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Post-translationally modified human lens crystallin fragments show aggregation *in vitro*

O.P. Srivastava*, K. Srivastava, J.M. Chaves, A.K. Gill

Department of Optometry and Vision Science, University of Alabama at Birmingham, Birmingham, AL 35294, United States

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ABSTRACT

Background: Crystallin fragments are known to aggregate and cross-link that lead to cataract development. This study has been focused on determination of post-translational modifications (PTMs) of human lens crystallin fragments, and their aggregation properties.

Methods: Four crystallin fragments-containing fractions (Fraction I [~3.5 kDa species], Fraction II [~3.5–7 kDa species], Fraction III [~7–10 kDa species] and Fraction IV [> 10–18 kDa species]), and water soluble high molecular weight (WS-HMW) protein fraction were isolated from water soluble (WS) protein fraction of human lenses of 50–70 year old-donors. The crystallin fragments of the Fractions I–IV were separated by two-dimensional (2D)-gel electrophoresis followed by analysis of their gel-spots by mass spectrometry. The Fractions I–IV were examined for their molecular mass, particle-diameters, amyloid fibril formation, and for their aggregation by themselves and with WS-HMW proteins.

Results: Crystallin fragments in Fractions I–IV were derived from α-, β- and γ-crystallins, and their 2D-gel separated spots contained multiple crystallins with PTMs such as oxidation, deamidation, methylation and acetylation. Crystallin fragments from all the four fractions exhibited self-aggregated complexes ranging in M_r from 5.5×10^5 to 1.0×10^8 Da, with diameters of 10–28 nm, and amyloid fibril-like formation, and aggregation with WS-HMW proteins.

Conclusion: The crystallin fragments exhibited several PTMs, and were capable of forming aggregated species by themselves and with WS-HMW proteins, suggesting their potential role in aggregation process during cataract development.

General significance: Crystallin fragments play a major role in human cataract development.

1. Introduction

Vertebrate lens contains long lived crystallins (classified α-, β- and γ-crystallins) as the major structural proteins. While α- and β-crystallins exist as oligomers, only the γ-crystallin exists as a monomer. Alpha-crystallin comprises of two related subunits (αA and αB), which are derived by gene duplication and divergence, and both have chaperone function. Beta- and γ-crystallins are also derived by gene duplication and are referred as a superfamily. They share common core protein structures, with two similar domains, each composed of two characteristic-modified Greek key motifs. Beta-crystallins are subdivided into acidic and basic subunits, and while the acidic β-crystallins have N-terminal extension whereas the basic β-crystallins have both N- and C-terminal extension besides the core structure. Although the lens crystallins have been shown to be long-lived with very little turnover [1], several reports have shown extensive truncations of lens α-, β-, and γ-crystallins in aging and cataractous human lenses [2–8]. Also, it is well established that specific regions of crystallins are more susceptible to *in vivo* truncations, *e.g.* the C-terminal region of both αA- and αB-crystallins showed a greater susceptibility to truncation *in vivo* than did the N-terminal region [9,10]. Our previous reports and those of others have shown that the crystallin fragments not only showed insolubilization but also formed aggregates *in vitro* [11,12], and are part of *in vivo*-existing covalent complexes of human lenses [13–15]. Additionally, the truncation or mutation in the C-terminal extension of α-crystallin has been shown to result in myopathies [16,17]. It has been shown that *in vivo* generated crystallin peptides also interact with crystallins to enhance their aggregation and cross-linking [18,19].

Certain crystallin fragments also affected α-crystallin chaperone activity, and suppressed aggregation of proteins [20]. Further, the mini-chaperones derived from α-crystallin chaperone region suppressed the aggregation of proteins, block*ed* amyloid fibril formation, stabilized mutant proteins, sequester metal ions, and exhibit anti-apoptotic properties [20]. Together, the above reports suggest a

* Corresponding author.
E-mail address: srivasta@uab.edu (O.P. Srivastava).

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potential role of crystallin fragments in aggregation and cross-linking *in vivo, and* also as therapeutic chaperones.

Crystallin fragments increase with aging in human lenses in both water soluble-high molecular weight (WS-HMW) proteins (~5% of total protein in 16- to 19-year-old lenses, and 27% in 60- to 80-year-old lenses), and also in water insoluble (WI)-proteins (up to 20% of total protein) [4]. Selective aggregation of fragments of βA3- and βB1-crystallins in the WS-HMW proteins and WI- proteins of cataractous lenses relative to normal lenses has been reported [21,22]. Furthermore, the crystallin fragments of cataractous lenses also exhibited relatively increased post-translational modifications (PTMs) such as truncation, deamidation of Asn residues to Asp, and oxidation of Trp residues. On a comparative analyses of proteins of water insoluble-urea soluble and water insoluble-urea insoluble fractions from normal and cataractous lenses, only the cataractous lenses showed an absence of αA- (but not of αB-crystallin), and preferential insolubilization of β-crystallins and their fragments [15]. This finding suggested a greater role for αB-crystallin in the process of aggregation and insolubilization relative to αA-crystallin. On a similar comparison of HMW-proteins from normal aging and cataractous human lenses, multi-protein complexes were observed that were composed of intact α-, β-, and γ-crystallins and their fragments, beaded filament proteins (filensin and/or phakinin), and aldehyde dehydrogenase [15]. Further, the age-related increasing aggregation was also supported by the sizes of polydispersed spherical protein particles, *i.e.* their sizes in the WS-HMW proteins were relatively bigger in 60- to 70-year-old normal human lenses compared to younger 20-year-old normal lenses, and their sizes were further increased in the 60- to 70-year-cataractous lenses.

Truncation of specific regions of crystallins also affect their structural stability and solubility. For example, the homomer aggregates of αA-crystallin with C-terminal extension (residue no. 140-173)-deletion became water insoluble, whereas similar aggregates of αA with deletion of the N-terminal domain (residue no. 1-63) remained water soluble [11]. A similar altered solubility property was also observed in our report on deletion of either N-terminal domain or C-terminal extension of αB-crystallin [23,24]. The crystallin fragments complexes have also been observed. For example, the WI-proteins of 25- and 41-year-old normal human lenses contained two types of covalent multimers (M_r > 90 kDa) of crystallins [25]. The first type was composed of fragments of eight different crystallins (*i.e.*, αA, αB, βA3, βA4, βB1, βB2, γS, and γD), and the second type contained α-, β-, and γ-crystallins (possibly fragments) and two beaded filament proteins (phakinin and filensin). The study further showed that αA-crystallin fragments with three post-translational modifications (*i.e.*, oxidation of M and W residues, conversion of S residue to dehydroalanine, and formylation of H residue) that are known to lead to cross-linking of proteins. An *in vivo*-generated 9 kDa γD-crystallin polypeptide (residue no. 87-173) showed covalent cross-linking by themselves and also with individual α-, β-, and γ-crystallins [26]. In summary, the above studies have provided evidence of aggregation and cross-linking of crystallin fragments *by* themselves and with intact crystallins, their insolubilization and *in vivo* existence as complex of crystallin fragments.

Although the molecular mechanism of aggregation in crystallin fragments is presently unclear, it is suspected that the aggregation could be due to either abnormal conformation of the truncated species compared to their parent crystallins, and/or induced by their post-translational modifications. In this regard, the PTMs of intact crystallins have been extensively characterized in the literature; however, the PTMs of crystallin fragments remain largely uncharacterized. We hypothesize potential roles of truncated crystallin fragments with additional PTMs might cause cumulative effects by making these species to be more prone to aggregation. Additionally, the interactions of crystallin fragments with WS-HMW proteins (believed to a precursor of aggregated and cross-linked crystallins [27]), have not been investigated. The present study was undertaken with the aim to identify crystallin fragments of four different M_r ranges and their PTMs, and characterize their properties of *in vitro* self-aggregation and aggregation with WS-HMW proteins. The study shows that the majority of crystallin fragments with M_r between 3 and 18 kDa from 50 to 70-year old normal human lenses contained a variety of PTMs, and possibly exist as covalent multi-crystallin fragments complexes. These fragments show self-aggregation and also aggregation with WS-HMW proteins to form amyloid fibril-type aggregated products.

2. Methods

2.1. Materials

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involved human lenses. Normal human lenses with no apparent opacity were obtained from Dr. Robert Church (Emory University, Atlanta, GA). The retrieved lenses were stored at −20 °C until used. The pre-stained and unstained protein molecular weight markers were from GE Biosciences (Piscataway, NJ). All chemicals used in the 2D-gel electrophoresis were from either GE Biosciences or BioRad (Hercules, CA). Unless indicated otherwise, all other chemicals used in this study were purchased from Sigma (St. Louis, MO) or Fisher (Atlanta, GA) companies.

2.2. Isolation of crystallin fragments-containing fractions from water soluble (WS) protein fraction of human lenses

The WS-protein fraction from 20-pooled human lenses of 50–70-year-old donors was prepared as previously described [21,22]. All procedures were performed at 5 °C unless described otherwise. Briefly, lenses after their retrieval were immediately frozen at −20 °C in medium 199 without phenol red and were stored frozen −20 °C. The lenses were kept frozen until utilized. Lenses were thawed on ice, decapsulated, suspended (2 ml/lens) in buffer A (50 mM Tris-HCl, pH 7.9, containing 1 mM dithiothreitol [DTT], 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride), and homogenized using a tissue grinder (Polytron, model PT-1200C). The lens homogenate was centrifuged at 25,000×*g* for 15 min. The supernatant was recovered and the pellet was homogenized in buffer A and centrifuged twice as above. The supernatants recovered after each centrifugation were pooled and designated as the WS-protein fraction, and the pellet as the water insoluble (WI)-protein fraction. The WS-protein fraction was subjected to a preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE, 15% acrylamide gel) by the Laemmli's [28] method using the BioRad Prep Cell (Model 491, Hercules, CA). The eluted individual crystallin fragments-containing fractions were collected on their exit from gel using a fraction collector, and analyzed by SDS–PAGE. Next, based on the M_r, four individual fractions (designated Fractions I–IV) were collected with increasing molecular weights between 3 and 18 kDa (Fig. 1). The four crystallin fragments-containing fractions were: Fraction I [~3.5 kDa species], Fraction II [~3.5–7 kDa species], Fraction III [~7–10 kDa species] and Fraction IV [> 10–18 kDa species]. Each fraction was dialyzed against 50 mM phosphate buffer, pH 7.5 using 1000-Da molecular cut-off dialysis tubing with change of the buffer every 8 h for up to 48 h. Next, the fractions were dialyzed against deionized water with changes every 4 h during 24 h of dialysis, which was followed by their lyophilization. The amount of SDS present in each fraction was quantified using the stain-all solution as described [29]. Briefly, 5 µl of sample were dissolved in 1 ml buffer containing 90 µM stains-all, 2.5% (v/v) isopropanol, and 5% (v/v) formamide and their absorbance at 438 nm was compared to a standard curve prepared with known concentrations of SDS. If any fraction contained more than 0.05% SDS, it was passed through a Detergent-OUT™ SDS-300 spin micro column using the manufac-