Formation of a 55 000-Weight Cross-Linked β Crystallin Dimer in the Ca^{2+}-Treated Lens. A Model for Cataract†

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ABSTRACT: Incubation of lens in Ca^{2+}-containing media, considered by several investigators to be a useful model of cataract formation, gave rise to significant alterations in the covalent structures of various proteins. In rabbit lens, when sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used after reduction of disulfides in urea, the most readily observable changes were (i) disappearance of 210K, 95K, and 60K proteins, (ii) modifications of α crystallin subunits, (iii) alterations of β_H crystallins, and (iv) de novo production of 55K and higher molecular weight polymers. The addition of leupeptin inhibited the disappearances of 210K, 95K, and 60K proteins and the alteration of α crystallins, suggesting that all these were caused by a Ca^{2+}-activated protease. The proteolytically sensitive 60K species was identified as vimentin, a component of intermediate filaments. Formation of the 55K material and of higher molecular weight polymers during Ca^{2+} treatment of the lens could be prevented by histamine, a compound known to inhibit the transglutaminase-mediated cross-linking of proteins by ε-(γ-glutamyl)lysine peptide bonds in other biological systems. It could also be shown by immunoblotting that an antibody raised against the 55K material reacted selectively with β crystallins of normal lens. This indicates that the 55K product is in all likelihood an essential intermediate toward higher polymers and that the 55K product is a cross-linked dimer of certain polypeptides of β crystallin. The present findings are in full accord with earlier results from this laboratory [Lorand, L., Hsu, L. K. H., Siefring, G. E., Jr., & Rafferty, N. S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1356–1360] that β crystallin subunits are the main endogenous amine acceptor substrates for lens transglutaminase.

Two experimental systems proved to be particularly useful in studying posttranslational alterations of protein structures in relation to phenomena of cell aging. One is the human erythrocyte, treated with Ca^{2+} in the presence of ionophore A23187 (Lorand et al., 1976; Siefring et al., 1978); the other is the tissue culture keratinocyte, treated with high-salt solution or ionophore (Rice & Green, 1979). Since these treatments are thought to mobilize Ca^{2+} ions from cellular stores in the keratinocyte, elevation in the concentration of free Ca^{2+} appears to be the common denominator. This is in accord with measurements that senescent erythrocytes have a Ca^{2+} content as high as 2×10^{-4} M (Eaton et al., 1973; Palek, 1973). In the human erythrocyte, such a concentration of Ca^{2+} is sufficient to trigger transglutaminase-catalyzed cross-linking reactions (Lorand et al., 1978) as well as some proteolytic events (Lorand et al., 1983), whereas in keratinocytes only cross-linking could be detected thus far (Simon & Green, 1984). At the molecular level there are a number of differences between the responses of the two cellular systems. Activation of the intrinsic transglutaminase in the human erythrocyte generates cross-linked clusters comprising a number of membrane proteins [band 3, spectrin, band 2.1 (ankyrin), and band 4.1; Bjerrum et al., 1981] and some cytoplasmic components (hemoglobin and catalase; O. J. Bjerrum, M. Hawkins, M. Griffin, and L. Lorand, unpublished results), whereas in keratinocytes, it causes the formation of a cornifying envelope underneath the plasma membrane. Though several proteins may actually contribute to this fused polymeric structure, a special role was suggested for the cytoplasmic envelope precursor, called involucrin, as the substrate (Green, 1979; Watt & Green, 1981). Significant degradation of two major transmembrane proteins (band 3 and glycophorin) was observed in Ca^{2+}-enriched human erythrocytes in the presence of either ionophore A23187 (Lorand et al., 1983) or the calmodulin inhibitors stelazine and R24571 (Michalska et al., 1984).

This work represents a study of another model of cell aging: the alteration of protein structures in rabbit lens following incubation in Ca^{2+}-containing media. Such a treatment is known to result in the opacification of lens. Thus, this system has been used to study cataract formation [e.g., Hightower & Dering (1984), Hightower & Reddy (1982a), and Clark et al. (1980)]. Using inhibitors that differentiate between transglutaminase-mediated cross-linking and proteolytic reactions, we were able to show that both protein-modifying phenomena occurred in the Ca^{2+}-treated lens.[1]

Materials and Methods

Reagent-grade chemicals were obtained from the following suppliers: sodium chloride, calcium chloride, sodium acetate, sodium metabisulfite, methanol, and sodium phosphate from Mallinckrodt (St. Louis, MO); magnesium chloride and sodium azide from Fisher (Pittsburgh, PA); amido black, ethylenediaminetetraacetic acid, tris(hydroxymethyl)aminomethane, glycine, histamine dihydrochloride, Coomassie Brilliant Blue, bromophenol blue, 3-amino-9-ethylcarbazole, Tween-20, and dithiothreitol from Sigma (St. Louis, MO); sucrose from Schwarz/Mann (Spring Valley, NY); sodium dodecyl sulfate and urea from Aldrich (Milwaukee, WI); acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine, and ammonium persulfate from Bio-Rad (Richmond, CA); molecular weight standards from Sigma and Bio-Rad.

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[1] For preliminary reports, see Conrad et al. (1983, 1984).

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Leupeptin was a gift from the U.S.-Japan Cooperative Cancer Research Program. Vimentin (decamin), purified from baby hamster kidney HK 21 (BHK) cells, was a gift from Dr. R. Goldman of Northwestern University Medical School, as was the rabbit antiserum to this intermediate filament protein (Green & Goldman, 1983).

Frozen lenses from young rabbits were obtained from Pel-Freez (Rogers, AK); they were stored at −20 °C and were thawed just before use. Fresh lenses were excised from male albino rabbits after sacrificing the animals by intraperitoneal injection (1 mL/2 kg of body weight) of 10% pentobarbital sodium (Holmes Serum Co., Inc., Springfield, IL); the lenses were immediately placed on ice, rinsed with a solution of 50 mM Tris-HCl–100 mM NaCl at pH 7.4, and used for experiment without decapsulation.[2]

When preincubation was required, each lens was immersed separately for 1 h at 37 °C in 500 μL of 50 mM Tris-HCl–100 mM NaCl buffer of pH 7.4 that at times, contained 1.2 mM leupeptin. In studies using histamine (75 mM), the NaCl concentration of the medium was reduced. At the end of the 1-h period, 100 μL of the 50 mM Tris-HCl–100 mM NaCl buffer was added that depending on the experiment, contained 50 mM $CaCl_2$, 50 mM $MgCl_2$, or 10 mM EDTA. Incubation at 37 °C was then continued for periods up to 20 h.

In the absence of preincubation, each lens was immersed at 37 °C up to 20 h in 600 μL of 50 mM Tris-HCl–100 mM NaCl, pH 7.4, containing either 8.3 mM $CaCl_2$ or $MgCl_2$ or 1.7 mM EDTA.

Changes in the behavior of protein chains heavier than 33K could be examined simply by electrophoresis, following solubilization of lens samples at the end of the incubation periods. Each lens was solubilized in a 4-mL solution, containing 20 mM sodium phosphate, 9 M urea, 40 mM DTT, and 2% SDS, pH 7.1, by warming for about 2 h at 37 °C. Insoluble material was removed by filtration through a 0.45-μm HAWP membrane (Millipore Corp., Bedford, MA). Urea solutions were always prepared fresh from analytical-grade urea and were freed from cyanate by passing 50 mL of a 10 M solution through a Bio-Rad AG501-X8 column at room temperature at a flow rate of about 60 mL/h (White, 1967).

SDS–PAGE was performed with the discontinuous buffer system of Laemmli (1970) on 1.5 mm thick gels in a Bio-Rad Protean slab gel apparatus. A stacking gel of 3% acrylamide, a resolving gel of 10% acrylamide, and a running buffer of 50 mM Tris-HCl, 0.38 M glycine, and 0.1% SDS (pH 8.6) were employed, and 400–750 μg of protein samples per lane was applied. Protein determinations were carried out with the Bio-Rad Coomassie Blue dye binding assay (Bradford, 1976). Gels were stained with Coomassie Brilliant Blue and were calibrated with either Bio-Rad or Sigma molecular weight (M_r) standards of β-lactoglobulin (14.2K), lysozyme (14.3K), soybean trypsin inhibitor (20.1–21.4K), phenylmethanesulfonyl fluoride treated trypsinogen (24K), carbonic anhydrase (29–31K), glyceraldehyde-3-phosphate dehydrogenase (36K), ovalbumin (45K), bovine serum albumin (66K), phosphorylase B (92.5–97.4K), β-galactosidase (116K), and myosin (205K).

In order to analyze changes affecting the covalent structures of crystallins with chain weights lighter than approximately 40K, it was necessary to follow a more elaborate procedure that required the preparation of an aqueous extract from each lens, fractionation of this extract by chromatography on Sepharose 6B, and finally, examination of selected fractions by SDS–PAGE. Thus, following appropriate incubation, the lens was transferred to a 2.5-mL solution that contained 50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, and 5 mM DTT at pH 7.4 and was homogenized by four consecutive bursts (700 rpm, 10-s duration each, 4 °C) in the Potter-Elvehjem apparatus. Following centrifugation (20000g × 30 min), 1 mL of the supernatant was applied at 4 °C to a Sepharose 6B column (1.6 × 96 cm) by using the homogenization buffer as effluent with a flow rate near 6 mL/h (Garber & Gold, 1982). Protein was monitored by measuring absorbency at 280 nm on small aliquots. Selected fractions were then processed for electrophoresis by taking 50-μL fractions and treating them with 50 μL of 20 mM sodium phosphate, 9 M urea, 40 mM DTT, and 2% SDS, pH 7.1, at 37 °C for about 2 h. Electrophoresis was again carried out according to Laemmli (1970) but with 3.5% polyacrylamide in the stacking and 12% polyacrylamide in the running gels. Protein loads of 10–50 μg/lane were applied.

To prepare the polyclonal antibody to the 55K protein, frozen rabbit lens was incubated with Ca^{2+} (37 °C, 20 h), solubilized, and subjected to SDS–PAGE in double thick (3-mm) 10% gels, applying approximately 10 mg of material to each gel. After Coomassie Blue staining, the 55K band was cut out and a fine suspension of the gel was made in 0.15 M NaCl and 5 mM sodium phosphate, pH 8.0 (PBS), by using two bursts (5 s each) of a Willems Polytron (Model PT20). The material was dialyzed overnight against PBS and stored at −20 °C. A single gel provided a total of 4 mL of material.

The polyclonal antiserum was prepared by subcutaneously injecting a New Zealand White rabbit (male, albino, ~2.5 kg; Lesser Rabbitory, Union Grove, WI) with 1 mL of the 55K protein gel suspension that was emulsified in 0.5 mL of PBS and 1.5 mL of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Booster shots were administered 8 and 12 weeks after the initial injection with a similar emulsion made with Freund's incomplete adjuvant. The rabbit was bled by ear puncture 2 weeks after the third injection and every 4 weeks thereafter, with a booster shot applied 2 weeks prior to each bleeding. The blood was allowed to coagulate for 2 h at room temperature and overnight at 4 °C. Serum was obtained by centrifugation (3000g, 20 min, 4 °C) and stored at −20 °C. IgG was obtained by the method of Harboe & Ingild (1973).

For immunoblots, SDS–PAGE was carried out as previously described and the gels were transblotted onto nitrocellulose (0.2-μm pore size; Schleicher & Schuell, Keene, NH) with a Bio-Rad Trans-Blot apparatus. Transfers were essentially by the method of Towbin et al. (1979), using a transfer buffer of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3, and were run overnight at ~0.3 A and 10 °C. Amido black staining was performed with 0.2% amido black in 50% methanol and 10% acetic acid and destained with 25% methanol and 10% acetic acid. Immunoblotting was according to the method of Bjerrum et al. (1983), blocking unbound sites on the nitrocellulose with 2% (w/v) Tween-20, followed by overnight incubation with the primary antibody. The blot was then incubated for 2 h with peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins (Dako, Santa Barbara, CA) and the color developed with 3-amino-9-ethylcarbazole as the substrate.

Results

Changes Regarding Proteins with Chain Weights above 33K in Ca^{2+}-Treated Lens. The first lanes on panels A–C of Figure 1 show typical electrophoretic profiles obtained for lens proteins

[2] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-aminomethane; M_r, relative mass; K, 10^3; BHK cells, baby hamster kidney cells.

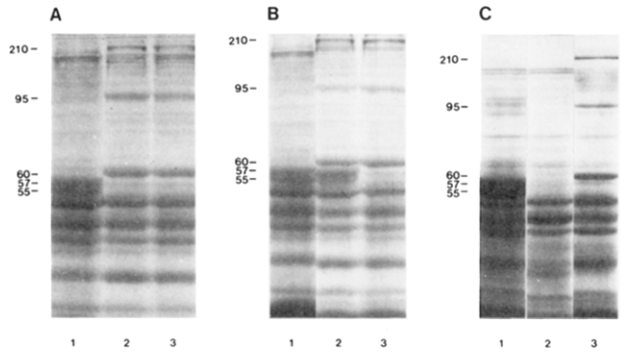


FIGURE 1: Changes in the protein profile of Ca^{2+}-treated lens, as shown by direct analysis with SDS–PAGE. Rabbit lenses (Pel-Freez) were incubated as described below. Following solubilization and reduction, SDS–PAGE was carried out in 10% gels, as described under Materials and Methods. $M_r \times 10^{-3}$ calibrations are shown on the left of each panel. Panel A: Lenses were incubated for 3 h at 37 °C in either 8.3 mM $CaCl_2$ (lane 1), 8.3 mM $MgCl_2$ (lane 2), or 1.7 mM EDTA (lane 3). Panel B: Lenses were preincubated at 37 °C for 1 h. The preparation in lane 2 contained 1.2 mM leupeptin. Then 8.3 mM $CaCl_2$ (lanes 1 and 2) or 1.7 mM EDTA (lane 3) was added for a further 3-h incubation at 37 °C. Panel C: Lenses were preincubated for 1 h. The preparation in lane 2 contained 75 mM histamine dihydrochloride. Then either 1.7 mM EDTA (sample in lane 3) or 8.3 mM $CaCl_2$ (lanes 1 and 2) was added for a further 20-h incubation.

in the 33–200K range following incubation of the tissue in Ca^{2+}-containing medium. A number of differences were evident between Ca^{2+}-treated lens and controls that were incubated with either Mg^{2+} (lane 2 of panel A) or EDTA (lanes 3 of panels A–C). In the 55–60K region, the Ca^{2+}-specific alterations proved to be very reproducible. Two major changes were observed: (i) a 60K band, present in the controls, disappeared; (ii) new 55K material, which could be frequently resolved into a doublet, was generated. Both changes were readily observable at a 2 mM concentration of Ca^{2+} in as short as 3 h of incubation.

Disparities in relative staining intensities suggested that the assumption that the 55K band arose from limited hydrolysis of the 60K band protein was not tenable. Furthermore, by addition of either leupeptin or histamine to the incubation medium prior to and during Ca^{2+} treatment, it could be shown that two unrelated chemical reactions took place. Leupeptin significantly inhibited the disappearance of the 60K protein in Ca^{2+}-treated lens but did not prevent the formation of the 55K band (panel B, lane 2). Conversely, when histamine was added to the medium (panel C, lane 2), only the formation of the 55K band, but not the disappearance of 60K, was inhibited.

In the upper regions of the gels, proteins of M_r's of 95K and 210K present in the controls (lane 2 in panel A; lanes 3 in panels A–C) were absent from the Ca^{2+}-treated lens samples (lanes 1 in panels A–C). Addition of leupeptin, as seen in lane 2 of panel B, had a protective effect, however, for both proteins during Ca^{2+} treatment.

The photographs of Figure 2, which pertain to experiments with freshly dissected lens, show the stacking gels so as to be able to document the fact that in the presence of leupeptin the formation of high molecular weight, non-disulfide-bonded polymers (marked X) was enhanced.

Immunological Identification of the 60K Protein as Vimentin. The intermediate filament component vimentin has a M_r of about 58K in most cellular systems, quite similar to the 60K protein in the rabbit lens that disappeared upon Ca^{2+} incubation unless leupeptin was also present. In order to probe the identity of the 60K rabbit lens protein, an authentic sample of BHK cell vimentin (lane 2, Figure 3) was electrophoresed

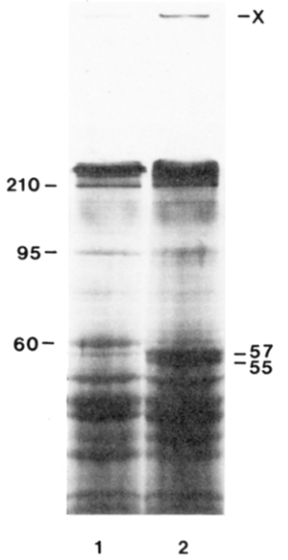


FIGURE 2: Leupeptin accentuated formation of high molecular weight polymers during Ca^{2+} treatment of lens. Freshly dissected lenses were preincubated for 1 h with 1.2 mM leupeptin. Then either 1.7 mM EDTA (lane 1) or 8.3 mM $CaCl_2$ (lane 2) was added for a further 20-h incubation. SDS–PAGE as in Figure 1. High molecular weight, non-disulfide-linked polymers on top of the stacking gel (lane 2) are marked X.

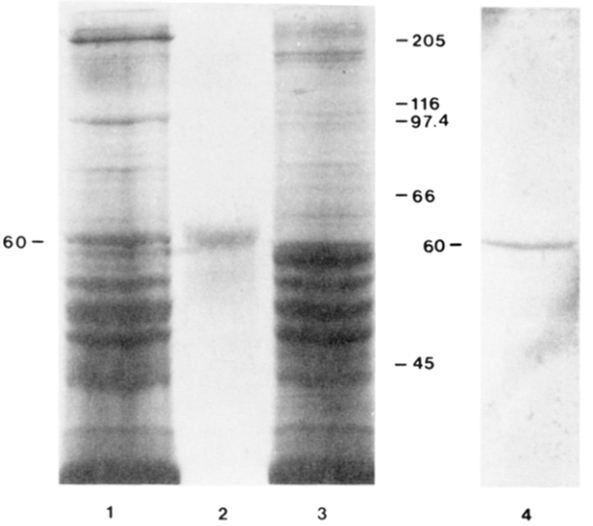


FIGURE 3: Identification of the 60K protein as vimentin. Freshly dissected lenses were incubated in either 1.7 mM EDTA (lane 1) or 8.3 mM $CaCl_2$ (lane 3) for 20 h. SDS–PAGE as in Figure 1. Lane 2 shows a parallel run with an authentic sample of vimentin. Lane 4 is the immunoblot of the gel corresponding to lane 1 by using specific rabbit antiserum against BHK cell vimentin.

in parallel with a control lens (lane 1) and a Ca^{2+}-treated specimen (lane 3). In the gel system employed, the migration of vimentin was rather similar to the 60K material that was present only in the control lens but not in the Ca^{2+}-treated one. More importantly, when a specific antibody against BHK vimentin was used, positive immune reaction for the 60K polypeptide (lane 4) could be obtained only by transblotting the control lens (as in lane 1) and not the Ca^{2+}-treated sample (as in lane 3).

Alterations in Structures of Crystallins during Exposure of Lens to Ca^{2+}. In order to examine changes in the state of aggregation of oligomeric crystallins, the low-salt extracts of individual lens homogenates were first passed through a Sepharose 6B gel filtration column, as described under Materials and Methods.

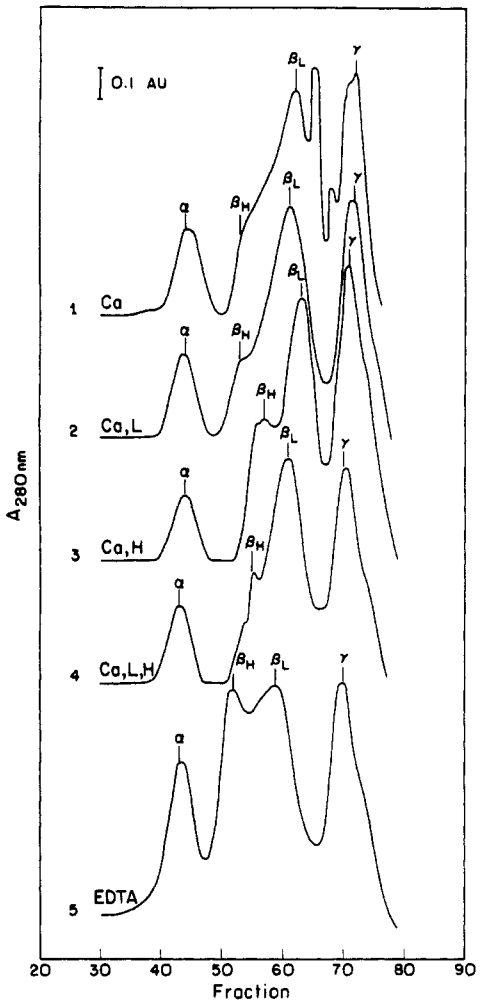


FIGURE 4: Isolation of crystallins from the low-salt extracts of lens preparations using gel filtration chromatography through Sepharose 6B. Individual lens samples (Pel-Freez) were treated for 1 h with 1.2 mM leupeptin (L) and/or 75 mM histamine (H) as marked. Then incubation for 20 h was carried out in the presence of either 8.3 mM $CaCl_2$ (Ca) or 1.7 mM EDTA. The lens samples were then homogenized and processed for gel filtration as described under Materials and Methods. The ordinate denotes absorbency values (AU) at 280 nm for the effluent, from which 2.2-mL fractions were collected. Positions of crystallins α, β_H, β_L, and γ are marked for each curve.

Elution profiles, presented in Figure 4, were rather similar in regard to α and γ crystallins. However, variations were found in the β crystallin region, relating mainly to the position and relative amount of β_H rather than the β_L fraction. It is interesting to point out that the gel filtration profile of crystallins in all experiments in which Ca^{2+}-treated lens was used (curves 1–4) was different from that of the EDTA control (curve 5). This was the case even when both leupeptin and histamine were present.

The peak fractions marked in Figure 4 were reduced with DTT in urea and analyzed by SDS–PAGE on 12% gels. Composite electrophoretic patterns are shown in Figure 5. Regardless of the conditions employed for the treatment of the lens, the γ crystallin bands remained invariant (see bottom right-hand panel in the figure). As far as stability was concerned, β_L crystallins (bottom left-hand panel) seemed to be next in resistance to calcium treatment. A shift in the positions of the β_L constituent units toward M_r's lower than in the control (lane 5) was seen only when the Ca^{2+} treatment was performed in the presence of histamine (lane 3). A similar observation was made in regard to α crystallins (top left-hand panel), where the degradation in Ca^{2+}-treated lens was accentuated by the presence of histamine (lane 3). A comparison of lanes 2 and 4 with lanes 1 and 3 for α, just as a comparison of lane 3 with lane 4 in the β_L panel, shows that leupeptin was effective in inhibiting the breakdown of both α and β_L crystallins.

Changes in the β_H crystallin fractions (top right-hand panel in Figure 5) were quite varied and difficult to interpret because they could not be blocked entirely even by the combined application of leupeptin and histamine when the lens was exposed to Ca^{2+} (compare lane 4 with the EDTA control in lane 5). The major difference between these two patterns was the diminution of 27K and 30K components in the controls and the appearance of a 33.5K band in the Ca^{2+} plus histamine treated lens. The approximately 23K and 21K components in β_H crystallin remained unchanged. The presence of histamine during Ca^{2+} treatment seemed to enhance proteolysis just as it did for α and β_L crystallins; lane 3 shows a prominent band of about 20K, smaller than found after any other treatment. A comparison between lanes 3 and 4 demonstrates that formation of this 20K material was blocked by the presence of leupeptin.

As described above in connection with the results given in Figure 1, a prominent and characteristic feature of Ca^{2+}-treated lens was the de novo generation of a new 55K species. Thus, it was of great interest that a protein with very similar mass could also be demonstrated to be associated with the β_H crystallin fraction of the lens that was incubated with Ca^{2+} and leupeptin (marked by the arrow in Figure 5, top right panel, lane 2). From the decrease of the relative staining intensity of the 27K component in this lane, the suggestion could be made that the 27K component was a building block for producing this material. As expected from the effect of histamine in blocking cross-linking, no 55K band was seen in the β_H crystallin fractions when the lens was treated with Ca^{2+} in the presence of histamine (top right panel, lanes 3 and 4).

Antibody to the Ca^{2+}-Induced 55K Material Cross-Reacts Specifically with β Crystallins. The finding that histamine could prevent formation of the 55K material in Ca^{2+}-treated lens (Figure 1, lane 2 of panel C) suggested that it arose by the cross-linking of smaller molecular weight units. Assuming that some immunological epitopes of the constitutive structures were conserved during cross-linking, it was thought to be possible to identify them by reactivity toward antibodies raised against the 55K material. We actually succeeded in obtaining an antiserum of suitably high titer to this cross-linked protein product. IgG's isolated from the antiserum were then used in immunoblotting experiments with the crystallin preparations from Figure 4. As seen in panel A, Figure 6, this antibody reacted primarily with the 30K component of β crystallins isolated from the control lens sample. With β crystallins from the Ca^{2+} plus leupeptin treated lens, however, there was an additional strong antibody-reactive band with the 55K material (panel B). The α and γ crystallin bands gave negative immunoblots with this specific antibody.

Discussion

The experiments presented in this paper show that treatment of lens in Ca^{2+}-containing media causes profound alterations in the covalent structures of several proteins. Very similar results were obtained when freshly excised rabbit lens rather than previously frozen specimens were used, and characteristic changes in the electrophoretic profile of proteins were readily noticeable by 3 h of incubation at 37 °C in a pH 7.5 buffer ($\mu \sim 0.15$), with approximately 2 mM of $CaCl_2$. Higher concentrations of Ca^{2+} (up to ca. 10 mM) and longer incu-

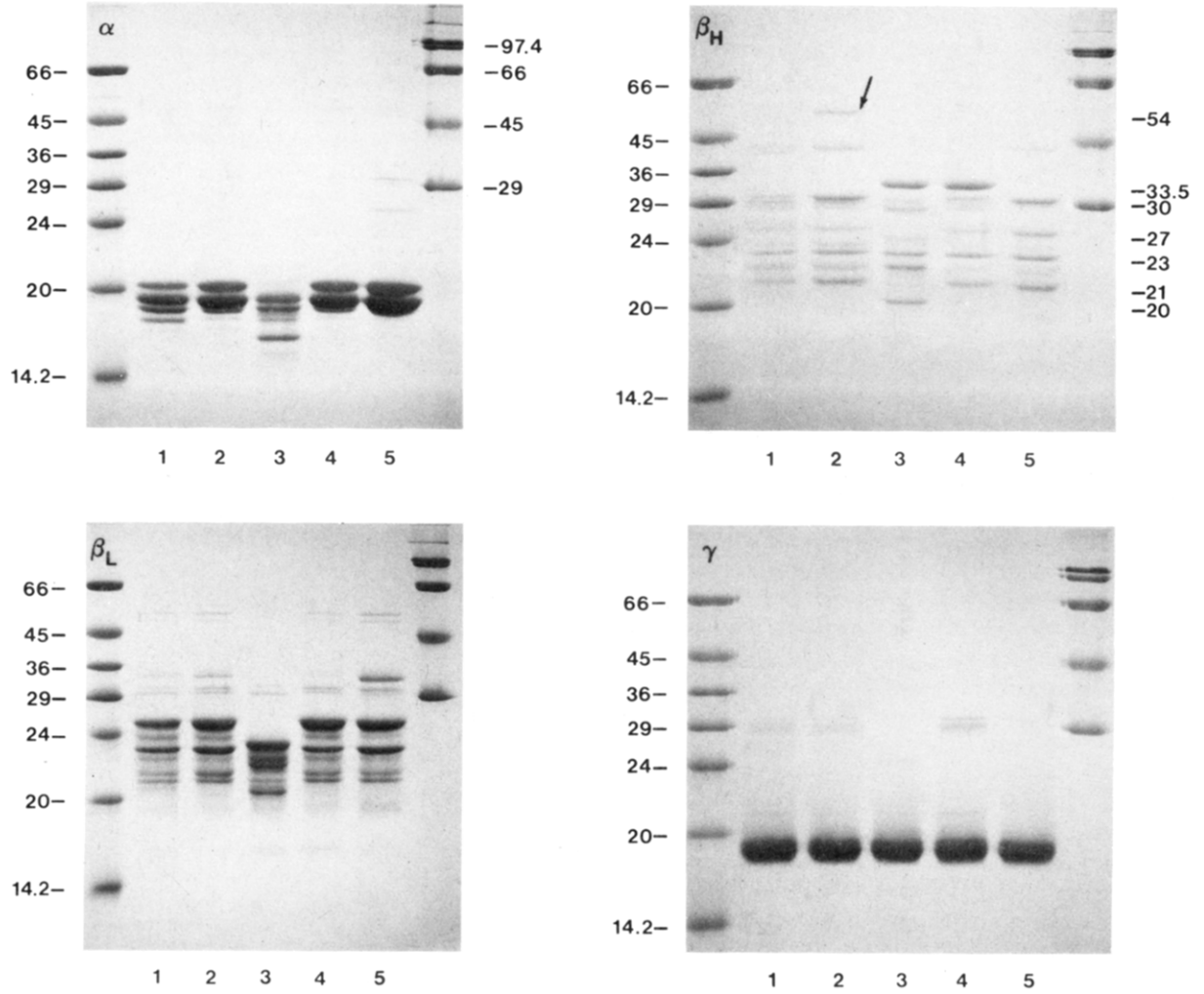


FIGURE 5: Electrophoretic analysis of the crystallin fractions of Ca^{2+}-treated lens. Fractions from the experiment in Figure 4, representing α, β_H, β_L, and γ crystallins, were examined by SDS–PAGE in 12% gels. Numbering of lanes in each panel corresponds to the chromatographic profiles of curves 1–5 in Figure 4. The arrowhead in lane 2 of the upper right panel marks the 55K cross-linked β crystallin species.

bation periods (up to 20 h) accentuated the protein changes even more.

Changes in lens proteins caused by treatment with Ca^{2+} fell essentially into the following categories: (a) disappearance of 210K, 95K, and 60K proteins; (b) changes in α crystallin units; (c) alterations of β_H crystallins, including the de novo appearance of a 55K material and the appearance of high molecular weight (>500K) polymers that did not enter the stacking and/or running gels.

Leupeptin, acetylleucylleucylargininal, is an inhibitor of trypsin-like enzymes and of some Ca^{2+}-activated, intracellular proteases (Umezawa, 1976; Malik et al., 1983). Thus, it was of interest to find that inclusion of this compound in the incubation medium greatly simplified the changes in the protein profile of Ca^{2+}-treated lens. Specifically, it prevented the fragmentation of α crystallin (lanes 1 and 2 in Figure 5, top left panel). Proteolysis of α crystallins has also been noted to occur in aged lens (Roy & Spector, 1976; van Kleef et al., 1976).

Leupeptin also protected the 210K, 95K, and 60K proteins from Ca^{2+}-activated proteolysis (Figure 1, panel B, lane 2). The latter material is thought to represent vimentin because, as shown in Figure 3, the two comigrated and cross-reacted immunologically. Vimentin has been identified as a component of intermediate filaments in the lens (Maisel & Perry, 1972; Rafferty & Goossens, 1978; Kibbelaar et al., 1979; Lieska et al., 1980; Geisler & Weber, 1980) and has been shown to be localized in the epithelial and cortical fiber cells; it is progressively lost from the deeper cortical cells and is absent from the nucleus (Ramaekers et al., 1980; Nasser et al., 1980; Ellis et al., 1984). Furthermore, in aqueous extracts of the lens, vimentin and some higher molecular weight proteins were found to undergo proteolysis in the presence of Ca^{2+} (Ireland & Maisel, 1983; Roy et al., 1984). Our experiments now show that the degradation of vimentin can also occur in the intact lens and that, more importantly, this can be prevented by the protease inhibitor: leupeptin. These findings also raise the possibility that vimentin might become degraded during cataract formation.

As in Ca^{2+}-enriched human red cell (Lorand et al., 1978), other major changes in Ca^{2+}-treated lens could be attributed to activating the intrinsic transglutaminase. The addition of a small molecular weight amine substrate of the enzyme histamine prevented the formation of the 55K species found in Ca^{2+}-treated lenses (Figure 1, panel C, lane 2). Histamine also inhibited the formation of large molecular weight polymers (marked X in Figure 2) that were particularly evident when leupeptin was added to the Ca^{2+}-containing incubation medium, suggesting that higher degrees of cross-linking by transglutaminase are favored by conditions when no proteolysis can occur. Previous research in regard to the involvement of transglutaminase in cataract (Lorand et al., 1981) focused on similar high molecular weight polymers, present only in pathological specimens.

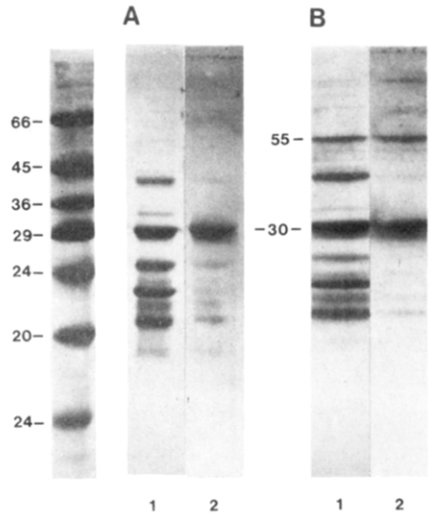
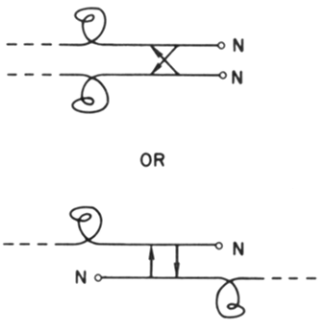


FIGURE 6: Antibody to the Ca^{2+}-induced 55K material cross-reacted specifically with β crystallins. SDS–PAGE gels of Sepharose 6B fractionated β_H crystallins from lenses, incubated with either Ca^{2+} and leupeptin or EDTA (see Figure 5, upper right panel, lanes 2 and 5), were transblotted onto nitrocellulose as described under Materials and Methods. Panel A represents the EDTA control; panel B is the Ca^{2+} and leupeptin treated lens. Lane 1 for each panel is the amido black stained nitrocellulose. Lane 2 for each panel is the immunoblot using the specific IgG produced against the 55K material from Ca^{2+}-treated rabbit lens.

From the inhibition experiments with histamine, it may be concluded that the 55K material is the primary and, in quantitative terms, probably the main intermediary product of cross-linking by transglutaminase in Ca^{2+}-treated lens. We raised an antibody against this protein and showed by immunoblotting that it cross-reacted selectively with β crystallin subunits of normal lens, its reaction with the 30K component being the strongest. Thus, it is reasonably certain that the 55K material represents cross-linked dimers of β crystallin subunits.

The finding that the 55K cross-linked product was related to β crystallin is fully in accord with earlier results (Lorand et al., 1981) that the M_r 26K and 30K subunits of β crystallin were the endogenous amine acceptor (i.e., acyl donor) substrates for transglutaminase in lens. Actually, formation of a 55K SDS–PAGE band could be readily demonstrated in experiments where β crystallin was allowed to react with transglutaminase in the presence of Ca^{2+} (L. Lorand, L. K. H. Hsu, and P. T. Velasco, unpublished results).

The γ-glutamine site-specific, transglutaminase-directed labeling of β crystallin subunits by extraneously added amine probes, such as [^{14}C]putrescine or dansylcadaverine (Lorand et al., 1981), opened the way for sequence work for delineating the cross-linking domains of the β crystallin molecules. This has recently been accomplished in bovine lens homogenates (Berbers et al., 1983) where amine incorporation was shown to take place near the N-terminal end of the βB_P chain at glutamine-7. Considering the fact that there are also lysine residues located in this region (Lys-10 and -17), the cross-linked β crystallin dimer described by us [see also Conrad et al. (1983)] could be generated by two reciprocal ϵ-(γ-glutamyl)lysine side chain bridges between the N-terminal segments of two β crystallin units. Measuring the frequency of ϵ-(γ-glutamyl)lysine cross-links in the 55K dimer of β crystallin will be needed to decide whether it is held together by one or two isopeptide bonds. Furthermore, the arrangement of the β crystallin units themselves in the cross-linked ensemble could be either parallel or antiparallel:



In some electrophoretic experiments, the cross-linked β crystallin dimer appeared as a doublet, resolvable into approximately 57K and 55K components, suggesting either that the β crystallin units that participate in the dimerization reaction are not identical or that the dimer may undergo minor proteolytic cleavages following cross-linking.

Some theories of cataract formation postulate that opacification of the lens arises from the aggregation of α crystallin units that, in vitro, would require about 10 mM Ca^{2+} (Jedziniak et al., 1973; Fein et al., 1979). Data regarding the concentration of free Ca^{2+} in cataract vary a great deal from investigator to investigator, and it is conceivable that not the bulk but the local, membrane-bound concentration of Ca^{2+} is important (Jedziniak et al., 1976; Hightower & Reddy, 1982b). Notwithstanding other cross-linking possibilities (Spector & Roy, 1978; Kramps et al., 1978; Stevens et al., 1978; Goosey et al., 1980; Garcia-Castineiras & Miranda-Rivera, 1983; Spector, 1984), when analyzing the problem of cataract formation, one must also take into account the fact that the activation of transglutaminase, in lens homogenates as well as in the intact lens, is brought about by Ca^{2+} concentrations 10–50-fold lower (Lorand et al., 1981) than would be necessary to cause aggregation of α crystallins. Thus, the transglutaminase-mediated cross-linking of β crystallins would take precedence over aggregation of α crystallins. An important question in regard to cataract formation then would be to ask how a change in state of the subunits of β crystallin to the ϵ-(γ-glutamyl)lysine cross-linked dimeric product would affect the packing and solubility of α crystallins.

Registry No. Ca, 7440-70-2; proteinase, 9001-92-7; transglutaminase, 80146-85-6; histamine, 51-45-6.

REFERENCES

Berbers, G. A. M., Bentlage, H. C. M., Brans, A. M. M., Bloemendal, H., & de Jong, W. W. (1983) *Eur. J. Biochem. 135*, 315–320.

Bjerrum, O. J., Hawkins, M., Swanson, P., Griffin, M., & Lorand, L. (1981) *J. Supramol. Struct. Cell Biochem. 16*, 289–301.

Bjerrum, O. J., Larsen, K. P., & Wilken, M. (1983) in *Modern Methods in Protein Chemistry—Review Articles* (Tschesche, H., Ed.) pp 79–124, de Gruyter, Berlin.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Clark, J. I., Mengel, L., Bagg, A., & Benedek, G. B. (1980) *Exp. Eye Res. 31*, 399–410.

Conrad, S. M., Velasco, P. T., Michalska, M., Hsu, L., & Lorand, L. (1983) *Invest. Ophthalmol. Visual Sci. (Suppl.) 24*, 157.

Conrad, S. M., Velasco, P. T., & Lorand, L. (1984) *Invest. Ophthalmol. Visual Sci. (Suppl.) 25*, 141.

Eaton, J. W., Shelton, T. D., Swofford, H. L., Kolpin, C. E., & Jacob, H. S. (1973) *Nature (London) 246*, 105–106.

Ellis, M., Alousi, S., Lawniczak, J., Maisel, H., & Welsh, M. (1984) *Exp. Eye Res. 38*, 195–202.

Fein, M., Pande, A., & Spector, A. (1979) *Invest. Ophthalmol. Visual Sci. 18*, 761–765.
Garber, A. T., & Gold, R. J. M. (1982) *Exp. Eye Res. 35*, 585–596.
Garcia-Castineiras, S., & Miranda-Rivera, M. N. (1983) *Invest. Ophthalmol. Visual Sci. 24*, 1181–1187.
Geisler, N., & Weber, K. (1980) *Eur. J. Biochem. 111*, 425–433.
Goosey, J. D., Zigler, J. S., Jr., & Kinoshita, J. H. (1980) *Science (Washington, D.C.) 208*, 1278–1280.
Green, H. (1979) *Harvey Lect. 74*, 101–139.
Green, K. J., & Goldman, R. D. (1983) *Cell Motil. 3*, 283–305.
Harboe, N., & Ingild, A. (1973) *Scand. J. Immunol. 2 (Suppl. 1)*, 161–164.
Hightower, K. R., & Reddy, V. N. (1982a) *Invest. Ophthalmol. Visual Sci. 22*, 263–267.
Hightower, K. R., & Reddy, V. N. (1982b) *Exp. Eye Res. 34*, 413–421.
Hightower, K. R., & Dering, M. (1984) *Invest. Ophthalmol. Visual Sci. 25*, 1108–1111.
Ireland, M., & Maisel, H. (1983) *Invest. Ophthalmol. Visual Sci. (Suppl.) 24*, 29.
Jedziniak, J., Kinoshita, J. H., Yates, E. M., Hacker, L. O., & Benedek, G. B. (1973) *Exp. Eye Res. 15*, 185–192.
Jedziniak, J., Nicoli, D. F., Yates, E. M., & Benedek, G. B. (1976) *Exp. Eye Res. 23*, 325–332.
Kibbelaar, M. A., Selten-Versteegen, A. M. E., Bloemendal, H., Dunia, I., & Benedetti, E. L. (1979) *Eur. J. Biochem. 95*, 543–549.
Kramps, J. A., Hoenders, H. J., & Wollensak, J. (1978) *Exp. Eye Res. 27*, 731–735.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lieska, N., Chen, J., Maisel, H., & Romero-Herrara, A. E. (1980) *Biochim. Biophys. Acta 626*, 136–153.
Lorand, L., Weissmann, L. B., Epel, D. L., & Bruner-Lorand, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4479–4481.
Lorand, L., Siefring, G. E., Jr., & Lowe-Krentz, L. (1978) *J. Supramol. Struct. 9*, 427–440.
Lorand, L., Hsu, L. K. H., Siefring, G. E., Jr., & Rafferty, N. S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1356–1360.
Lorand, L., Bjerrum, O. J., Hawkins, M., Lowe-Krentz, L., & Siefring, G. E., Jr. (1983) *J. Biol. Chem. 258*, 5300–5305.
Maisel, H., & Perry, M. M. (1972) *Exp. Eye Res. 14*, 7–12.
Malik, M., Fenko, M. D., Iqbal, K., & Wisniewski, H. M. (1983) *J. Biol. Chem. 258*, 8955–8962.
Michalska, M., Hawkins, M., & Lorand, L. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1078 (Abstr. 4640).
Nasser, S., Bradley, R., Alcala, J., & Maisel, H. (1980) *Exp. Eye Res. 30*, 109–113.
Palek, J. (1973) *Blood 42*, 988.
Rafferty, N. S., & Goossens, W. (1978) *Exp. Eye Res. 26*, 177–190.
Ramaekers, F. C. S., Osborn, M., Schmid, E., Weber, K., Bloemendal, H., & Franke, W. W. (1980) *Exp. Cell Res. 127*, 309–327.
Rice, R. H., & Green, H. (1979) *Cell (Cambridge, Mass.) 18*, 681–694.
Roy, D., & Spector, A. (1976) *Biochemistry 15*, 1180–1188.
Roy, D., Chiesa, R., Li, L.-K., & Spector, A. (1984) *Invest. Ophthalmol. Visual Sci. (Suppl.) 25*, 80.
Siefring, G. E., Jr., Apostol, A. B., Velasco, P. T., & Lorand, L. (1978) *Biochemistry 17*, 2598–2604.
Simon, M., & Green, H. (1984) *Cell (Cambridge, Mass.) 36*, 827–834.
Spector, A. (1984) *Invest. Ophthalmol. Visual Sci. 25*, 130–146.
Spector, A., & Roy, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3244–3248.
Stevens, J. V., Rouzer, C. A., Monnier, V. M., & Cerami, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2918–2922.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Umezawa, H. (1976) *Methods Enzymol. 45*, 678–695.
Van Kleef, F. S. M., Willems-Thijssen, W., & Hoenders, H. J. (1976) *Eur. J. Biochem. 66*, 477–483.
Watt, F. M., & Green, H. (1981) *J. Cell Biol. 90*, 738–742.
White, F. J., Jr. (1967) *Methods Enzymol. 11*, 481–484.