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Disulfide cross-links in the interaction of a cataract-linked αA-crystallin mutant with βB1-crystallin

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ABSTRACT

A number of αA-crystallin mutants are associated with hereditary cataract including cysteine substitution at arginine 49. We report the formation of affinity-driven disulfide bonds in the interaction of αA-R49C with βB1-crystallin. To mimic cysteine thiolation in the lens, βB1-crystallin was modified by a bimane probe through a disulfide linkage. Our data suggest a mechanism whereby a transient disulfide bond occurs between αA- and βB1-crystallin followed by a disulfide exchange with cysteine 49 of a neighboring αA-crystallin subunit. This is the first investigation of disulfide bonds in the confine of the chaperone/substrate complex where reaction rates are favored by orders of magnitude. Covalent protein cross-links are a hallmark of age-related cataract and may be a factor in its inherited form.

1. Introduction

Cataract is one of the leading causes of blindness worldwide [1]. Its most common form, age-related nuclear cataract (ARN), is characterized by the formation of protein aggregates that scatter light [2]. Lens proteins undergo covalent modifications of their sequences during aging that may reduce their stability [3–6]. In the absence of repair and turnover, concomitant with continual depletion of the binding capacity of the α-crystallin chaperones, damaged proteins nucleate aggregation through unfolding or solubility changes. This is the prevailing molecular model of age-related cataract [1].

A hallmark of protein aggregates in cataractous lenses is their extensive cross-linking primarily through the formation of disulfide bonds [7]. One of the least understood facets of lens chemistry is the relatively rich cysteine and methionine content of its proteins [8,9]. Protein sulfhydryl concentration is about 50 mM, an order of magnitude higher than that of the main reducing agent, glutathione (GSH). While the oxidation potential of a sulfhydryl is a complex function of a number of factors including topological location in a protein fold [10], the observation of disulfide-linked protein aggregates in old and cataractous lenses leads to the unavoidable conclusion that these sulfhydryls are targets for age-related oxidation [11–14]. The mechanism of disulfide-linked protein aggregate formation continues to be investigated although a consensus model is emerging [9,15–18]. Protein–thiol mixed disulfides, involving either glutathione or cysteines (PSSG or PSSC), are the intermediates in the formation of disulfide-linked proteins (PSSP). Oxidative stress simultaneously inactivates the dethiolation machinery in the lens and damages protein SH groups both of which accelerate protein aggregation and cataract formation. In ARN, depletion of GSH or reduction of its concentration below the threshold of 1 mM may be mediated by the formation of a barrier between the lens nucleus and the cortex where a more healthy 3 mM concentration is maintained [19,20]. A shift to high ratio of oxidized to reduced glutathione (GSSG/GSH) promotes formation of protein mixed disulfides [14], allows ascorbate to become reactive with proteins [21] and increases the deleterious effects of methylglyoxal [22,23].

Although ARN is the most prevalent form of the disease, a class of inherited cataract phenotypes has proven relevant to understanding the underlying molecular mechanism. The phenotypes are associated with single point mutations in the two resident chaperones of the lens αA- and αB-crystallin [24–26]. We have advanced the hypothesis that these mutations activate substrate binding leading to an effective unfolding by the chaperones [27]. The implied mechanism is an accelerated aggregation and

Abbreviations: sHSP, small heat-shock proteins; T4L, T4 lysozyme; αB-D3, αB-crystallin S19D/S45D/S59D; WT, wild-type

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precipitation of lens proteins in complex with the chaperones. Absent in this model is a consideration of the role of cross-linking and the fate of protein sulfhydryls. This is particularly relevant considering that a number of cataract-linked mutations introduce cysteine residues into the sequence. Although the environment of the young lens is highly reducing, entropic advantage conferred by the effective high local concentration of sulfhydryls in the protein fold or by proximity in a complex, can drive the formation of disulfide bonds [28–30]. Moreover, the unfolded conformation of substrate proteins in chaperone complexes releases steric constraints on sulfhydryls reactivity [31].

This paper explicitly considers the formation of affinity-driven disulfide bonds between lens crystallins. Specifically, we report that interaction of the αA-crystallin mutant R49C with βB1-crystallin leads to the formation of disulfide-linked α-crystallin subunits. Because the oxidizing equivalent is transferred from βB1-crystallin, the intermediate of this reaction is a transient disulfide bond between αA- and βB1-crystallin. These results add a new dimension to our understanding of the mechanism of cataract.

2. Materials and methods

2.1. Materials

Antibodies to α- and βB-crystallin were generous gifts from Dr. J.S. Zigler (National Eye Institute, National Institutes of Health, Bethesda, MD, USA).

2.2. Protein expression and purification

βB1-crystallin was expressed, purified as previously described [32] and reacted with (2-pyridyl)dithiobimane (PDT-Bimane) (Scheme 1). αA- and αB-crystallin were expressed and purified as previously described [33,34].

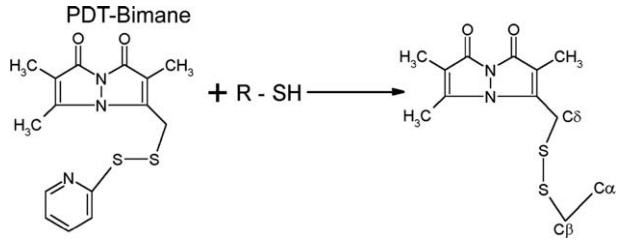
2.3. Binding isotherms of βB1-crystallin to α-crystallin

Binding isotherms were constructed at fixed concentrations of bimane-labeled βB1-crystallin. Samples containing a fixed concentration of βB1-crystallin and varying amounts of α-crystallin were incubated for 2 h at 37 °C. The fluorescence emission of the samples was obtained using a PTI L-format spectrofluorometer equipped with an RTC2000 temperature controller. Bimane excitation wavelength was 380 nm and the fluorescence emission was recorded in the 420–500 nm range. The bimane intensity at 480 nm was plotted versus the molar ratio of α- to βB1-crystallin to construct a binding isotherm.

2.4. Fluorescence anisotropy measurements

The fluorescence anisotropy (r) was measured using a PTI T-format spectrofluorometer and calculated according to the equation:

$$r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$$



Scheme 1.

where I_{vv} and I_{vh} refer to the amplitude of fluorescence emission parallel and perpendicular to the plane of light excitation, respectively. The G-factor was determined for each sample to correct for bias in each channel. Samples for anisotropy measurements containing 30 μM bimane-labeled βB1-crystallin and varying concentrations of α-crystallin were incubated at 37 °C for 2 h. The samples were excited at 380 nm and the emission collected at 465 nm. Each measurement represents an average of 10 readings.

2.5. Gel electrophoresis and Western blot

Purified α-crystallin and its mutants (15 μg) were incubated with or without bimane-labeled βB1-crystallin (15 μg) in a substrate binding buffer at 37 °C for 2 h. At the end of reaction, sample buffer that does not contain DTT was added and protein mixtures (5 μg/lane) were separated on 4–16% gradient SDS–PAGE gels. For reactions to reduce disulfide bonds prior to gel electrophoresis sample buffer was supplemented with 20 mM DTT. For Western blots, protein mixtures (1 μg/well) were separated as described above and then transferred onto nitrocellulose membranes (Millipore). The membranes were blocked by incubating for 30 min with 5% skim milk. For the detection of the crystallin proteins, membranes were incubated for 1 h with the rabbit anti-αA and anti-βB-crystallin polyclonal antibodies (1:5000) and extensively washed with PBS containing 0.1% Tween 20 before incubating with peroxidase-conjugated anti-rabbit IgG (Sigma–Aldrich, St. Louis, MO) as a secondary antibody. After the membranes were washed, the proteins bound to the antibody were visualized using the ECL chemiluminescent kit (GE Healthcare Biosciences, Piscataway, NJ).

3. Results and discussion

3.1. The interaction of αA-R49C with βB1-crystallin releases the bimane probe

To detect complex formation, a bimane probe is introduced at site 79 of βB1-crystallin. For this work, the bimane is attached to the protein via a reversible disulfide bond as illustrated in scheme 1. The original rationale for using a disulfide linkage for the probe is its higher reactivity compared to linkages via thioether bonds extensively employed in previous studies of T4 lysozyme (T4L) binding to small heat-shock proteins (sHSP) [35,36]. The reversibility of the linkage between the protein and the probe is a critical factor in the discovery of the tendency of the αA-R49C mutant to form disulfide bonds.

We have previously demonstrated that binding of βB1-crystallin to α-crystallin reduces the emission intensity of the bimane at this site [37]. By following the change in intensity as a function of the molar ratio between the two proteins, binding isotherms were constructed and analyzed to determine the affinity and the number of available binding sites. Parameters of αA- and αB-crystallin interactions with destabilized βB1-crystallin mutants were determined [37]. Because the postulated interactions between the αA mutants and lens proteins occur in the early stages of lens development, they are not expected to involve destabilization of the substrates. Therefore, the binding assays presented in this paper focus on wild-type (WT)-βB1 labeled at cysteine 79. The introduction of the bimane probe reduces the overall stability of the protein [32].

Fig. 1 compares the change in bimane fluorescence upon binding of βB1-crystallin to WT αA-crystallin and the two mutants R116C and R49C. The curves show a decrease in emission intensity as previously reported for WT αA-crystallin and the phosphorylation mimic of αB-crystallin, αB-crystallin S19D/S45D/S59D

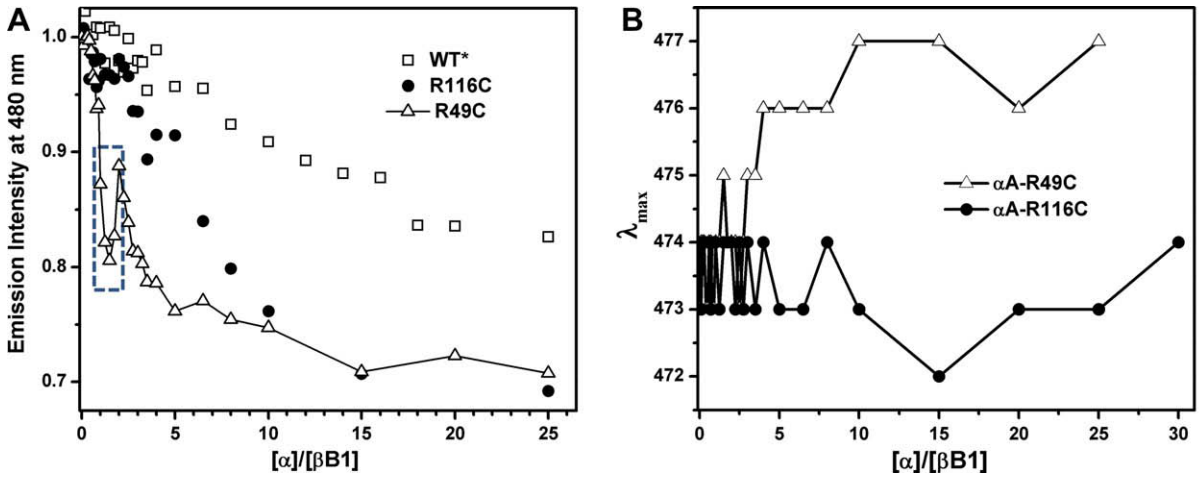


Fig. 1. (A) Binding isotherms of bimane-labeled βB1-crystallin to αA-WT* and mutants linked to hereditary cataract at 37 °C. The concentration of βB1 was 30 μM. Each curve was reproduced at least two times and the data points for R49C are the average of two measurements. (B) Changes in the maximum emission wavelength of the bimane probe upon binding. The data points are the average of two independent measurements. The dashed box highlights a region of discontinuity in the emission intensity presumably caused by the release of the bimane probe.

(αB-D3) [37]. At similar molar ratios of αA to βB1, the intensity is lower for R49C suggesting an increase in binding for this mutant consistent with previous results obtained with T4L.

Binding of βB1-crystllin to R49C leads to two distinguishing spectral characteristics. A reproducible break in the binding curve (Fig. 1A dashed box) is observed at low molar ratio of αA to βB1 accompanied by a shift in the wavelength of the emission maximum (λ_{max}) to higher values (Fig. 1B). The λ_{max} shift is not observed for binding to R116C (or αA-WT*) suggesting that it is not due to the environment of the bimane in the binding site of the chaperone. Indeed, red shifts are usually associated with increased accessibility to the aqueous environment [38]. Consistent with this interpretation, DTT reduction of the disulfide bond linking the bimane to the probe leads to a similar red shift and a change in the emission intensity. The latter is likely the origin of the abrupt intensity change in the binding curve (Fig. 1A dashed box). In that range of molar ratio, the intensity has contribution from bound and unbound βB1-crystallin as well as free (released) bimane which hinders a quantitative analysis of binding. Because the samples were thoroughly desalted prior to the binding assay, the only mechanism for bimane release involves a nucleophilic attack by the thiolate of R49C on the disulfide bond of the linking arm. The product is a disulfide-linked complex of αA- and βB1-crystallin.

A binding-induced release of the bimane is expected to decrease the steady state anisotropy of the bimane probe as a function of added αA-R49C. Fig. 2 shows that the anisotropy of the bimane is significantly reduced at high concentration and the values are consistent with the tumbling of a small molecule. In contrast, binding to αA-R116C leads to an increase in the anisotropy (not shown) due to the formation of the large complex [27]. As a reference, Fig. 2 also shows the increase in anisotropy of βB1-crystallin L207A [32], a destabilized mutant of βB1-crystallin bimane-labeled at position 79, upon binding to the phosphorylation mimic of αB-crystallin, αB-D3, which is the α-crystallin variant with the highest affinity to substrates [35].

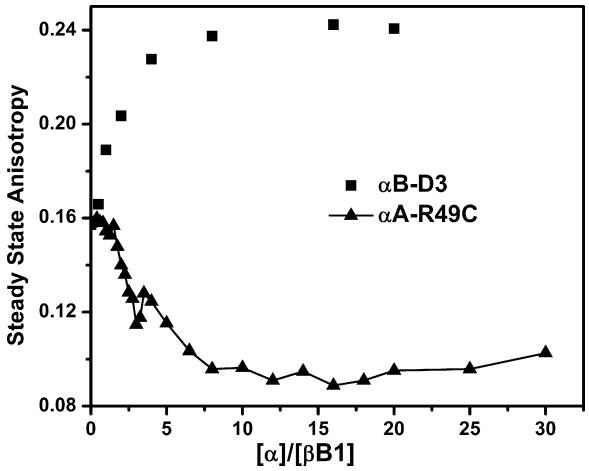


Fig. 2. Binding-induced change in the anisotropy of bimane-labeled βB1-crystallin. In the presence of αA-R49C, the steady state anisotropy decreases to a value characteristic of small molecule tumbling. In the presence of αB-D3 (or αA-R116C) the bimane anisotropy increases as expected upon the formation of a large molecular weight complex.

3.2. Disulfide crosslinks in the interaction of αA- and βB1-crystallin

To probe for the formation of stable covalent products, the samples were analyzed by SDS–PAGE under reducing and non-reducing conditions (Fig. 3A). The results show the formation of a high molecular weight population upon incubation of bimane-labeled βB1-crystallin and αA-R49C. This population is not observed in solutions of βB1-crystallin or αA-R49C incubated separately (Fig. 3B) suggesting that it results from the interaction of the two proteins. The loss of the high molecular weight band upon addition of DTT indicates that this population is linked by a disulfide. The lack of a red shift in the bimane emission following binding to αA-R116C correlates with the absence of high molecular weight, disulfide-linked products on the gel. Furthermore, the high molecular weight band is not observed for αA-C131, a native cysteine in the human protein highlighting the specificity of the αA-R49C/βB1-crystallin interaction. The formation of this intermediate requires bimane labeling of βB1-crystallin consistent with the disulfide bond in the linking arm acting as the oxidizing agent (not shown).

The molecular weight of the disulfide-linked species suggests that it consists of two αA-crystallin monomers. To verify this conclusion we probed the gel with both α-crystallin and βB-crystallin polyclonal antibodies. Fig. 3 shows that the major covalent product

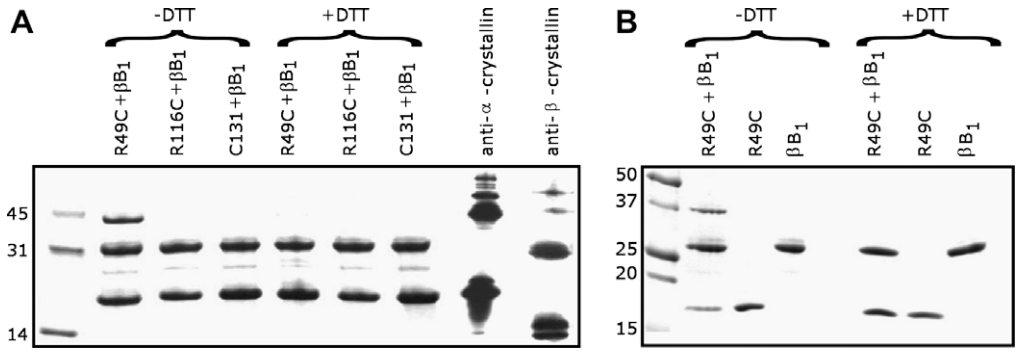


Fig. 3. SDS–PAGE analysis of mixtures of αA and bimane-labeled βB1-crystallin incubated at 37 °C for 2 h. (A) In the absence of DTT, a high molecular weight species is observed upon incubation of αA-R49C and βB1-crystallin. Western blot analysis indicates the formation of α-crystallin dimers. (B) Incubation of αA-R49C and βB1-crystallin separately does not lead to disulfide cross-links.

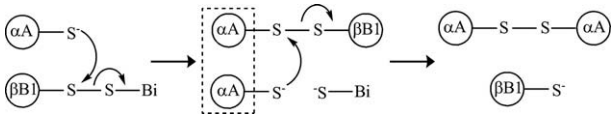


Fig. 4. Scheme describing the disulfide bond rearrangement in the interaction of αA-R49C and βB1-crystallin. A nucleophilic attack on the disulfide bond linking βB1-crystallin and the bimane (Scheme 1) leads to the formation of a transient disulfide link between βB1- and αA-R49C. Another disulfide exchange occurs leading to the formation of a covalently linked αA-crystallin dimer.

is a disulfide-linked αA-crystallin. Staining by the β-crystallin antibody reveals a minor band consisting of α- and β-crystallin.

Because the formation of the stable α-crystallin dimer involves the disulfide bond of the bimane as an oxidizing agent, it implies that the reaction mechanism must involve the formation of an unstable mixed α/β intermediate. The decay of this intermediate must be due to a rapid attack on the disulfide bond by the R49C of a neighboring subunit in the α-crystallin oligomer. Fig. 4 depicts the postulated disulfide rearrangement. We have carried out a more extensive analysis at different molar ratios of αA to βB1 and in the presence of GSSG/GSH buffer. The formation of the αA-crystallin dimer is unaffected by 1 to3 molar excess of GSSG over GSH or vice versa as long as βB1-crystallin is modified by the bimane (data not shown).

4. Concluding remarks

Although loss of free sulfhydryls and protein cross-linking are a hallmark of ARN [7], in vitro analysis of disulfide bonds between various crystallins has received relatively little attention. In principle, there are two important factors that promote the formation of disulfide bonds in the α-crystallin/substrate complex. First the proximity of the cysteines in the complex leads to a significant entropic advantage. A prominent example of the magnitude of this effect is the effective concentration between pairs of cysteines in a protein that can reach 500 000 M [28]. Second, we have demonstrated that bound substrates to α-crystallin are extensively unfolded [31]. Therefore, the cysteines lose steric protection by the fold and are unhindered for reaction. Finally, the formation of disulfide bonds requires an oxidizing agent. In the α-crystallin/βB1-crystallin interaction reported here, this role is fulfilled by the sulfhydryl in the bimane linking arm. In the lens, protein mixed disulfides (P-CH, P-GSH) are likely to be the intermediates [15]. The specificity of the cross-link to R49C likely reflects the location of this residue in a substrate binding region. The formation of the αA-crystallin dimer suggests that residues R49C from different subunits are in close proximity in the oligomer. On the basis of the structure of Hsp16.5 and its variants [39–41], R49C is likely to be located in the buried core of the oligomer.

Much remains to be explored in terms of the oxidizing potential and the functional consequence of the disulfide bonds on the structure and chaperone activity of αA-crystallin. One of the fascinating aspects of congenital cataract is that an age-related process, effectively limited by kinetics, becomes instantaneous. If formation of disulfide-linked aggregates is a causative event in age-related cataract, then mutants such as R49C may time-compress the process. Through their increased affinity, they induce complex formation with substrates without the need for age-related modifications and consequently lead to early exhaustion of chaperone capacity. Subsequent disulfide cross-linking may have further deleterious functional consequences.

Acknowledgments

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