**Sequence Analysis of Lens β-Crystallins Suggests Involvement of Calpain in Cataract Formation**

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Abnormal activation of the protease calpain in the lens may be a cause of cataracts. Cataracts were induced in 10-day-old rats by a single overdose of sodium selenite. The water-insoluble protein from the opaque lens nucleus was separated by two-dimensional electrophoresis, electroblotted onto membranes, and the NH2-terminal sequence of partially degraded β-crystallin polypeptides determined. Selenite cataractous lenses contained four major structural proteins, BB1, βB3, βA3/A1, and βA4 crystallins, missing from 5 to 49 amino acids from their NH2 termini. Incubation of intact β-crystallins with calpain II in vitro produced identical cleavage sites. This provided further evidence for the role of calpain in the production of light scattering protein in cataractous lenses and also suggested that a similar process may lead to lens protein insolubilization during aging.

Calpains are non-lysosomal calcium-dependent cysteine proteases found in nearly all animal tissues (1). Their normal functions in cells remain speculative. However, they likely function in specific and limited proteolytic events which alter cellular metabolism and structure, rather than in general protein turnover.

Three pieces of evidence support the idea that partial degradation of lens proteins by calpain is a cause of opacification and cataract in rat lens. 1) Lenses with experimental cataract, induced by a single overdose of selenite to young rats, underwent elevation in free calcium concentrations high enough to activate calpain II (2). These lenses contained partially degraded proteins with molecular weights similar to those produced by purified calpain II (3, 4). 2) Lenses cultured in the presence of cataract inducing agents such as the calcium ionophore A23187, selenite, diamide, and xylose underwent elevation in free calcium concentrations high enough to activate calpain I1 (2). These lenses contained NH2-terminal cleavage sites identical to those produced by calpain. The results further supported the role of calpain activation in cataracts and provided some of the most convincing evidence to date that loss of calpain regulation may occur during pathology.

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**MATERIALS AND METHODS**

**Incubation of β-Crystallin Aggregate with Calpain II**—The high molecular weight form of β-crystallin aggregate (βA4) was selected for incubation with calpain II because it contains all seven β-crystallin polypeptides. βA4 was isolated from the soluble protein of whole lenses of 12-day-old rats using gel filtration (9). Calpain II from rat skeletal muscle was purified as previously described (8). Proteolysis was carried out for 60 min at 30 °C in an incubation mixture containing 2 mg/ml βA4, 90 units (0.3 mg) calpain/ml, 20 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithioerythritol, 0.5 mM EGTA, 0.5 mM EDTA, and 3.0 mM CaCl2. Proteolysis was stopped by addition of excess EDTA, and samples were dried by vacuum centrifugation.

**Isolation of Insoluble Protein from Cataractous Lenses**—Cataracts were induced by a single subcutaneous injection of 30 μmol of sodium selenite/kg body weight into 10-day-old rat pups. Animals were sacrificed 4 days post injection following formation of dense nuclear opacities. Lenses from 14-day-old control and cataractous rats were dissected into cortical and nuclear regions as before (3), and 20 lens regions/ml were homogenized in 20 mM Tris, pH 7.5, 1.0 mM EDTA, and 1.0 mM EGTA. Insoluble proteins were isolated by centrifugation at 10,000 × g for 30 min at 4 °C, and the pellet was suspended and centrifuged again to remove soluble fractions. The pellet was then resuspended in water, a portion was assayed for protein content (bicinchoninic acid assay, Pierce Chemical Co.), and the remaining portion was dried by centrifugal evaporation.

**Two-dimensional Electrophoresis and Sequence Analysis**—Proteins were separated by two-dimensional electrophoresis (100 μg gel) as previously described (9), except that 3500 volt h, 1.5-mm × 12-cm gels, and 3.5-10 amphotole (Pharmacia LKB Biotechnology Inc.) were used for the first dimension, and a model SE 600 electrophoresis unit (Hoefer), and low molecular weight SDS-polyacrylamide gel electrophoresis standards (Bio-Rad) were used during the second dimension. Gels were electroblotted onto polyvinylidene difluoride membrane (Millipore) at 100 V for 45 min at 15 °C using a TE 42 blotting apparatus (Hoefer). Polypeptides were then stained, cut out, and material from one to three gels subjected to six cycles of NH2-terminal sequence analysis using a model 477 protein-sequenator and an on-line model 120 phenylthiohydantoin analyzer (Applied Biosystems) (10).

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This was identified as pA3 missing 11 amino acids from its NH₂ terminus. The seven major pH-crystallin subunits can be observed. When the crystallin aggregate incubated with purified calpain I1 and no calcium, crystallin aggregates. Panel polypeptide and cleavage sites identified as summarized in Table I. Only the portion of gels containing crystallin polypeptides is shown.

| Polypeptide  | NH₂-terminus | Identity  | NH₂-terminus | Identity  |
|--------------|--------------|-----------|--------------|-----------|
| 1            | Blocked      | Blocked   | Blocked      | Blocked   |
| 2            | GAPPEQA      | βB3 (~5)  | GAPPEQA      | βB3 (~5)  |
| 3            | GGLGGS       | βB3 (~17) | GGLGGS       | βB3 (~17) |
| 4            | TLPTTK       | βA3 (~11) | TLPTTK       | βA3 (~11) |
| 5            | MAQTNP       | βA3/βA1'  | TNPMPG       | βA3/βA1   |
| 6            | NPMPS        | (–17, –20)  | NPMPS        | (~20, 21) |
| 7            | Blocked      | Unknown   | Unknown      |           |
| 8            | ELPPGS       | βB1 (~49) | ELPPGS       | βB1 (~49) |
| 9            | TAPAPG       | βB1 (~27) | TAPAPG       | βB1 (~27) |

- Polypeptide numbers correspond to numbered polypeptides shown in Figs. 1B and 2B.
- The identity of the parent polypeptide and number of amino acids missing from the NH₂ terminus (parentheses) were determined by matching the sequences of the six NH₂-terminal amino acids to published sequences of whole βB1, βB3, βA1/βA3, and βA4-crystallin polypeptides (16–18). Similar cleavage sites suggested that calpain was activated during cataract formation.
- βA3 and βA1 are translated from the same mRNA, except βA3 contains 17 extra amino acids at its NH₂ terminus due to the use of an alternate AUG start codon (18). Therefore, if 17 or more amino acids were removed it was uncertain if the parent polypeptide was βA3 or βA1. In these cases the parent polypeptide was designated βA3/βA1.
- βA3/βA1 (~21, Asp) designates a polypeptide unique to cataracts identified as βA3/βA1 missing 21 amino acids from the NH₂ terminus and containing an NH₂-terminal deamidated asparagine.
- The NH₂-terminal amino acid of polypeptide 7 (labeled X) could not be determined due to the presence of cysteine at residue 19 in βA4.

Fig. 1. Two-dimensional electrophoresis of lens β-crystallin aggregates. Panel A, control group containing lens soluble β-crystallin aggregate incubated with purified calpain II and no calcium. The seven major β-crystallin subunits can be observed. When the NH₂ termini of these polypeptides were sequenced, all appeared blocked, with the exception of the polypeptide marked with an arrow. This was identified as pA3 missing 11 amino acids from its NH₂ terminus (18). Panel B, lens-soluble β-crystallin aggregate incubated with purified calpain II and calcium. Numbers refer to partially degraded β-crystallin polypeptides which were sequenced and parent polypeptide and cleavage sites identified as summarized in Table I. Only the portion of gels containing crystallin polypeptides is shown.

Fig. 2. Two-dimensional electrophoresis of insoluble protein from the nuclear region of lens. Panel A, insoluble proteins from the lens central nuclear region of normal 14-day-old rats. In addition to the higher molecular weight, β-crystallin polypeptides, the insoluble protein from the nuclear region of control lenses was also composed of α- and γ-crystallin polypeptides (9) (boxed regions). Panel B, insoluble proteins from the cataractous lens central nuclear region of 14-day-old rats, 4 days following a single dose of selenite to induce cataracts. Numbers refer to partially degraded β-crystallin polypeptides which were sequenced and parent polypeptide and cleavage sites identified as summarized in Table I. α-Crystallins were also partially degraded during cataract formation (boxed region) but did not contribute significantly to the composition of the insoluble protein (9).

RESULTS

Compared to intact βB1-crystallins (Fig. 1A), calpain II in vitro produced nine partially cleaved β-crystallin fragments (Fig. 1B). NH₂-terminal sequence analysis allowed identification of these partially degraded polypeptides. Calpain removed 5 to 49 amino acids from the NH₂ terminus of βB1, βB3, βA1/βA3, and βA4 polypeptides (Table I). These partially degraded polypeptides were mostly absent from non-calpainized lens proteins (Fig. 1A). An exception was a polypeptide corresponding to βA3/βA1 missing 11 amino acids from its NH₂ terminus (Fig. 1A, arrow). This partially degraded polypeptide was normally present in the lens of 14-day-old animals, but the concentration was increased by calpain incubation (Fig. 1B, 4). However, all major non-calpainized β-crystallin polypeptides shown in Fig. 1A appeared blocked. This was consistent with earlier reports that intact bovine β-crystallin polypeptides are NH₂ terminally blocked (11). Surprisingly, evidence for cleavage of βB2, a major β-crystallin polypeptide in lens, was not found. The apparent molecular weights of the cleaved β-polypeptides suggested that calpain cleaved at only the NH₂ terminus. Whether calpain also removed small regions of the COOH terminus will require determination of the exact molecular weight by mass spectrometry.

We next determined if the same NH₂-terminal cleavage sites were found in lenses undergoing cataract formation following an overdose of selenite. The proteins from the central opaque region of cataractous lenses (Fig. 2B) contained new polypeptides which migrated to positions similar to partially degraded βH-crystallins produced by calpain in
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**B3**

Met-Ala-Glu-Gln-His-Gly-Ala-Pro-Glu-Gln-Ala-Ala-Ala-Lys-Ser-His-Gly-Leu-Gly-Gly-Ser-Globular Domains

**A3/A1**

Met-Glu-Thr-Gln-Thr-Glu-Leu-Glu-Thr-Leu-Pro-Thr-Thr-Lys-Met-Ala-Gln-Thr-Ala-Pro-Met-Pro-Gly-Ser-Leu-Gly-Pro-Globular Domains

**B1**

Met-Ser-Gly-Met-Phe-Ser-Gly-Ser-Ile-Ser-Glu-Thr-Ser-Gly-Met-Ser-Leu-Gln-Cys-Thr-Lys-Ser-Ala-Gly-Globular Domains

**Fig. 3.** The amino acid sequences of the NH$_2$ termini extending from the globular domains of the four $\beta$-crystallin polypeptides cleaved by calpain. Arrowheads indicate calpain cleavage sites produced in vitro which are identical to cleavage sites occurring in selenite overdose cataracts. The sequences of $\beta$B3 and $\beta$B1 are from rat (17), $\beta$A3/A1 from mouse (18), and $\beta$A4 from cow (16).

vitro (Fig. 1B). These polypeptides were largely absent from insoluble proteins of normal lens from 14-day-old rats (Fig. 2A), and compared to the lens soluble protein fraction, were most abundant in the insoluble fraction of cataractous lenses. When sequenced, eight of these polypeptides contained the same shortened NH$_2$ terminus as found in $\beta$-crystallin polypeptides incubated with calpain II (Table I, Fig. 3). Unique to cataractous lenses was the appearance of a shortened $\beta$A3/A1 polypeptide containing an NH$_2$-terminal deaminated asparagine. This polypeptide was not detected following calpain incubation in vitro.

**DISCUSSION**

The similarity between sites where $\beta$-crystallin polypeptides were cleaved by calpain and where cleavage occurred during cataract suggested that calpain was responsible for the majority of the proteolysis observed during formation of selenite-induced cataract. The probability that a protease other than calpain could produce identical cleavage sites appeared remote.

As in other studies of calpain cleavage sites (12), the amino acids at the cleavage sites in the present study were quite variable, suggesting that calpain cleaved due to conformation or accessibility rather than recognition of sequence. The locations of the cleavage sites were all within nonconserved regions of varying length found at the NH$_2$ terminus of $\beta$-crystallin polypeptides (Fig. 3). These regions extend beyond the more highly conserved globular domains, and their function remains unknown. It has been postulated that they may contain interaction sites that stabilize oligomerization of $\beta$-crystallin polypeptides (13). However, crystallographic analysis indicates that $\beta$B2 extensions in $\beta$B2 dimers do not occupy fixed positions (14). This suggested that NH$_2$-terminal extensions do not contribute to dimer formation, and may have a random structure. This random structure may be the property that targets the NH$_2$-terminal extensions for cleavage by calpain. The only similarity recognized in cleavage sites was in $\beta$B1, $\beta$B3, and $\beta$A4. In these polypeptides, the NH$_2$ termini were removed 6 residues from the start of the globular domains (Fig. 3). In $\beta$A3/A1 the innermost cleavage was 9 residues from the globular domain, perhaps due to proline at the preferred site.

How removal of the NH$_2$ terminus of these proteins by calpain induces insolubilization remains unknown (8). However, this process may not be restricted only to selenite-induced cataracts. Recently, insoluble lens proteins with electrophoretic patterns similar to the partially degraded $\beta$-crystallin polypeptides characterized in the present study were observed in 7-day-old cataractous mouse lenses following injection of the whole animal with buthionine sulfoximine, an inhibitor of glutathione synthesis. Insoluble proteins from the nuclear region of normal rats greater than 14 days of age also contained significant amounts of these partially degraded $\beta$-crystallin polypeptides. Note that these polypeptides were in low concentration but could still be observed in the insoluble protein of the lens nuclear region from 14-day-old control rats (Fig. 2A). If a cataractogenic agent were given to rats older than 14 days, the appearance of the partially degraded polypeptides would not have been observed because they would have also been present in age matched control lenses.

We hypothesize that proteolysis by calpain contributes to the slow insolubilization of crystallins that occurs with normal aging (15) and that cataract formation results from calpain-induced insolubilization of crystallins that occurs too rapidly. It is unknown if a similar process may occur during aging and cataract formation in humans since partially degraded crystallins corresponding to calpain cleavage produces have never been measured in human lens.

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