched peptide mixtures (1 \( \mu \)l) were separated on an Agilent HPLC chip (43 mm, Zorbax C18 Chip) and directly coupled to Agilent 6520 Accurate-Mass Quadrupole time-of-flight (Q-TOF) LC/MS. Elution was done using gradients with initial conditions: 3% B to 10% B over 1 min, 10% B to 40% B over 22.5 min, 40% B to 90% B over 1 min, hold at 90% B for 5 min and back to initial conditions at 3% B for 4 min, with a total run time of 35.5 min. Solvent A is 0.1% formic acid in water, solvent B is 99.9% acetonitrile and 0.1% formic acid. Flow rate was maintained at 600 nL/min. MS spectra of the eluting peptides were acquired in the range of 295–2500 m/z, 2 spectra/sec. For each cycle of MS scan (3.1 sec), the five most abundant peptides (>2500 counts) with a charge state of two, three or higher were selected and subjected to N2-induced CID peptide fragmentation (MSMS, 7–2500 m/z, 2 spectra/sec). Two internal reference mass compounds (methyl stearate 299.29 and hexaxis [1H, 1H, 4H-hexafluorobutyl]oxypoline 1221.99) were used to recalibrate mass spectra during acquisition.
Identification of cross-linked peptides

LC-MS raw data were processed into deconvoluted peptide peak lists with monoisotopic mass using “find compounds by molecular feature” algorithm of Mass Hunter Qualitative Analysis Software B.02.00. Theoretical mass lists of the cross-linked peptides were generated using GPMAW software. In GPMAW, lysines in αA-WT and αA-G98R crystallin are selected and tryptic cleavage products are generated. Since trypsin will not cleave at modified lysines (involved in cross-links), two missed cleavage sites is set as a parameter. A m/z mass difference of 4 amu is set as a criterion to create mass lists corresponding to cross-linked peptide pairs. The mass lists generated by GPMAW are compared with experimental mass lists obtained by LC-MS analysis of tryptic digests of cross-linked αA-WT and αA-G98R (Table 1). Based on the match, a list of potential cross-linked peptides is created. Sequences of the corresponding cross-linked sites in proteins are identified in-silico and represented as a map. The links in the map represent cross-link sites within and between subunits in αA-WT and αA-G98R crystallins, respectively (Table 1).

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Results

Cross-linking of proteins

To identify the subunit–subunit interaction sites in αA-WT and αA-G98R crystallins, deuterated and non-deuterated form of a homobifunctional cross-linker BS2G were used to cross-link the proteins (Figure 1). BS2G reacts with protein N-terminal α-amino and lysine ε-amino groups. The analytical strategy used to characterize the intra- and inter-subunit interaction sites in αA-WT and αA-G98R crystallin is illustrated in Figure 2. To identify the initial inter-subunit interactions that initiate aggregation, we pre-incubated the αA-WT and G98R proteins at 37°C for 30 min, before adding the cross-linker. Mild thermal stress at 37°C induces the process of aggregation in the mutant G98R protein [9,10]. After incubation, cross-linker was added in 20-, 50- and 100-fold molar excess to determine the optimal cross-linker to protein ratio. The reaction mixtures were kept in ice for 1 hr to suppress subunit exchange during crosslinking. After termination of cross-linking reactions and removal of unreacted cross-linker by filtration, an aliquot of the sample was boiled and run in SDS-PAGE (Figure 3). We did not observe sufficient cross-linking in samples containing 1:10 protein to cross-linker ratio (data not shown). At proteins to crosslinker ratios of 1:20 and above, cross-linking of αA-WT crystallin was characterized by the appearance of ladder of bands corresponding to dimer, trimer, tetramer, etc. (Figure 3, Lane 5 and Figure S1A). With αA-WT, the extent of cross-linking and the gel pattern appeared the same at all protein to cross-linker ratios and at all-time points of cross-linking reaction tested. G98R crystallin, being an aggregation-prone mutant protein, formed high mass aggregates visible on top of the gel at all protein to cross-linker ratios tested (Figure 3, Lane 3 and Fig. S1B). For further analysis, a molar ratio of 1:20 was chosen in order to minimize nonspecific cross-links.

The cross-linked samples were digested in solution with trypsin prior to reversed-phase LC separation and MS/MS analysis. Tryptic cleavage results in peptides with a basic residue at the carboxy terminus, and if the basic lysine residue is cross-linked, it results in missed cleavage [24]. Protease digestion results in complex mixtures of mono-linked, loop-linked and interlinked peptides (Figure 2B). Among these the desired cross-linked product will be present in low abundance amidst the large excess of unwanted, unmodified peptide [19]. Strong cation exchange (SCX) chromatography has been successfully used to enrich the
cross-linked peptides [25–27]. SCX chromatography is based on the charge difference between the cross-linked and non-cross-linked peptides. Cross-linked peptides have a higher charged state than non-cross-linked peptides. Thus, the cross-linked peptides can be eluted from SCX chromatography using high salt concentrations [19,26,28]. Therefore, in our experiments we enriched the targeted cross-linked peptides using SCX-Stage tips. LC-MS analysis of enriched peptide mixtures from SCX chromatography was done and the mono-isotopic deconvoluted m/z values of 500 high intensity peaks were selected for comparison with theoretical mass lists generated by GPMAW software. Lysines in the proteins were chosen as reaction sites of the cross-linker in GPMAW. The cross-linking reagent and the potential amino acids in the proteins that can be cross-linked were defined. A-WT has seven lysines, which can potentially be modified by the cross-linker.

Peptides cross-linked with heavy forms (d4) appear with a characteristic mass shift of 100.045 amu, and peptides cross-linked with light forms (d0) show a mass shift of 96 amu. Thus the cross-linked peptides appear as doublet mass signals with a mass difference of 4.025 Da, 2.0123 Da and 1.341 Da for mono, double and triple protonated forms, thus facilitating identification by mass spectrometry. BS2G- d0 and d4 cross-linked ion pairs with the same retention time and a delta m/z of 4.0247 were used as filters to identify and generate the list of peptide doublets in LC-MS data after comparison with GPMAW data (Table 1). A map of linkage sites between subunits in A-WT crystallin and A-G98R crystallin was developed based on the match (Figure 4). In A-WT crystallin, the major site of cross-linking is at K88, and only one linking site is seen at K99. In contrast, in mutant A-G98R crystallin, the major interaction point shifts to K99, and only one linking site is seen at K88. This difference in linkage sites is significant as the structural arrangement and conformation of WT and mutant crystallins are different [10,29], and therefore different cross-linked products are expected. Cross-linked peptide ion pairs were subjected to MS/MS for identification of cross-linked sites. Except for one, other cross-linked peptides identified in MS were not amenable to MS/MS analysis due to low abundance and changes in their ionization potential [19]. Figure 5A shows the cross-linked peptides with heavy forms (d4) and light forms (d0) eluting from SCX chromatography at 9.7 min in HPLC. The signal region circled in the chromatogram is expanded to show the cross-linked peptide pairs (light and heavy, m/z 638.7 for d0 species and m/z 639.5 for d4 species) having a charge state of +5 and differing by a mass (m/z) of 0.804. (Please note that 638.7 m/z is the +5 charged species whose neutral monoisotopic (+1) mass is 3189.57 (Highlighted in Table 1). The signal highlighted in the inset was analyzed by MS/MS. B— Product ion mass spectrum of +5 charged precursor (m/z 638.7 for d0 species) of the inter-protein cross-linked A-98R crystallin. The spectrum represents two separate peptides from A-G98R comprising amino acids 99–103 and 79–98 cross linked at Lys88 and Lys99. The spectrum exhibits peaks corresponding to b and y ion series from fragmentation of each peptide. Peptide sequences with cross-linked site and the identified fragment ions are indicated in the upper right. The b and y ions are labeled in the spectra and indicated on the peptide sequence in upper right.) MS/MS data were collected in the m/z range 70–2500 Da. Larger fragment ions are not observed as the MS/MS signal significantly drops above 2000 Da. doi:10.1371/journal.pone.0065610.g005

Figure 5. LC-MS and MS/MS analysis of a cross-linked aA-G98R peptide. A— ESI mass spectrum of peptides from tryptic digest of A-A-G98R crystallin cross-linked with 20-fold molar excess of BS2G d0/d4, eluting at 9.7 min in HPLC. The signal region circled in the chromatogram is expanded to show the cross-linked peptide pairs (light and heavy, m/z 638.7 for d0 species and m/z 639.5 for d4 species) having a charge state of +5 and differing by a mass (m/z) of 0.804. B— Product ion mass spectrum of +5 charged precursor (m/z 638.7 for d0 species) of the inter-protein cross-linked A-G98R crystallin. The spectrum represents two separate peptides from A-G98R comprising amino acids 99–103 and 79–98 cross linked at Lys88 and Lys99. The spectrum exhibits peaks corresponding to b and y ion series from fragmentation of each peptide. Peptide sequences with cross-linked site and the identified fragment ions are indicated in the upper right. The b and y ions are labeled in the spectra and indicated on the peptide sequence in upper right.) MS/MS data were collected in the m/z range 70–2500 Da. Larger fragment ions are not observed as the MS/MS signal significantly drops above 2000 Da. doi:10.1371/journal.pone.0065610.g005

Subunit Interactions in aA- and aAG98R Crystallins
Table 1. αA-WT and αA-G98R crystallin cross-links identified based on increase in mass corresponding to peptides cross-linked with deuterated and non-deuterated reagents.

| d0/d4 Pair | Experimental data | GPMAW analysis |
|------------|-------------------|-----------------|
| RT(min)    | (MH+)<sup>a</sup> | (MH+)<sup>b</sup> | αA-wt<sup>c</sup> | αA-wt<sup>d</sup> | Type<sup>e</sup> | ppm<sup>f</sup> |
| 1          | 5.593             | 3312.705        | 3312.705         | 158–173          | 1–12            | X-link          | 0             |
| 2          | 5.598             | 3308.684        | 3308.673         | 158–173          | 1–12            | X-link          | −3            |
| 3          | 9.256             | 4195.113        | 4195.119         | 158–173          | 79–99           | X-link          | 2             |
| 4          | 9.261             | 4191.090        | 4191.088         | 158–173          | 79–99           | X-link          | 0             |
| 5          | 9.831             | 3076.437        | 3076.512         | 79–103           |                |                |               |
| 6          | 9.843             | 3072.416        | 3072.481         | 79–103           |                |                |               |
| 7          | 13.796            | 4097.042        | 4097.059         | 79–99            | 1–12            | X-link          | 4             |
| 8          | 13.797            | 4093.026        | 4093.028         | 79–99            | 1–12            | X-link          | 0             |
| 9          | 14.541            | 4657.343        | 4657.369         | 89–103           | 66–88           | X-link          | 5             |
| 10         | 14.546            | 4653.330        | 4653.337         | 89–103           | 66–88           | X-link          | 2             |
|            |                   |                 | 4653.369         | 79–103           |                |                |               |
|            |                   |                 | 4653.377         | 79–103           |                |                |               |
|            |                   |                 | 4653.377         | 79–103           |                |                |               |
|            |                   |                 | 4653.377         | 79–103           |                |                |               |
| 11         | 11.461            | 3214.629        | 3214.644         | 1–12             | 1–12            | X-link          |               |
| 12         | 11.466            | 3210.611        | 3210.613         | 1–12             | 1–12            | X-link          | 1             |
| 13         | 15.025            | 3238.703        | 3238.708         | 66–78            | 1–12            | X-link          | 2             |
| 14         | 15.031            | 3234.674        | 3234.677         | 66–78            | 1–12            | X-link          | 1             |

| d0/d4 Pair | RT (min) | (MH+)<sup>a</sup> | (MH+)<sup>b</sup> | αA-G98R<sup>c</sup> | αA-G98R<sup>d</sup> | Type<sup>e</sup> | ppm<sup>f</sup> |
|------------|----------|-------------------|-------------------|---------------------|-------------------|-----------------|-----------------|
| 1          | 2.684    | 2434.249          | 2434.232          | 158–173             | 99–103            | X-link          | −7              |
| 2          | 2.696    | 2438.235          | 2438.263          | 158–173             | 99–103            | X-link          | 3               |
| 3          | 4.475    | 3410.763          | 3410.765          | 158–173             | 158–173           | X-link          | 1               |
| 4          | 4.492    | 3406.741          | 3406.734          | 158–173             | 158–173           | X-link          | −2              |
| 5          | 3.759*   | 3189.587          | 3189.571          | 99–103              | 79–98             | X-link          | −3              |
| 6          | 15.759   | 3193.614          | 3193.603          | 99–103              | 79–98             | X-link          | −4              |
| 7          | 9.987    | 1657.847          | 1657.852          | 1–12                | Int. X-link       | 3               |
| 8          | 9.993    | 1653.824          | 1653.821          | 1–12                | Int. X-link       | −2              |
| 9          | 2010     | 2537.243          | 2537.235          | 99–103              | 99–112            | X-link          | −3              |
| 10         | 2028     | 2533.227          | 2533.204          | 99–103              | 99–112            | X-link          | −9              |
| 11         | 2540     | 3608.710          | 3608.709          | 99–112              | 99–112            | X-link          | 0               |
| 12         | 2542     | 3604.685          | 3604.677          | 99–112              | 99–112            | X-link          | −2              |
| 13         | 2709     | 3505.724          | 3505.705          | 158–173             | 99–112            | X-link          | −5              |
| 14         | 2736     | 3509.790          | 3509.737          | 158–173             | 99–112            | X-link          | −4              |
| 15         | 3078     | 3509.732          | 3509.737          | 158–173             | 99–112            | X-link          | 1               |
MS/MS fragmentation spectra of a distinct cross-linked peptide observed in mutant G98R protein, but not in αA-WT protein. At the elution time point of 9.7 min, the average mass spectrum of the mutant G98R crystallin exhibit the signal of an inter-subunit cross-linked product at 3189.58 [MH]+. MS/MS analysis of the precursor ion shows the cross-link between K99 of one G98R subunit and K98 of another G98R subunit (Figure 5B). This interaction site is attributed to a conformational rearrangement within the mutant protein due to replacement of neutral glycine to positively charged arginine.

**Discussion**

G98R-mutated αA-crystallin exhibits structural and functional differences from αA-WT crystallins [9,10]. These include secondary and tertiary structural perturbations resulting in larger oligomeric size, decreased stability, altered chaperone ability and folding defects in the mutant protein, as reported earlier [9,10]. Increased aggregation propensity of the mutant protein underlies the molecular basis for the lens turbidity and cataract formation. Subunit interactions determine the self-assembly and organization of native proteins. Therefore, it is imperative that these interactions be altered in order for mutant proteins to have increased oligomeric size and aggregation propensity. [9,30,31].

We hypothesized that determining the subunit interaction sites in αA-WT crystallin and αA-G98R crystallin would provide key insights into the molecular basis for the structural conversions leading to the crystallin aggregation. We used chemical cross-linking combined with mass spectrometry to identify the subunit interaction regions in αA-WT and αA-G98R crystallins. Such an approach has been successfully used to identify subunit-subunit interactions in native α-crystallin [32,33], phage proteins [34], quaternary domain interactions in Hsp90 chaperones [35], interaction sites in soluble aggregates of monoclonal antibody [36] and sHSP21 and substrate interactions [37], etc.

We report hitherto unreported inter-subunit crosslinks in α-crystallin domain of αA-WT and G98R crystallins. Our cross-linking studies reveal that majority of the inter-subunit crosslinking is clustered in the K88 region in αA-WT and in the K99 region in mutant αA-G98R protein (Figure 4). K99 in αA-WT crystallin is solvent exposed and is not proximal to any other amino group, as has been shown using DTSSP cross-linker [32,33]. In agreement with these studies, we observed no inter-subunit interactions involving K99 in αA-WT, but did note inter-subunit interactions in the K99 region in mutant G98R protein. The β-sheet assembly comprising β2–β9 strands in the α-crystallin domain [38] forms an anti-parallel (AP) interface comprising the dimer, which forms the basic assembly unit for the higher order oligomerization of the wild-type protein [39–41].

The oligomerization involves the formation of various inter-chain interactions involving ion pairs between topologically equivalent residues [8,36]. G98R mutation in αA-crystallin introduces a charged amino acid, which could have resulted in the gain of ion pairs in the interface not seen in WT proteins. The cross-link observed in G98R mutant proteins between K88–K99 in the B5 strand could have resulted from the close proximity of the two lysines as a result of a conformational change different from the WT protein. Such new and altered interface interactions in G98R could have affected the subunit exchange dynamics and the structural organizations required for protein stability and lens transparency. Our findings support the earlier view that mutant proteins have increased and different interfacer interactions not found in wild-type proteins [11,29,42] and has uncovered the specific changes in α-crystallin domain. Further analysis of subunit
arrangements in proteins could help in constructing a structural model and therefore the long-range consequences of such mutations.

In conclusion, the results reveal a new, previously unknown interaction site between G98R subunits. The difference in the cross-linking pattern between the αA-WT and G98R crystallin likely reflects the different oligomerization of the proteins due to altered subunit interaction regions. Our studies demonstrate the use of chemical cross-linking and mass spectrometry as a tool for expanding our understanding of the interactions and conformational changes in mutant proteins that contribute to their aggregation.

Supporting Information

Figure S1  SDS-PAGE of BS²Gd₀ cross-linked αA-WT and G98R crystallin (50- and 100-fold molar excess of cross-linker). Cross-linking reactions contained 25 μM of protein in 50 mM Phosphate buffer (pH 7.4) (final volume 500 μl). The reactions were carried out in ice for 2 hr, and 100 μl aliquots were drawn at 5, 15, 30, 60 and 120 min. The reactions were terminated by adding Tris (final concentration 50 mM) to each aliquot. A—SDS-PAGE of cross-linked αA-WT crystallin - 1:50 and 1:100 at different time points. B—SDS-PAGE of cross-linked G98R crystallin- 1:50 and 1:100 at different time points. Although the cross-linking occurs in both WT and G98R αA-crystallin in 5 min the profiles are distinct on SDS-PAGE. (TIF)

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Author Contributions

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

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