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Lens transglutaminase and cataract formation

[ε-(γ-glutamyl)lysine crosslinks/aging lens/β-crystallins]

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ABSTRACT **A protein polymer characteristically present in human cataract was shown to contain significant amounts of γ-glutamyl-ε-lysine isopeptides. It is proposed that these crosslinks are produced by the action of transglutaminase (R-glutaminyl-peptide:amine-γ-glutamyl-yltransferase, EC 2.3.2.13), which is all the more plausible because lens contains the enzyme and endogenous protein substrates for it. The enzyme is similar to that obtained from liver and is Ca^{2+} dependent. Highest apparent activity is found in lens cortex. When cortex homogenate from the rabbit was incubated in the presence of Ca^{2+} with either [^{14}C]putrescine or with dansylcadaverine, a selective incorporation of the radioactive or fluorescent amine into the heavier subunits ($M_r \approx 26{,}000$ and 30,000) of β-crystallins could be demonstrated. Possible modes of regulating the crosslinking activity of this enzyme in lens are discussed.**

Loss of transparency of the aging lens, culminating in senile cataract, is thought to be related to posttranslational modifications affecting the cell proteins themselves. Deamidation, proteolytic degradation, changes in properties of aggregation, and polymerization of constituent proteins by disulfide bonds have been observed (1, 2). However, for the purpose of the present paper, the most pertinent finding is that cataract tissue contains polymeric structures that cannot be dissociated into smaller components by solubilization in sodium dodecylsulfate and urea after reduction of disulfides (3, 4). Thus, the possibility exists that these unusual polymers may have been assembled under the catalytic influence of a Ca^{2+}-dependent transglutaminase (R-glutaminyl-peptide:amine-γ-glutamyl-yltransferase, EC 2.3.2.13) (i.e., an endo-γ-glutamine:ε-lysine transferase; ref. 5) and are held together by γ-glutamyl-ε-lysine side-chain bridges. In addition to promoting this crosslinking reaction, transglutaminases also catalyze the specific incorporation of amines into proteins and, in fact, these enzymes in tissues can be assayed readily by amine incorporation (6).

Recent work on human erythrocytes from this laboratory (7–9) and on cultured keratinocytes by Rice and Green (10, 11) suggests a general role for transglutaminases in some remodeling reactions during the process of cell aging. Usually an increase in Ca^{2+} concentration seems to trigger the activity of these otherwise latent enzymes, although the possibility of regulation by other factors also has to be considered. Accumulation of Ca^{2+} in the cytoplasm might be brought about by the failing of an outward-directed Ca^{2+} pump in the plasma membrane (as in the erythrocytes) or by the disintegration of mitochondria (as in the keratinocytes), but, whatever the cause, the ensuing activation of transglutaminase seems to be the common denominator for determining further events. The structural consequences of the polymerization reaction vary from cell type to cell type. In the case of erythrocytes, γ-glutamyl-ε-lysine crossbridging involves proteins in the membrane and in the infrastructure. In keratinocytes, a circularly arranged cornifying envelope forms just underneath the plasma membrane. However, the net result in both cases is the irreversible rigidification of the cell.

We sought to explore the possibility that polymer formation by covalent bonds other than disulfide in cataract might be due to transglutaminase action. Thus far we have been able to show that the polymer isolated from human cataract, indeed, contains significant amounts of γ-glutamyl-ε-lysine crosslinks. Furthermore, there is a Ca^{2+}-dependent transglutaminase in lens that acts on protein substrates in this tissue in a rather selective manner. An account of this work was presented in New Orleans at the meeting of the American Society of Biological Chemists (12).

MATERIALS AND METHODS

Freshly frozen rat, rabbit, guinea pig, and bovine lenses were purchased from Pel-Freez. Human cataract specimens were obtained through the courtesy of Drs. Margaret Gerber and Paul Hauser, Northwestern University. Materials were thawed out just prior to use by immersing in a solution of 50 mM Tris·HCl, pH 7.4/1 mM EDTA at room temperature and, if required, the capsule was removed to allow separation of cortex from nucleus. A hand-operated glass homogenizer was used to break up the cells.

Transglutaminase-specific fluorescent staining after electrophoresis of the homogenates on agarose, including photography under UV light, and assays for incorporating [^{14}C]putrescine (Amersham/Searle) into *N,N'*-dimethylcasein were performed as described (13, 14).

Purification of lens transglutaminase was carried out at 4°C after dissecting and homogenizing 15 rabbit cortex preparations (ca. 2.7 g wet weight) in 8 ml of 50 mM Tris·HCl, pH 7.4/50 mM NaCl/1 mM EDTA. After centrifugation at 30,000 × *g* for 30 min, the supernatant was applied to a Sepharose 6B column (2.5 × 88 cm) that had been equilibrated with the same solution. By using this buffer, enzyme activity (monitored by the incorporation of [^{14}C]putrescine into *N,N'*-dimethylcasein; ref. 14) emerged between 300 ml and 400 ml of effluent. Glycerol and solid NaCl were added to the collected enzyme fluid to final concentrations of 50% (vol/vol) and 100 mM, respectively. The material was then applied to a column of DEAE-cellulose (1.6 × 7 cm) equilibrated with 50 mM Tris·HCl, pH 7.4/100 mM NaCl/30% glycerol/1 mM EDTA. The same solution was used to remove unretained proteins. From measurements of absorbance at 280 nm, it was estimated that less than 5% of the applied proteins were retained by the column. Enzyme was then eluted with a linear gradient of 100–400 mM NaCl in the Tris/NaCl/glycerol/EDTA solution. The activity emerged in

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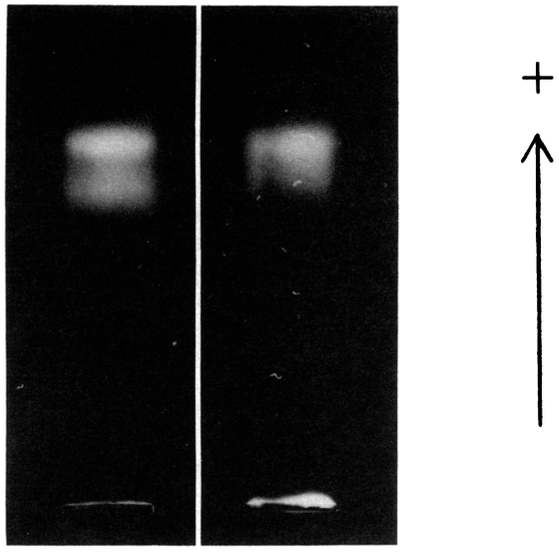

FIG. 1. Transglutaminase of guinea pig lens, shown by enzyme specific activity staining with fluorescent dansylcadaverine. (*Right*) Guinea pig lens homogenate. (*Left*) Liver homogenate. Both tissues were homogenized with an equal amount (wt/vol) of solution containing 50 mM Tris·HCl, pH 7.4/0.25 M sucrose/5 mM EDTA. Of each homogenate, 20 μl was applied to the agarose plate for electrophoresis. For details of electrophoresis, staining, and UV photography, see ref. 13.

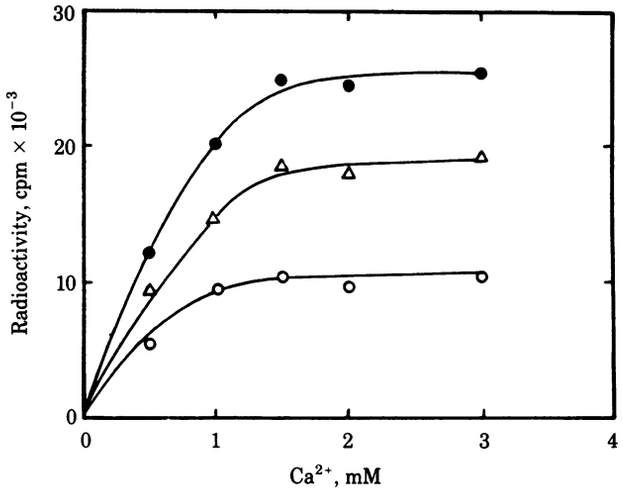

FIG. 2. Ca^{2+} dependence of purified rabbit lens cortical transglutaminase. The enzyme at 0.14 mg of protein per ml was in 50 mM Tris·HCl/50 mM NaCl/1 mM EDTA, pH 7.4, in which all reagents were also dissolved. Reaction mixtures (at 37°C) comprised 20 μl of this buffer, 10 μl of $CaCl_2$ (up to 32 mM), 20 μl of 2% *N,N'*-dimethylcasein, 10 μl of 12 mM [^{14}C]putrescine (11.6 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels), and 20 μl of enzyme. After 15 min (○), 30 min (△), and 60 min (●), 10 μl aliquots were withdrawn for measuring trichloracetic acid-insoluble radioactivity (14) given as cpm on the ordinate. The abscissa represents the calculated Ca^{2+} concentrations of the solutions (i.e., added Ca^{2+} minus 1 mM due to the presence of EDTA).

a peak at about 250 mM NaCl in a volume of about 30 ml. This was concentrated on a Amicon PM-30 filter and dialyzed against a solution of 50 mM Tris·HCl, pH 7.4/50 mM NaCl/1 mM EDTA. As judged by protein determination with crystalline bovine serum albumin as reference (15), approximately 0.28 mg of purified enzyme was obtained in 2 ml of solution.

Isolation of polymer crosslinked by covalent bonds other than disulfides from the nuclei of human cataracts was carried out essentially as described by Kramps *et al.* (3). Proteolytic digestion of the polymer and analysis of γ-glutamyl-ε-lysine were performed as described (8, 16, 17).

RESULTS

The Ca^{2+}-dependent incorporation of dansylcadaverine into *N,N'*-dimethylcasein, applied as activity staining to electrophoretic gels, provides a sensitive means of detection for transglutaminases (13). With this procedure, it was possible to demonstrate activities in rat, guinea pig, rabbit, bovine, and human lens homogenates. As shown for the guinea pig material (Fig. 1), the lens enzyme migrates similarly to that in liver. On a tissue wet-weight basis, however, the apparent activity in the lens homogenate was considerably less than it was in liver, and ex-

Table 1. Transglutaminase activity (cpm) in lens tissue

	15 min	30 min	60 min	90 min	120 min
Guinea pig*	2,000 (200)	4,000 (200)	6,000 (200)	—	7,000 (300)
Bovine†	2,000 (300)	4,000 (400)	6,000 (400)	8,000 (500)	
Rat‡	200 (100)	200 (200)	300 (200)	400 (200)	
Rabbit§					
Capsule	2,500 (100)	4,000 (200)	7,000 (200)	8,000 (300)	
Cortex	11,000 (200)	19,000 (300)	33,000 (200)	40,000 (300)	
Nucleus	3,000 (200)	5,000 (200)	9,000 (300)	11,000 (300)	
Human¶					
Cortex	1,000 (200)	1,500 (200)	2,500 (200)	3,000 (400)	
Nucleus	300 (100)	500 (100)	800 (200)	800 (200)	

Equal amounts of wet-weight of whole lenses, capsule, cortex, or nucleus were homogenized per vol of 50 mM Tris·HCl buffer containing 0.25 M sucrose and 5 mM EDTA (pH 7.4). The assay mixture (at 37°C) comprised 20 μl of solution of 50 mM Tris·HCl/0.1 M NaCl, pH 7.4; 20 μl of 2% (wt/vol) *N,N'*-dimethylcasein in 50 mM Tris·HCl, pH 7.4; 10 μl of 50 mM $CaCl_2$ in 50 mM Tris·HCl, pH 7.4; 10 μl of 1.67 mM [^{14}C]putrescine in 50 mM Tris·HCl, pH 7.4; and 20 μl of homogenate. After various times as indicated, 10-μl aliquots were treated with 10% (wt/vol) trichloroacetic acid, the precipitates were washed with the acid, and the radioactivity of the insoluble material was measured as counts per minute (cpm). (For general methodology, see ref. 14.) Values in brackets indicate incorporation without Ca^{2+}. Total (i.e., trichloroacetic acid-soluble plus -insoluble) radioactivity in 10-μl aliquots was 220,000 cpm.

* Sexually mature (300–400 g).

† Sexually mature.

‡ Sexually mature (200–500 g).

§ 8–12 wk.

¶ Cataract specimen.

Table 2. Incorporation (cpm) of [^{14}C]putrescine by rabbit lens cortex homogenate in the presence of various concentrations of glycerol, with and without *N,N'*-dimethylcasein.

Glycerol, % (vol/vol)	Incorporation, cpm	
	With	Without
—	21,000 (400)	2,000 (300)
10	24,000 (200)	5,000 (300)
20	26,000 (300)	9,000 (400)
30	27,000 (400)	10,000 (400)

Reactions were carried out at 37°C for 30 min. Apart from including glycerol or omitting *N,N'*-dimethylcasein, as indicated, the experiments were carried out as given in Table 1. Values in brackets indicate radioactive counts incorporated in the absence of added Ca^{2+}.

posure time for the former had to be increased to obtain the photographic comparison.

Table 1 provides a more detailed appraisal of potential transglutaminase activity in lens by measuring the incorporation of [^{14}C]putrescine into *N,N'*-dimethylcasein (14). Of all the species examined, rabbit showed the highest and rat the lowest apparent activity, whereas the bovine, guinea pig, and human specimens gave intermediate values.

With respect to the distribution of transglutaminase within the lens itself, the cortex gave the highest specific activity, with considerably lower values found in capsule and nucleus. Half-maximal velocities with cortex homogenates were obtained at Ca^{2+} concentrations between 0.2 and 0.4 mM, and the purified transglutaminases (rabbit and human) showed a similar requirement for Ca^{2+} (Fig. 2).

It was also important to ask whether endogenous proteins in the lens could serve as substrates for the transglutaminase in this tissue. Mixing [^{14}C]putrescine with homogenates in the presence of Ca^{2+} (without the addition of *N,N'*-dimethylcasein) would cause the labeling of proteins that contained the enzyme-reactive γ-glutamine sites. Experiments with rabbit cortex produced significant incorporation of the isotopic amine, and proteins soluble in the homogenizing buffer could be shown to be responsible for incorporating about 90% of all the bound radioactivity. Depending on the presence of some added solutes, the intrinsic rates of putrescine incorporation varied from 10% to 40% of those found when *N,N'*-dimethylcasein was also present. The effect of glycerol (Table 2) is particularly interesting because it enhances the reactivity of endogenous tissue proteins in a rather selective manner.

Identification of transglutaminase substrates in the buffer-soluble extract of rabbit cortex was carried out by chromatography after exposure to [^{14}C]putrescine and Ca^{2+}. Fractionation by Sepharose 6B column (Fig. 3) revealed that, of all the resolved protein peaks, only the crystallin regions marked as β_1 and β_2 contained bound radioactivity. Moreover, $NaDodSO_4$ electrophoresis of the chromatographically separated proteins in polyacrylamide gels showed (Fig. 4) that the isotope was localized preferentially in the 26,000 molecular weight subunits of β_1 crystallin and in both the 26,000 and 30,000 weight subunits of β_2. In addition, radioactivity also was present in a high molecular weight component that chromatographed with β_1. Identical labeling patterns were obtained when fluorescent dansylcadaverine was used as an amine marker instead of [^{14}C]putrescine.

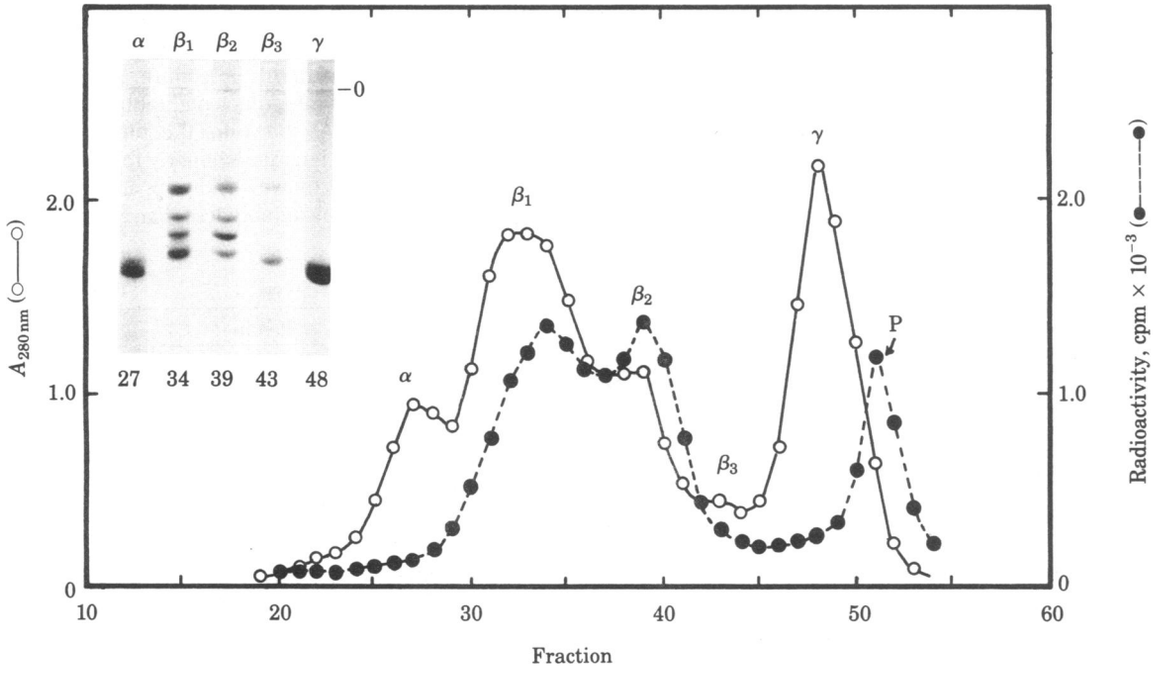

FIG. 3. β-Crystallins incorporate [^{14}C]putrescine preferentially by the action of transglutaminase in cortex homogenates. Rabbit cortex was homogenized with an equal amount (wt/vol) of a solution containing 50 mM Tris·HCl, 50 mM NaCl, and 5 mM EDTA (pH 7.4). A 200-μl aliquot was then mixed with 400 μl of 60% glycerol (dissolved in 50 mM Tris·HCl/50 mM NaCl/1 mM EDTA, pH 7.4) and with 100 μl each of 12 mM [^{14}C]putrescine (11.6 mCi/mmol) and of 50 mM $CaCl_2$. After 2 hr of incubation at 37°C, the reaction was stopped by the addition of 100 μl of 50 mM EDTA and the mixture was centrifuged (30,000 × *g* for 30 min). The supernatant was dialyzed against a solution of 50 mM Tris·HCl/50 mM NaCl/1 mM EDTA, pH 7.4, and was applied to a 1.6 × 96 cm Sepharose 6B column equilibrated with the same buffer solution (at 20°C). Fractions (3.2 ml each) were assayed directly for absorbancy at 280 nm (○, ordinate on left) and also for isotope content (●, ordinate on right) by withdrawing 10 μl and mixing with 10 ml of Aquasol-2 for counting radioactivity. Assignments of α, β and γ-crystallin regions are shown. P, free [^{14}C]putrescine. (*Insert*) Coomassie blue-stained electrophoretic profiles for fractions 27, 34, 39, and 43. Aliquots of 25 μl were mixed with 100 μl of a solution containing 2% (wt/vol) $NaDodSO_4$, 40 mM dithiothreitol, 9 M urea, and 20 mM NaH_2PO_4 (pH 7.1) and were electrophoresed on 12% (wt/vol) Laemmli gels (18).

Approximately 10 mg of polymer of the type described by Kramps *et al.* (3) and by Pirie (4) were obtained from the nuclei of seven specimens of human cataract. Digestion of this polymer by the sequential application of five proteolytic enzymes (subtilisin, pronase, leucine aminopeptidase, prolidase, and carboxypeptidase Y) yielded approximately 48 nmol of γ-glutamyl-ε-lysine isopeptide by the analytical procedure described (8).

DISCUSSION

The present investigation was prompted by reports of isolation of high molecular weight polymers from cataract tissue, stabilized by covalent bonds other than disulfides (3, 4). Up to now, no attempts have been made to clarify the reactions by which these polymers are assembled from their constituent proteins. We were particularly interested in exploring apparent similarities with transglutaminase-catalyzed crosslinking reactions that are known to occur in a variety of biological systems where either polymer formation *per se* or the stabilization of an already existing polymer is due to the bridging of protein units by γ-glutamyl-ε-lysine isopeptides (7–11, 16, 17).

In order to make the idea of enzyme-catalyzed transamidation in lens plausible, the presence of transglutaminase and of endogenous substrates had to be demonstrated.

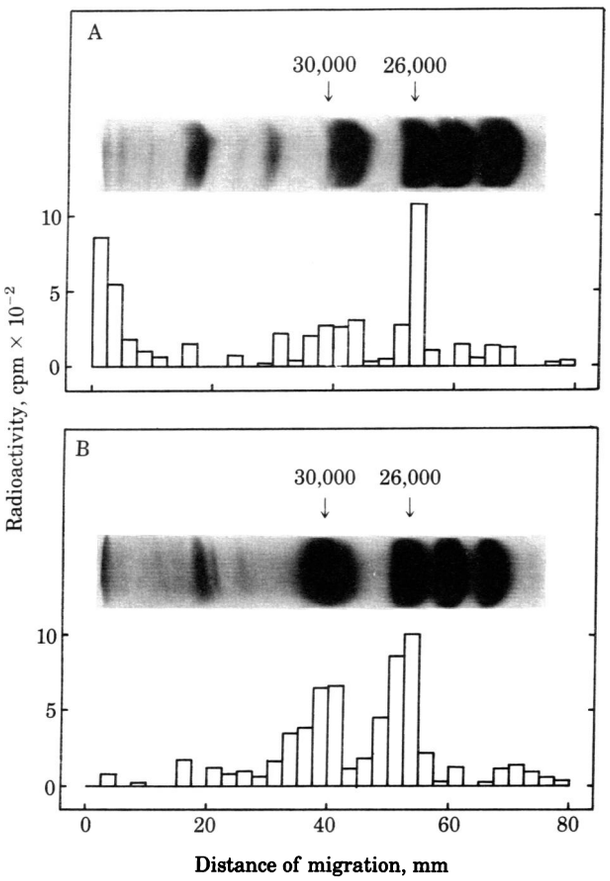

FIG. 4. Pattern of subunit labeling of β-crystallins with [^{14}C]putrescine by the action of transglutaminase. Aliquots (100 μl) of fraction 34 β_1 crystallin (*A*) and fraction 39 β_2 crystallin (*B*), corresponding to the experiment presented in Fig. 3, were mixed with 100 μl of a solution containing 2% $NaDodSO_4$, 40 mM dithiothreitol, 9 M urea, and 20 mM NaH_2PO_4 (pH 7.1) and were electrophoresed on 12% Laemmli gels (19). After Coomassie blue staining, the gels were sliced for measurement of radioactivity (18), given as cpm on the ordinate. Positions of 26,000 and 30,000 molecular weight markers are indicated.

The results show that lens, and mainly the cortex, contains a Ca^{2+}-dependent transglutaminase (Table 1), but we have no explanation for the interspecies variations observed in crude homogenates. They may reflect real differences in enzyme concentration or simply could be due to a variety of kinetic factors, such as different turnover rates or different affinities of the enzymes for the *N,N'*-dimethylcasein or putrescine test substrates. The possible presence of various amounts of inhibitors (e.g., Zn^{2+}) or competing substrates (e.g., polyamines) in the homogenates might have to be taken into account also. Actually, some interference of this sort may well be the reason for the slow incorporation of [^{14}C]putrescine in the rat tissue because, after electrophoretic separation, staining with dansylcadaverine indicates the presence of an activity not unlike that found in bovine lens.‡

We have succeeded in purifying the enzyme from rabbit and, though not described in detail, also from human lens. By the method of incorporation of [^{14}C]putrescine into *N,N'*-dimethylcasein, half-maximal velocities were obtained between 0.2 and 0.4 mM Ca^{2+}. How could this enzyme be regulated in lens?

On the basis of analytical data (21), one may assume that overall there is sufficient Ca^{2+} in lens to allow the enzyme to become fully activated. If Ca^{2+} and the transglutaminase were sequestered in the tissue from one another, activation could be triggered by the merging of the two compartments. On the other hand, if they coexisted in the same phase, alternate mechanisms of regulation must be explored, and antagonism between Zn^{2+} and Ca^{2+} is a possibility worth considering. Zn^{2+} competes very effectively against Ca^{2+} in the activation of similar enzymes derived from guinea pig liver and from human plasma. For the inhibition of the latter enzyme by Zn^{2+}, an apparent K_i of about 6×10^{-7} M was obtained (22). In senile cataract there is a significant decline in the concentration of Zn^{2+} and an increase in that of Ca^{2+} (23). Such an inverse change in the ionic composition of the lens could play a role in tipping the balance for activating transglutaminase.

In searching for mechanisms of regulating the activity of lens transglutaminase, some other possibilities could be mentioned. One of the prostatic transglutaminases of the guinea pig is activated by small organic solutes such as tosylglycine (24). Whereas this and similar compounds enhance the activity of this particular enzyme from immeasurably low to very high velocities (without affecting K_M for substrates), they inhibit the transglutaminase from the liver of the same species. It is possible that the lens enzyme might be activated by some metabolite in a similar fashion.

Naturally occurring polyamines (e.g., spermine, spermidine, putrescine) are good substrates for transglutaminases (25) and, if present, they could exert a controlling influence on protein crosslinking by competition against ε-lysine residues. Cystamine can compete as an amine, and it can also block the active center cysteine residue of transglutaminase (9). Other disulfide compounds in the cell might react likewise.

The finding in regard to glycerol (Table 2) hints at the possibility of yet another interesting type of control at the substrate level. The polyol specifically enhanced the reactivities of β-crystallins in lens homogenates without having much effect on amine incorporation into the *N,N'*-dimethylcasein test protein. A similar effect was demonstrated for acidic polyanions on the reaction of rat prostatic transglutaminase with seminal vesicle secretion protein (26). Regarding the situation in lens, the main question is whether a metabolite could be found that would

‡ A recent attempt (20) to purify transglutaminase from rat lens resulted in isolating a product that rapidly lost activity.

mimic the action of glycerol but in a more specific manner. One should also focus on the possibility that age and disease-related posttranslational modifications, such as the glycosylation and sulfhydryl oxidation observed in diabetic cataract (27), might render the lens proteins themselves more reactive towards transglutaminase.

Of the soluble components of lens, certain β-crystallins act as selective substrates for endogenous transglutaminase, and even within the reacting β-crystallin structures significant differences exist among the various subunits. [^{14}C]putrescine or dansylcadaverine incorporation could be demonstrated only with two of the β-crystallin subunits (molecular weight ≈ 26,000 and 30,000), and as such these carry reactive γ-glutamine residues that could participate in the formation of isopeptide crossbridges. By recalling previous work with fibrin, the site-specific labeling of the residues with a radioactive or a fluorescent marker should greatly facilitate the exploration of the potential crosslinking domains in the β-crystallin molecules (18, 28, 29).

In this paper we have demonstrated that lens contains a transglutaminase that reacts with protein components in the tissue. As such, whenever the enzyme becomes activated, formation of various γ-amides and polymerization of proteins ($P,P'\ldots$) by γ-glutamyl-ε-lysine crosslinks (X), will certainly occur:

$$\underset{(\mathrm{TGase_i})}{\text{Inactive transglutaminase}} \rightarrow \underset{(\mathrm{TGase_a})}{\text{Active transglutaminase}}$$

$$mP + nP'\ldots \rightarrow X(P_mP'_n\ldots)$$

Isopeptide crosslinks were isolated from a polymer fraction of human cataract tissue. This makes it all the more plausible that the proposed transglutaminase-mediated polymerization of proteins in the aging lens indeed could play a significant role in the development of cataract. Available inhibitors (8, 9, 16, 22, 25) of this enzyme-catalyzed crosslinking process might be of interest.

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