α-Crystallin Chaperone Activity Is Reduced by Calpain II in Vitro and in Selenite Cataract*

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This study reports the first demonstration of a marked reduction in α-crystallin chaperone activity in an experimental model of cataract, and the study implicates activation of the cysteine protease calpain II (EC 3.4.22.17) as the in vitro protease responsible for decreased chaperone activity. Chaperone activity of normal α-crystallin from lenses of young rats was assayed by measuring attenuation of heat-induced aggregation and scattering of β-crystallin. α-Crystallin from the nucleus of lenses with selenite cataract showed specific proteolytic cleavage, and chaperone activity was diminished. Proteolysis of α-crystallin from selenite cataract lenses was mimicked by incubating normal α-crystallin with calpain II, and this also resulted in loss of chaperone activity. Two-dimensional gel electrophoresis and peptide mapping were used to identify four partially degraded αA- and αB-crystallin polypeptides following incubation of normal α-crystallin with calpain. Similar partially degraded αA and αB polypeptides were found in selenite cataract. Previous experiments indicated that α-crystallin chaperone activity decreases because of removal of the COOH terminus. Our experiments support this observation and suggest that calpain proteolysis of α-crystallin at the COOH terminus may result in a loss of chaperone activity in selenite cataract.

The transparency and refractive power of the lens in mammalian eyes are maintained by a highly ordered array of structural proteins designated α-, β-, and γ-crystallins. Based on sequence homologies, β- and γ-crystallins belong to the same evolutionary superfamily, whereas the α-crystallins are related to the small heat shock protein family (1-4). The chaperone activity of α-crystallin was found to be as effective as some small heat shock proteins in preventing heat denaturation of proteins (5). A recent hypothesis therefore states that the chaperone activity of α-crystallin may be important in helping to prevent denaturation of crystallins in lens (6, 7). Such denatured insoluble crystallins are a hallmark of experimental cataracts in lens (8). Opaque cataract is the universal response of the lens to a wide variety of insults including excess sugar, UV irradiation, trauma, toxic chemicals, and aging. Protein denaturation during cataract formation is particularly detrimental to the lens because the lens has little protein turnover. Thus, it is important to determine which factors influence α-crystallin chaperone activity in lens.

Aging in bovine lenses was recently shown to reduce α-crystallin chaperone activity (9). α-Crystallins undergo cleavage at their COOH termini during aging of the lens (10); however, the specific in vitro protease causing the loss of the COOH terminus of α-crystallin are unknown. Furthermore, no data have been published on whether or not α-crystallin chaperone activity is reduced in cataract.

The experimental model of cataract produced by an overdose of selenite in the young rat was used in the present study to investigate changes in chaperone activity during cataract formation, because the model involves proteolysis of α- and β-crystallins (11). Loss of NH2-terminal extensions on β-crystallins may lead to their insolubilization and to production of light scattering opacity (12). Calpain II, a calcium-dependent nonlysosomal cysteine protease found in all vertebrate cells studied, is believed to be responsible for most of the proteolysis in selenite cataract (13), and this protease has also been implicated in other models of cataract in rat lenses utilizing calcium ionophore A23187 (14), galactose (15), xylose (14), and diamide (16). Although α-crystallins are not insolubilized during formation of selenite cataract, they do undergo partial degradation, both during cataract formation (17) and after incubation with calpain II (18). Therefore, calpain II is a likely endogenous protease that may contribute to loss of chaperone activity in rat lens. Thus, the purposes of the present study were to determine if the chaperone activity of α-crystallin is lost during the formation of the selenite cataract and to determine if in vitro incubation with purified calpain II can also cause the loss of chaperone activity of α-crystallin.

MATERIALS AND METHODS

Production of Selenite Cataract—Cataracts were induced in 10-day-old Sprague-Dawley rats from B & K International (Fremont, CA) following a single subcutaneous injection of 30 μmol of sodium selenite/kg of body weight. Five days after injection, cortex and nucleus of lenses were isolated by dissection, as described previously (17). Animals used in this study were treated in accordance with the Declaration of Helsinki and appropriate National Institutes of Health recommendations.

Gel Filtration of Crystallins—To obtain α- and β-crystallins, cortical and nuclear regions from 40 lenses were homogenized in buffer I containing 20 mM imidazole (pH = 6.8), 0.1 mM EGTA, 2 mM dithioerythritol, and 0.01% azide. Homogenates were centrifuged at 8000 × g for 15 min at room temperature to prevent cold precipitation of crystallins. Following centrifugation, the supernatant was applied to a 2.5 × 90-cm Bio-Gel A1.5 m gel filtration column (Bio-Rad) and eluted with 20 mM Tris buffer (pH = 7.5) containing 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 100 mM NaCl.
Reduction of Chaperone Activity by Calpain II

at 25 ml/h flow rate. After pooling various fractions (Fig. 1), α- and βL-crystallins were concentrated by ultrafiltration (YM-5 membrane, Amicon, Beverly, MA), and the gel filtration buffer was exchanged for buffer I. Protein concentrations were determined by the Bio-Rad dye-binding reagent (Bio-Rad) using bovine serum albumin as standard.

Proteolysis of Lens Proteins with Calpain—Calpain II was purified from porcine cardiac muscle as described previously (12). Use of porcine cardiac muscle calpain II was appropriate, because it degraded crystallin at similar sites as calpain purified from rat lens (19). To proteolyze α-crystallin, 17 units of purified calpain II were incubated with 0.48 mg of normal α-crystallin and 0.4 ml of buffer I at 37°C. The reaction was stopped after 1 h by the addition of 0.1 ml of 25 mM EGTA.

Chaperone Experiments—Heat-induced aggregation of βL-crystallin was measured by the increase in light scattering at 340 nm in an enzyme-linked immunosorbent assay plate reader. Normal βL-crystallin (200 µg/ml) was incubated at 64°C in a total volume of 0.25 ml of buffer I containing 0.08 M NaCl. The wells were normalized to correct for uneven heating across the plate. The measurement of the light scatter of the same βL-crystallin solution in all of the individual wells (columns 1 and 12 not used) was the basis of this normalization. To test chaperone effects, either 25, 50, or 75 µg/ml α-crystallin were added to the βL-crystallin prior (5 min at 37°C) to heat-induced aggregation.

Electrophoresis—One-dimensional SDS-PAGE1 (20) of lens proteins was performed on 12% (0.75 mm thick) gels. Low and broad range molecular weight standards were used (Bio-Rad) at 6.2 µg of protein/lane. Two-dimensional electrophoresis using a nonequilibrium pH gradient in the first dimension and SDS-PAGE with 12% gels in the second was conducted as described previously (13, 21). Gels were stained with Coomassie Blue, and blots were stained with Ponceau S before being subjected to computer image analysis (Image 1.44, National Technical Service, Springfield, VA) following digital flat bed scanning (LaCie Silverscanner, Beaverton, OR) using green lamp illumination, 200 dots/inch resolution, and 256 gray levels/pixel.

Tryptic Mapping—Following incubation with calpain, putative breakdown products of αA- and αB-crystallins were identified by tryptic mapping. The lenses of 14-day-old rats were dissected and homogenized at a ratio of 1 mg of lens wet weight/2.5 µl of buffer I. The lens homogenates were centrifuged at 8000 × g for 15 min at 25°C to remove the insoluble protein. Soluble lens protein (30 mg/ml) was incubated for 60 min at 37°C in buffer I containing 2.4 units of calpain II/mg of lens protein and 1.2 mM CaCl2 and centrifuged at 8000 × g for 15 min. The supernatant was removed, dried in a vacuum centrifuge, and frozen at −70°C. Aliquots (250 µg) of the lens proteins proteolyzed with calpain and nonproteolyzed control samples were then subjected to two-dimensional gel electrophoresis. The proteins from four gels were blotted onto PVDF membranes (Immobilon P, Millipore, Bedford, MA), stained, excised, subjected to in situ digestion with trypsin, and separated by reverse phase HPLC (19). Amino-terminal sequence analysis was performed as described previously (13). The molecular weights of αA-crystallin tryptic fragments were measured using fast atom bombardment mass spectroscopy, as described previously (22).

RESULTS

Chaperone Activity of Rat Lens α-Crystallins—α- and βL-Crystallins were isolated from the soluble protein fraction of 14–15-day-old rat lens cortex by gel filtration (Fig. 1). SDS-PAGE demonstrated that α-crystallin was composed of three major polypeptides (Fig. 2, lane 2), and βL-crystallin was composed of two major and two minor polypeptides (Fig. 2A, lane 8). Based on their migration during SDS-PAGE and two-dimensional electrophoresis, the α-crystallin polypeptides

![Figure 1](image1.png)

**Fig. 1.** Gel filtration chromatography of water-soluble proteins from lens cortex of 14-day-old rats. Heavy vertical bars on this representative chromatogram enclose α-crystallin and βL-crystallin peaks. The inset shows a portion of a two-dimensional electrophoretic gel of the proteins in the α-crystallin peak, indicating αA, αB, and αA insert polypeptides.

![Figure 2](image2.png)

**Fig. 2.** A, SDS-PAGE of α-crystallin preparations. Lane 1, molecular mass standards labeled in the margin in kilodaltons; lane 2, α-crystallin from control cortex (CC); lane 3, α-crystallin from selenite cortex (SC); lane 4, α-crystallin from control nucleus (CN); lane 5, α-crystallin from selenite nucleus (SN); lane 6, α-crystallin from control cortex incubated with calpain (CALP); lane 7, α-crystallin from control cortex incubated with both calpain and a synthetic protease inhibitor, E64 (E64); lane 8, βL-crystallin (βL) isolated from the control cortex by gel filtration and used in the heat-induced aggregation and scattering assay. All sample lanes were loaded with 10 µg of protein/lane. Note the appearance of proteolytic fragments below the arrow, especially in lanes 5 and 6. B, percentage of α-crystallin fragments. Percentages were calculated by densitometric scanning of the gel shown in A and dividing the density of bands below 20 kDa by the total density of each lane × 100.

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1. The abbreviations used are: PAG, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HPLC, high performance liquid chromatography.
were identified as αA, αB, and αA insert (Fig. 1, box) (23). The α-crystallin fractions from lens cortex and nucleus were first tested for their ability to act as chaperones. Heating βL-crystallin from the cortex of rat lens in the absence of α-crystallin caused aggregation as measured by an increase in light scattering at 340 nm (Fig. 3, beta-L). The extent of aggregation of βL-crystallin was directly related to both the concentration of βL-crystallin and duration of heating (Fig. 3, inset).

Addition of normal α-crystallin from the lens cortex of young rats caused a marked protective effect against the heat-induced aggregation and scattering of βL-crystallin (Fig. 3, beta-L/CC). This was similar to the chaperone activity of α-crystallin observed in other species (6, 7). The results of the chaperone activity of α-crystallin depended upon two factors, the temperature, and the purity of the preparation. If the temperature was too high, or if the preparation was not very pure, then the α-crystallin sample was a less effective chaperone. The chaperone activity of α-crystallin from the cortex of rat lens shown in Fig. 3 was 91% effective against heat-induced aggregation and scattering of βL-crystallin at 30 min and 82% effective even after 60 min of heating at 64 °C (Fig. 3). The proportions of α- and βL-crystallin subunits in Fig. 2 were used to calculate a weighted average molecular mass of the α-crystallin monomer to be 21 kDa and of the βL-crystallin monomer to be 29 kDa. By using these values, the molar ratio of βL to α-crystallin subunits in Fig. 3 was approximately 2:1, but the chaperone effect of cortical α-crystallin was appreciable even at a molar ratio of βL to α-crystallin of 6:1. Note that α-crystallin from the nucleus of rat lens was also an effective chaperone for the first 30 min of heating of βL-crystallin (Fig. 3, beta-L/CN). However, after 30 min, α-crystallin from the nucleus tended to lose chaperone effectiveness faster than α-crystallin from the cortex.

**Chaperone Activity of α-Crystallins from Selenite Cataract**—In order to test if the chaperone effect of α-crystallin was altered in a model of cataract, α-crystallins were also isolated from the lens cortex and nucleus of rats with dense nuclear selenite cataracts. After 30 min at 64 °C, the α-crystallin from the nucleus of lenses with selenite cataract showed markedly reduced chaperone activity (Fig. 4A, SN). By contrast, the α-crystallin from control cortex (CC), selenite cortex (SC), and control nucleus (CN) were all effective chaperones (Fig. 4A), although the α-crystallin from the control cortex (CC, Fig. 4A) was slightly more effective than the others at lower concentrations.

Correlations between proteolysis and chaperone activity were made by subjecting the α-crystallin from the lens cortical and nuclear regions of control and selenite treated rats to SDS-PAGE (Fig. 2). The chaperone-defective α-crystallin aggregates from the cataractous nucleus of selenite-treated (SN) rats showed specific selective proteolysis of αA, αB, and αA insert polypeptides. In addition, new proteolytic fragments appeared at approximately 18 and 19 kDa (Fig. 2A, lane 5). The proteolytic fragments of α-crystallins at 18 and 19 kDa comprised approximately 58% of the total α-crystallin isolated from the selenite cataractous nucleus (Fig. 2B). Control nucleus (CN) and selenite cortex (SC), which exhibited slightly reduced chaperone activity, showed lesser levels of proteolysis (Fig. 2A, lanes 3 and 4) with fragments comprising only 25 and 33% of the total protein (Fig. 2B).

**Chaperone Activity of α-Crystallin Incubated with Purified E64**

![Fig. 3. Rate of heat-induced aggregation and scattering of βL-crystallin (200 μg/ml) in the presence (75 μg/ml) and absence of α-crystallin isolated from the lens cortex and nucleus of control 14-day-old rats. Control cortical α-crystallin (CC) was more effective than control nuclear α-crystallin (CN) in preventing the heat-induced aggregation and scattering of βL-crystallin. βL-Crystallin without α-crystallin aggregates and precipitates most rapidly. The inset shows a time course of heat-induced aggregation and scattering of successively higher concentrations of βL-crystallin at 64 °C. The y axis (optical density (mOD) increase 1000) is the increase in light scattering measured at 340 nm and the x axis is incubation time (min) for both figures. The higher concentrations of βL-crystallin showed increased precipitation and light scatter during heating. For all subsequent aggregation assays, 200 μg/ml βL-crystallin was used. Mean range for multiple determinations (n = 2–4) for all points on this figure was ± 10%. At each time point, none of the individual values within a group overlapped individual values in another group.

![Fig. 4. A, comparison of the chaperone activity of normal and selenite cataract α-crystallins. α-Crystallins from selenite nucleus (SN), selenite cortex (SC), control nucleus (CN), and control cortex (CC) were used at the concentrations indicated to suppress the heat-induced aggregation and scattering of 200 μg/ml βL-crystallin at 64 °C. α-Crystallins from selenite nucleus (SN) were ineffective chaperones when compared with the other groups shown. The mean range for duplicate determinations for each point within this experiment was ± 8%. None of the individual values at 25, 50, and 75 μg of α-crystallin from the selenite nucleus overlapped with any value from the control nucleus, selenite cortex, or control cortex groups. B, treatment of α-crystallin with calpain decreased chaperone activity. Prior to incubation with 200 μg/ml βL-crystallin at 64 °C for 30 min, α-crystallin was incubated for 1 h at 37 °C in the presence of calpain, both with and without the protease inhibitor E64. The α-crystallin treated with calpain proved to be an ineffective chaperone of βL-crystallin. E64, however, inhibited calpain proteolysis and allowed the α-crystallin to act as a chaperone to prevent precipitation of βL-crystallin. The mean range for replicates within this representative experiment was ± 3%. None of the individual values at 25, 50, and 75 μg of α-crystallin overlapped between the calpain and E64 groups.**
Calpain II—Proteolysis observed in selenite cataract is caused by activation of calcium-activated protease, calpain II, present in rat lens (13). Thus, it was conceivable that the loss of chaperone activity observed in selenite cataract was due to proteolysis of α-crystallin by calpain. In order to test this hypothesis, normal α-crystallin from the clear cortical region of rat lenses was incubated with purified calpain II (Fig. 2, lane 6). This resulted in a pattern of proteolysis similar to that observed in selenite cataract (Fig. 2, lane 5), with similar proportions of proteolytic fragments below 20 kDa (Fig. 2B).

As a control, the cysteine protease inhibitor E64 was added at the beginning of the incubation in order to inhibit calpain. This resulted in an α-crystallin preparation which showed only minor proteolysis (Fig. 2A, lane 7 and Fig. 2B), resembling normal α-crystallin. The α-crystallin preparation resulting from incubation of α-crystallin with calpain showed markedly reduced chaperone activity (calpain curve, Fig. 4B). This was compared with the chaperone activity of α-crystallin incubated with calpain inhibited by E64 (E64 curve, Fig. 4B), which retained effective chaperone activity.

Unlike βL-crystallin, normal intact α-crystallin showed no appreciable increase in light scattering when heated to 64 °C. However, the α-crystallin incubated with calpain showed increased light scattering when heated (data not shown). Some loss of thermal stability was also noted for α-crystallin from the cataractous nucleus of rats with selenite cataract.

Nature of α-Crystallin Cleavage—In order to gain insight into specific changes occurring on α-crystallin leading to loss of chaperone activity, control soluble protein from rat lens, and soluble protein incubated with calpain II were separated by two-dimensional electrophoresis, and the polypeptides were electrotransferred onto PVDF membranes. Since αA and αB were NH2-terminally blocked, the identity of the intact αA and αB polypeptides (Fig. 5A) was confirmed by in situ trypsinization on the membrane surface and separation of the resulting tryptic fragments by HPLC (Fig. 6, αA and αB). Sequence analysis of two of the resulting peaks confirmed the identity of these polypeptides as αA and αB. A peak eluting at 48 min from the αA digest (Fig. 6, αA) was subjected to eight cycles of amino acid sequence analysis. The peak contained two polypeptides with the sequences ALGPFYPS and APSXIDTGLS. This sequence corresponded to residues 57–66 of hamster αB (25). The identity of the residue marked X could not be determined because of histidine at this position. Mass spectroscopy confirmed the identity of these fragments and detected peptides with the masses of 1006.5 and 1171.5 in this peak. These masses differed from the predicted masses of residues 13–20 and 79–86 of αA by 0.0 and 0.9 atomic mass units, respectively. A peak eluting at 56 min from the αB digest (Fig. 6, αB) contained a fragment with the sequence APSXIDTGLS. This sequence corresponded to residues 57–66 of hamster αB (25). The identity of the residue marked X could not be determined because of the presence of tryptophan at this position. Following incubation with calpain, the spots corresponding to degraded αA and αB decreased in concentration and several fragments appeared which were tentatively identified as αA and αB breakdown products (Fig. 5B, αA-1, αA-2, αA-3, and αB-1). These partially degraded polypeptides were trypsinized on the membrane surface and the resulting fragments were separated by HPLC (Fig. 6, αA-1, αA-2, αA-3, and αB-1). The tryptic maps of αA-1, αA-2, and αA-3 were similar to the tryptic map of undegraded αA. Similarly, the tryptic map of the partially degraded polypeptide αB-1 was similar to the tryptic map of undegraded αB. This confirmed that the polypeptides αA-1, αA-2, and αA-3 in Fig. 5B were derived from partial cleavage of αA and that the polypeptide αB-1 was derived from partial cleavage of αB. NH2-terminal sequence analysis of polypeptides αA-1, αA-2, αA-3, and αB-1 indicated that all remained NH2-terminally blocked. This is consistent with earlier reports that calpain cleaved αA and αB at their COOH terminus (26). Polypeptides migrating to the same positions as αA-1, αA-2, αA-3, and αB-1 were also found in the nucleus of rats with selenite cataract (Fig. 5C). Therefore, the loss of residues on the COOH terminus of αA and αB was related to loss of chaperone activity, both in vitro following calpain incubation, and in vivo following cataract formation.

DISCUSSION

The major findings in the present study were that chaperone activity of α-crystallin isolated from selenite cataract was reduced and that the lens protease calpain may be responsible for the diminished chaperone activity. We hypothesize that α-crystallins may stabilize β-crystallins and thereby help maintain lens transparency. Previous work has shown that α-
Several pieces of evidence from the present investigation support the hypothesis that calpain II is probably responsible for proteolysis and the loss of chaperone activity in selenite cataract. First, incubation of intact α-crystallin with calpain markedly reduced chaperone function, and addition of E64, a calpain inhibitor, blocked this effect (Fig. 4B). Second, the pattern of limited proteolysis of α-crystallins from selenite cataract was similar to that produced by incubation of calpain with α-crystallin in vitro. In both cases, the proteolysis was characterized by decreases in αA and αB crystallin polypeptides and appearance of three fragments of αA and one fragment of αB that migrated similarly during two-dimensional electrophoresis (Fig. 5).

Calpain appeared to decrease chaperone activity in a manner similar to that occurring with aging by removal of a portion of the COOH terminus of α-crystallins (10, 27). The three partially degraded αA and one partially degraded αB fragments remained NH₂-terminally blocked following calpain proteolysis. To obtain these fragments, a 1–2-kDa decrease in molecular mass must have occurred by removal of a portion of the COOH terminus. Yoshida et al. (26) found that calpain caused limited degradation of bovine α-crystallins by cleaving αA at the carboxyl side of residues 162 and 163 and αB at the carboxyl side of residues 163 and 170. This suggested that removal of 11–12 residues or less at the COOH terminus of αA and αB would decrease chaperone activity. Limited digestion with trypsin, in a separate study, also suggested that removal of 16 and 12 residues from the COOH terminus of αA and αB, respectively, caused loss of chaperone activity (28). The reason why removal of such a limited number of residues produces loss of chaperone function is unknown. It is possible that removal of the COOH terminus causes a loss of chaperone activity by destabilizing α-crystallin aggregates.

To our knowledge, these are the first data to show that chaperone function is reduced in a model of cataract and to attribute the effect to a specific enzyme found in the lens. Activity of calpain II in young rat lens is much higher than in humans (29). Therefore, partial degradation of α-crystallin in human lenses during aging and cataract could involve a protease other than calpain or a mechanism other than proteolysis. The present data, however, do confirm that a loss of chaperone activity accompanies cataract formation in an animal model. They also provide a rationale to determine if a similar process occurs during human cataract.

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REFERENCES


![HPLC separation of tryptic fragments of αA- and αB-crystallins and their partial degradation products produced by calpain. Polypeptides marked αA and αB in Fig. 5A, and αA-1, αA-2, αA-3, and αB-1 in Fig. 5B, were transferred to PVDF membrane. The polypeptide spots were excised, trypsinized on the membrane surface, and the resulting fragments were separated by HPLC. Intact αA and αB polypeptides were identified by partial amino acid sequence of the indicated tryptic fragments. Fragments with the sequence ALGPFYPS and XFSPQDLT corresponding to residues 57–66 of hamster αB (25) were present in the peak eluting at 56 min. The residue indicated by the asterisk denotes the tryptic peak, and the numbers on the right side of each chromatogram are the absorbance values of the largest peak, excluding the tryptic peak.](image)
Reduction of Chaperone Activity by Calpain II