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Acceptor–Donor Relationships in the Transglutaminase-Mediated Cross-Linking of Lens β-Crystallin Subunits†

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ABSTRACT: Following the isolation of the N^{ϵ}-(γ-glutamyl)lysine-containing polymers from human cataracts, our efforts were directed to induce such cross-links experimentally in rabbit lens, and evidence was obtained for the selective reactivities of certain β-crystallin subunits in this transglutaminase-catalyzed event. In the present work, we examined the enzymatic cross-linking of purified crystallins individually (α, β_H, β_L, and γ) and in combinations, with particular emphasis on forming the approximately 55K dimer. This species was the primary product in the cross-linking of β_H-crystallins; β_L also reacted with transglutaminase. Neither α- nor γ-crystallins formed appreciable amounts of cross-linked structures with transglutaminase. Dansylcadaverine, known to compete against the reactive lysines of proteins in forming N^{ϵ}-(γ-glutamyl)lysine cross-bridges, was shown to inhibit the generation of dimeric and higher ordered oligomers from β_H and β_L. The fluorescent amine specifically labeled only two subunits in β_H (~29–30K and ~26K) and one in β_L (~26K), identifying these substrates as possessing transglutaminase-reactive endo-γ-glutaminyl residues. An antiserum to bovine βBp recognized the ~23K subunit of rabbit β-crystallins and also the ~55K dimer, suggesting that the ~23K protein participates as a lysine donor in generating the cross-linked dimer with transglutaminase. Inasmuch as the same antiserum reacts with a ~50K material reported to appear in increasing amounts with age in human lens, the results lend added support to the physiological significance of transglutaminase in the aging of lens.

Isolating N^{ϵ}-(γ-glutamyl)lysine peptides from polymers which are characteristically present only in cataractous specimens called attention to the role of transglutaminase in lens (Lorand et al., 1981). Previous work focused on reactions catalyzed by the intrinsic enzyme, activated from its latent form by adding Ca^{2+} to homogenates of rabbit lens or to the whole organ. Using the enzyme-directed incorporation of amines ([^{14}C]putrescine or *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (dansylcadaverine))[1] as a strategy for identifying acceptor proteins [see Lorand and Conrad (1984)], it could be shown that, among all the crystallin components

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[1] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular weight; K, $\times 10^3$; dansylcadaverine, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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in the lens, only two subunits of β-crystallin (with relative molecular weights of approximately 30K and 26K) carried transglutaminase-reactive γ-glutaminyl functionalities. Another important observation was that a dimer at about 55K was an early product in the endogenous cross-linking process and that an antibody to the dimer also recognized the ~30K subunit in β_H-crystallin (Lorand et al., 1985).

In order to elucidate molecular aspects of acceptor–donor relationships in forming N^{ϵ}-(γ-glutamyl)lysine-bridged β-crystallin dimers, it was essential to examine the reactivities of isolated lens crystallins with purified transglutaminase. The present paper analyzes the generation of cross-linked species in the absence as well as in the presence of a competing amine, dansylcadaverine, and also the concomitant incorporation of the latter into protein substrates and products. Studies were carried out with each crystallin group (α, β_H, β_L, and γ) either individually or in combination with others. Apart from relying on the fluorescent properties of dansylcadaverine to demonstrate the enzyme-directed labeling of proteins (Lorand et al., 1972), we also employed immunoblotting with anti-dansyl antibody (Lorand et al., 1986). Furthermore, the presence of βBp-type crystallin subunit in the cross-linked dimer of about 55K was shown by its immunoreactivity toward antiserum specific for βBp [McFall-Ngai et al., 1986; for the definition of βBp, see Driessen et al. (1981)].

Materials and Methods

Reagent-grade chemicals were obtained from the following suppliers: sodium chloride, calcium chloride, potassium chloride, and methanol from Mallinckrodt (St. Louis, MO); amido black, ethylenediaminetetraacetic acid, tris(hydroxymethyl)aminomethane, glycine, Coomassie Brilliant Blue R, bromophenol blue, 4-chloro-1-naphthol, and dithiothreitol from Sigma (St. Louis, MO); 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.

Leupeptin was a gift from the U.S.–Japan Cooperative Cancer Research Program. Dansylcadaverine hemifumarate was a gift from Kabi (Stockholm, Sweden).

Transglutaminase was isolated by Dr. P. Turner of this laboratory from frozen guinea pig livers (Pel-Freez; Rogers, AK). The livers (250 g) were homogenized in 960 mL of 25 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM EDTA, and 150 mM NaCl by using a Waring blender (5 × 20 s bursts), followed by centrifugation at 8500*g* for 30 min at 5 °C. The supernatant was loaded through glass wool onto a DEAE-cellulose column which had been equilibrated with homogenization buffer. After application of the sample, the column was washed with 700 mL of homogenization buffer, followed by 1 L of the same buffer containing 180 mM NaCl. The enzyme was eluted with 25 mM Tris-HCl, pH 7.5, 2 mM DTT, 1.5 mM EDTA, and 230 mM NaCl. Selected fractions were pooled and precipitated by 80% saturation of ammonium sulfate, centrifuged, and dialyzed against 15 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 5 mM DTT. The purified enzyme was stored at −20 °C.

Crystallins were isolated from frozen lenses from young rabbits (Pel-Freez) by gel filtration chromatography through Sepharose 6B. Two lenses were homogenized in 4 mL of 50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, and 5 mM DTT at pH 7.4 by four consecutive bursts (700 rpm, 10-s duration each, 4 °C) in the Potter–Elvehjem apparatus. Following centrifugation (20000*g* × 30 min), 4 mL of supernatant was applied at 4 °C to a Sepharose 6B column (2.5 × 90 cm) by using the homogenization buffer as effluent with a flow rate of 9.7 mL/h (Garber & Gold, 1982). Protein was monitored by measuring absorbancy at 280 nm. Peak fractions representing α-, β_H-, β_L-, and γ-crystallins were dialyzed against two changes of deionized water and lyophilized. Crystallins were redissolved in 50 mM Tris-HCl and adjusted to pH 7.5. Final concentrations of crystallin stocks were adjusted to approximately 2 mg/mL for α- and γ-crystallins and 4 mg/mL for β-crystallins.

Incubation of crystallins was carried out at 37 °C in a total volume of 100 μL of 50 mM Tris-HCl, pH 7.5, which included 50 μL of crystallin, 1 mM leupeptin, 2–3 μg/mL transglutaminase, and either 8 mM $CaCl_2$ or 2 mM EDTA. For incubations involving more than one type of crystallin, stock solutions were mixed in equal volumes and the incubations carried out as described. Amine incorporation studies included either 0.2 or 2.0 mM dansylcadaverine.

Changes involving crystallin subunits were examined by electrophoresis, following solubilization and reduction of the incubation mixtures. Forty microliters of sample was solubilized in 1–2 volumes of 20 mM sodium phosphate, 9 M urea, 40 mM DTT, and 2% SDS, pH 7.1, and warmed at 37 °C for 2 h. 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 3.5% acrylamide and a resolving gel of 12% acrylamide and a running buffer of 50 mM Tris, 0.38 M glycine, and 0.1% SDS (pH 8.6) were employed. Gels were stained with Coomassie Brilliant Blue R and were calibrated with molecular weight (M_r) standards of soybean trypsin inhibitor (20.1K), trypsinogen (24K), carbonic anhydrase (29K), glyceraldehyde-3-phosphate dehydrogenase (36K), ovalbumin (45K), and bovine serum albumin (66K). Gels containing dansylcadaverine-labeled crystallins were photographed under UV light (366 nm) prior to Coomassie Blue staining.

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 an LKB Model 2005 Transphor Electroblotting unit. Transfers were 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 for 2 h at 0.6 A and 5 °C. Amido black staining was performed with 0.2% amido black in 50% methanol and 10% acetic acid and destained with 20% methanol and 10% acetic acid. Immunostaining was essentially as described by Lorand et al. (1986) utilizing a Vectastain ABC kit specific for rabbit IgG (Vector Laboratories, Burlingame, CA). Rabbit IgG to dansylated bovine γ-globulin was prepared as described by Lorand et al. (1986) and used at a dilution of 1:4000. Rabbit antiserum to bovine βBp was a generous gift from Dr. J. Horwitz of the Jules Stein Eye Institute, University of California, Los Angeles, CA (McFall-Ngai et al., 1986), and was used at a dilution of 1:4500. Peroxidase labeling was developed with a solution containing 10 mL of 3 mg/mL 4-chloro-1-naphthol dissolved in ice-cold methanol, 50 mL of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.5, and 30 μL of 30% hydrogen peroxide.

Results

Generation of Cross-Linked Products in the Reaction of Crystallins with Transglutaminase. Purified crystallins of the α, β_H, β_L, and γ class were allowed to react with transglutaminase in the presence of Ca^{2+} ions (or EDTA in the controls) either individually (Figure 1) or in various combinations with each other (Figures 2 and 3). Reactions were terminated by

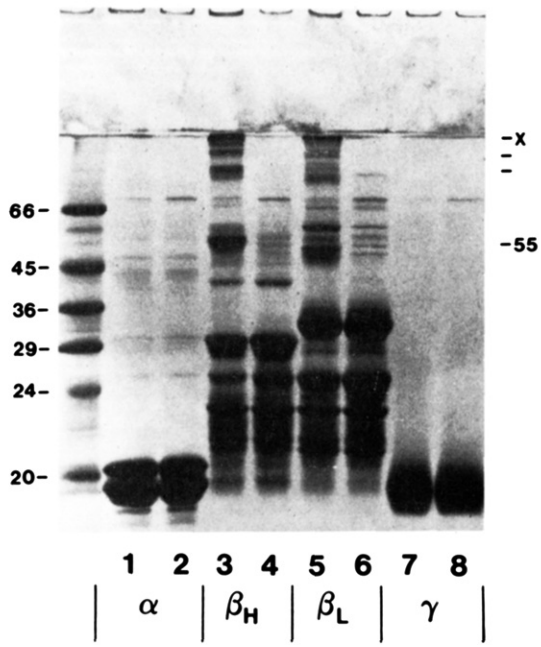


FIGURE 1: Cross-linking of individual classes of lens crystallins (α, β_H, β_L, and γ) by transglutaminase, demonstrated by SDS–PAGE profiles. For details, see Materials and Methods; reaction times were 90 min with Ca^{2+} (odd-numbered lanes) or with EDTA (even-numbered lanes, as controls). Molecular weight markers are shown on the left. Positions of the ~55K dimer and those of the major cross-linked products of β_H, including that at the stacking gel interface (x), are marked on the right.

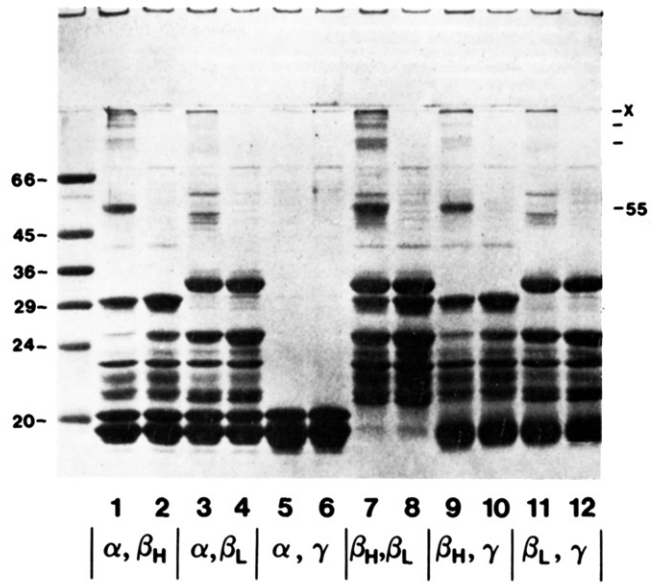


FIGURE 2: Cross-linking of binary mixtures of lens crystallins ($\alpha\beta_H$, $\alpha\beta_L$, $\alpha\gamma$, $\beta_H\beta_L$, $\beta_H\gamma$, and $\beta_L\gamma$) by transglutaminase. Details as described under Materials and Methods and as given for Figure 1. Odd-numbered lanes represent reactions with Ca^{2+} and even-numbered lanes the controls with EDTA.

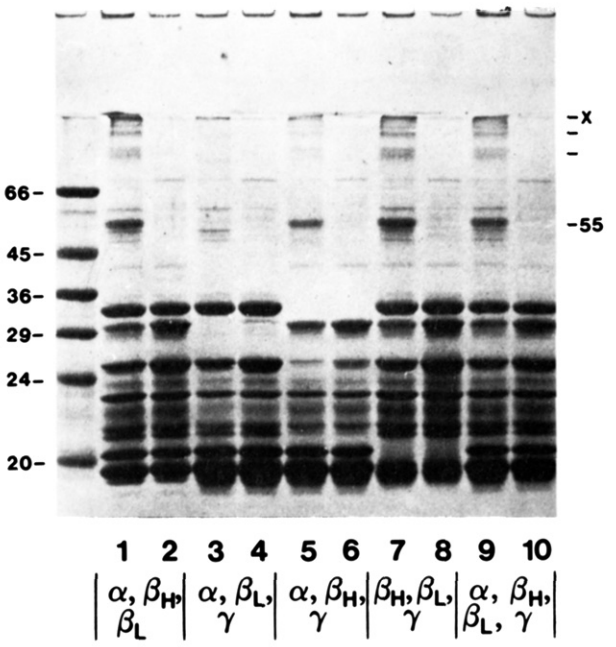


FIGURE 3: Cross-linking of ternary ($\alpha\beta_H\beta_L$, $\alpha\beta_L\gamma$, $\alpha\beta_H\gamma$, and $\beta_H\beta_L\gamma$) and quaternary ($\alpha\beta_H\beta_L\gamma$) mixtures of lens crystallins by transglutaminase. Details as described under Materials and Methods and as given for Figure 1. Odd-numbered lanes represent reactions with Ca^{2+} and even-numbered lanes the controls with EDTA.

adding a mixture of SDS–DTT–urea, and samples were analyzed by SDS–PAGE. As seen in the figures, cross-linked products could be elicited in the Ca^{2+}-dependent enzymatic reactions with only β_H- and β_L-crystallins as substrates (lanes 3 and 5 in Figure 1). Regarding de novo formed bands in the β_H system, that around 55K had the strongest staining intensity, followed by bands at higher molecular weights. The material at the stacking gel interface (x) may well represent various orders of polymers. In the region of β_H monomers, diminished intensities were observed at about 29K, 26K, and 23.5K (compare lanes 3 and 4 in Figure 1). Cross-linking of β_L-crystallin showed a doublet near 60K and 54K (or a triplet, with the 52K band included) and some higher oligomers (lane 5 in Figure 1). Neither α- nor γ-crystallins could be cross-linked by transglutaminase (lanes 1 and 7 in Figure 1).

When crystallins were used in pairs of $\alpha\beta_H$, $\alpha\beta_L$, $\alpha\gamma$, $\beta_H\beta_L$, $\beta_H\gamma$, or $\beta_L\gamma$ as substrates for transglutaminase, cross-linked products were found only in the reaction mixtures which contained β_H- or β_L-crystallins (Figure 2). Furthermore, there was no indication that α- or γ-crystallins could, in any way, inhibit or promote the cross-linking of β-crystallins. The same conclusions could be drawn from experiments with ternary ($\alpha\beta_H\beta_L$, $\alpha\beta_L\gamma$, $\alpha\beta_H\gamma$, $\beta_H\beta_L\gamma$) and even with quaternary ($\alpha\beta_H\beta_L\gamma$) combinations of crystallins (Figure 3), pointing to the unique selectivity of β, among all classes of crystallins, as a transglutaminase substrate.

Inhibition of the Transglutaminase-Mediated Cross-Linking of β-Crystallins by Dansylcadaverine, with Concomitant Incorporation of the Fluorescent Amine into Protein Substrates and Products. Inhibiting cross-linking by dansylcadaverine, with simultaneous incorporation of the fluorescent amine into proteins, is a powerful experimental approach for studying transglutaminase-catalyzed reactions in biological systems (Lorand & Conrad, 1984). When β_H- or β_L-crystallins were allowed to react with transglutaminase in the presence of dansylcadaverine (lanes 3 and 6, Figure 4, panel A), there was a significant reduction in the amount of cross-linked species formed (in comparison to lanes 2 and 5, respectively). Photographing the gels under UV light, prior to staining with Coomassie Blue, revealed two fluorescent bands at about 30K and 26K in β_H-crystallin, and one (~26K) in β_L (Figure 4, panel B). Labeling was also evident in some of the cross-linked dimers generated from β_H and β_L, as well as in the higher polymers (x) at the stacking gel interface in both cases.

When examined at different initial concentrations of dansylcadaverine, an interesting pattern of inhibition and labeling ensued. The lower concentration of dansylcadaverine (0.2 mM) still allowed for the generation of some cross-linked species (lane 2 in Figure 5, panel A) whereas, in the presence

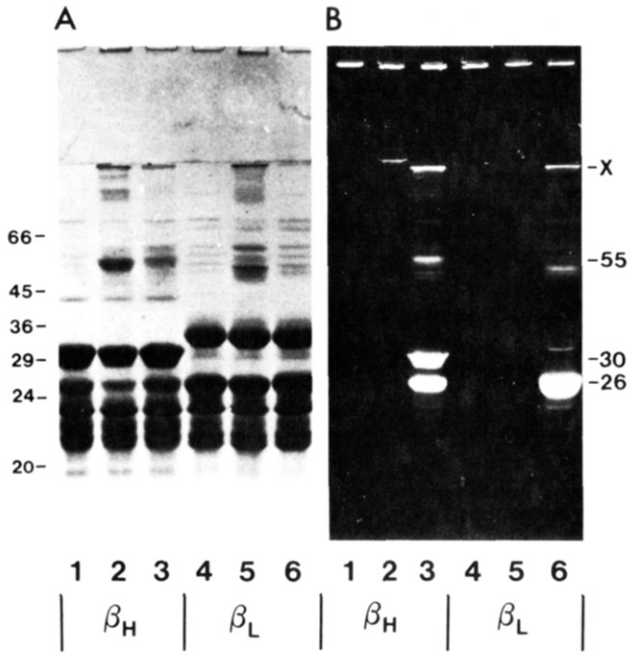


FIGURE 4: Inhibition of the transglutaminase-mediated cross-linking (90 min) of β_H- and β_L-crystallins by dansylcadaverine with concomitant incorporation of the fluorescent amine into proteins. (Panel A) Coomassie Blue stained protein profiles; (panel B) photograph of the gel under UV light prior to protein staining. Lanes 1 and 4 represent EDTA controls in the absence of dansylcadaverine; lanes 2 and 5 correspond to reaction mixtures with Ca^{2+} but without dansylcadaverine; for lanes 3 and 6, in addition to Ca^{2+}, dansylcadaverine (2 mM) was also present.

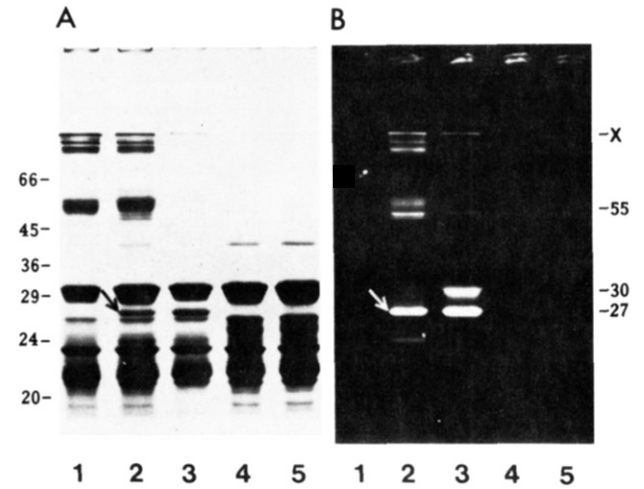


FIGURE 5: Transglutaminase-specific labeling of β_H-crystallin subunits by dansylcadaverine. Reactions were carried out for 5 h at two different concentrations of the fluorescent amine: 0.2 mM for lane 2 and 2 mM for lane 3. (Panel A) Coomassie Blue stained protein profiles; (Panel B) photograph of the gel under UV light prior to protein staining. Lane 1 corresponds to the enzymatic mixture in the absence of dansylcadaverine; lanes 4 and 5 represent reactions in the absence of Ca^{2+} containing 0.2 and 2 mM dansylcadaverine, respectively. The latter two controls were included to check for nonspecific uptake of dansylcadaverine by proteins.

of the higher concentration of the amine (2 mM), barely any 55K or bands of higher molecular weight were seen by protein staining (lane 3, Figure 5, panel A). At the monomeric region of subunits, with 0.2 mM dansylcadaverine most of the label was present in an approximately 27K band, while with 2 mM dansylcadaverine an appreciable amount of label was associated also with the position around 30K (lanes 2 and 3, Figure 5, panel B). The 27K band (marked by an arrow in lane 2 in Figure 5) can be assumed to represent proteins displaced

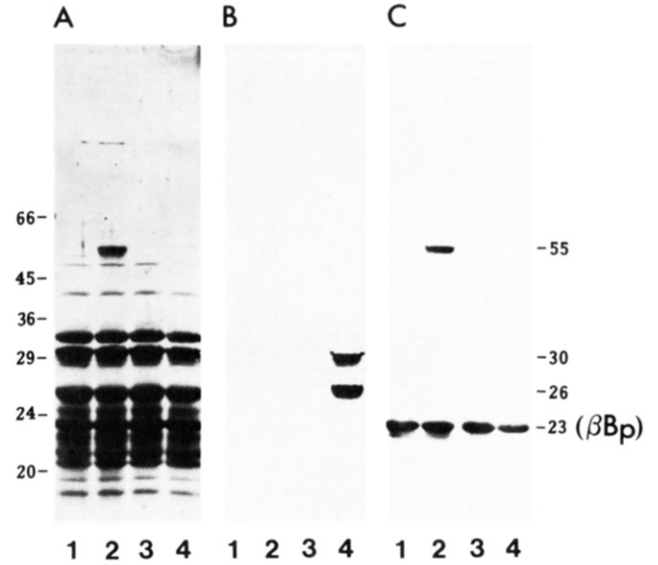


FIGURE 6: Antiserum to the βBp recognizes the cross-linked β-crystallin dimer generated by reaction with transglutaminase. An equal mixture of β_H- and β_L-crystallins was reacted for 90 min with transglutaminase. Following SDS–PAGE, transfers to nitrocellulose were made. (Panel A) Amido black staining; odd-numbered lanes are the EDTA controls in the absence (lane 1) and presence of 2 mM dansylcadaverine (lane 3); even-numbered lanes are the Ca^{2+}-containing reaction mixtures in the absence (lane 2) and presence of 2 mM dansylcadaverine (lane 4). (Panel B) Transblot probed with anti-dansyl IgG. (Panel C) Transblot probed with anti-βBp antiserum. Molecular weight calibrations are marked on the left.

from the 24–26K region upward by the added mass of incorporated dansylcadaverine.

Antiserum to the βBp Type of Subunit Recognizes the Cross-Linked β-Crystallin Dimer Generated by Reaction with Transglutaminase. With the use of a combination of β_H- and β_L-crystallins as substrates (described also in Figure 2) and transblotting to nitrocellulose after SDS–PAGE, amido black staining produced the protein profiles shown in Figure 6, panel A. Lanes 1 and 3 are EDTA controls, in the absence and presence of dansylcadaverine; lanes 2 and 4 are the corresponding Ca^{2+}-containing reaction mixtures. When a parallel transblot was probed with IgG to the dansyl moiety, only lane 4 gave a positive result (panel B), showing the incorporation of dansylcadaverine only into subunits near M_r ~30K and 26K. Another transblot was tested for immunoreactivity toward the antiserum against βBp type of crystallin subunit. As seen in panel C, the antiserum recognized the M_r ~23K subunit of β-crystallin, and, even more significantly, it reacted with the cross-linked dimer of approximately 55K generated by transglutaminase.

Discussion

Though it was previously known (Lorand et al., 1981) that, by the criterion of amine incorporation, two subunits in β-crystallin were specially reactive toward transglutaminase when the enzyme was activated by addition of Ca^{2+} to lens homogenates, in order to gain further insights into the molecular mechanisms of cross-linking of proteins in lens, a more penetrating analysis of the reactions of purified crystallins with transglutaminase was warranted.

Four classes of rabbit crystallins were used as substrates (α, β_H, β_L, and γ) either individually or in binary, ternary, and quaternary combinations. Results of the transglutaminase-catalyzed reaction, monitored by SDS–polyacrylamide gel electrophoresis, were analyzed in the context of (i) formation of oligomers and polymers of $M_r \gtrsim 50K$ which could not be

dissociated by reduction with DTT in SDS–urea; (ii) inhibition of the generation of such high molecular weight species by dansylcadaverine, an amine known to compete effectively against the reactive lysines of proteins in the formation of N^{ϵ}-(γ-glutamyl)lysine cross-bridges; (iii) concomitant incorporation of dansylcadaverine into protein subunits and partially cross-linked products, revealing those structures which carry transglutaminase-reactive γ-glutaminyl residues by fluorescence under UV light (Lorand et al., 1972) or by immunoblotting against anti-dansyl antibody (Lorand et al., 1986); and (iv) participation of a 23K βBp-like subunit in the formation of cross-linked dimers, previously shown to arise in whole lens upon exposure to Ca^{2+} ions (Lorand et al., 1985) but now elicited also by treatment of purified β-crystallin with transglutaminase. This point was proved by immunoreactivity of the dimeric species against the antiserum specific for βBp.

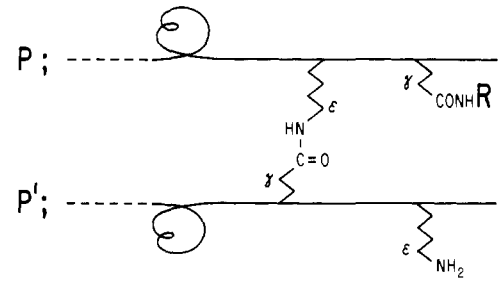
As seen from the results in Figures 1–3, β-crystallins seem to have been uniquely selected during evolution to react with transglutaminase. Neither α- nor γ-crystallins could form cross-linked structures when mixed with the enzyme in the presence of Ca^{2+}. Moreover, we could not find evidence that addition of these crystallins, singly or together, to β_H and β_L could influence in any way the production of the cross-linked β material. Thus, our attention was turned to the reactions of β-crystallins alone.

The cross-linked products derived from β_H were different from those generated in the reaction of β_L with transglutaminase (Figure 1). In the region of dimeric bands, staining around 55K was strongest in the β_H system, whereas a doublet of close to 60K and 54K (possibly a triplet, including 52K) was evident in β_L. Since the next higher order of cross-linked polymers of β_H was found at molecular weights of about 110K, it is possible that the enzyme-induced polymerization proceeds by multiples of cross-linked dimers, i.e., $2\beta \rightarrow \beta_2$; $2\beta_2 \rightarrow \beta_4$, and $3\beta_2 \rightarrow \beta_6$; however, a more accurate analysis in a different electrophoretic system will be needed to decide the issue. In mixtures of β_H- and β_L-crystallins (Figures 2 and 3), the cross-linked products characteristic for β_H seem to predominate.

At a concentration of 0.2 mM, dansylcadaverine could not prevent the enzymatic cross-linking of β_H-crystallin, but the amine was almost fully inhibitory at 2 mM concentration (Figure 5, panel A, lane 3). It is important to recall that the extraneously added amine competes for the acyl-enzyme intermediate (Lorand et al., 1968; Lorand & Conrad, 1984), partitioning it into cross-linked and dansylcadaverine-labeled products. Considering the fact that apparent K_i's for dansylcadaverine with transglutaminase, measured against amine incorporation on casein substrates, are in the 10^{-4}–10^{-5} M range (Lorand et al., 1979), on the basis of the relatively high inhibitory requirement of 2 mM it may be suggested that in the case of β_H-crystallins, as in the case of fibrin cross-linking (Lorand et al., 1968), deacylation proceeds preferentially by cross-linking rather than by incorporation of the amine.

The propensity for cross-linking over amine incorporation is also reflected in the fluorescent photographs taken under UV illumination (Figure 5, panel B). There is an ordered reactivity in the transglutaminase-directed labeling of β_H subunits in the sense that, at 0.2 mM concentration of dansylcadaverine fluorescence was present only at around 27K (lane 2), whereas with 2 mM dansylcadaverine two bands near positions corresponding to ~27K and ~30K were fluorescent (lane 3). Furthermore, fluorescent labeling of bands in the 55K and higher molecular weight regions could be demonstrated mainly by employing 0.2 mM dansylcadaverine (lane 2) which still allowed for a certain degree of cross-linking to take place.

Scheme I



The fluorescent labeling of dimerically and multimerically cross-linked products by dansylcadaverine (as seen, for example, in Figure 5, panel B, lane 2) in the course of the reaction of β-crystallins with transglutaminase is of interest, because it suggests that in this situation cross-linking would only be partial. Incorporation of the amine (RNH_2) would depend on the availability of γ-glutaminyl residues not critically required for producing cross-linked, though perhaps less efficiently cross-linked, structures between two proteins (P and P′). There are a number of possible mechanisms, one of which is shown in Scheme I.

It is evident from the labeling experiments, with detection based either on the fluorescence properties of dansylcadaverine (Figure 4, panel B, lanes 3 and 6, and Figure 5, panel B, lane 3) or on immunoblotting against anti-dansyl IgG (Figure 6, panel B), that of all the β_H-crystallin components only subunits of approximately 29–30K and 26K, and of the β_L-crystallins only the ~26K protein, carry transglutaminase-reactive endo-γ-glutaminyl residues, reflecting a unique selectivity between substrates and enzyme. This is even more impressive if, indeed, β_H and β_L were to share the same 26K subunit. Using an anti-dansyl IgG affinity chromatographic procedure (L. Lorand et al., unpublished results), we hope to be able to isolate directly the dansylcadaverine-labeled subunits of β-crystallins and answer the question whether the labeled components account for the totality of proteins in the M_r 26K and 29–30K regions or represent subsets within these structures.

Regarding the composition of the band (or bands) at the cross-linked dimeric position near 55K, it is known that an antibody against the dimer recognized the 29–30K subunit of β_H-crystallin (Lorand et al., 1985). We have now shown (Figure 6, panel C, lane 2) that an antiserum specific for the βBp-like 23K subunit immunoblotted to the ~55K material produced in the transglutaminase-mediated cross-linking of purified β-crystallins. Given the fact that the ~23K βBp type of subunit could not be labeled with dansylcadaverine in the enzymatic reaction, it is reasonable to conclude that this subunit participates as a lysine donor in forming cross-linked dimers. This, of course, does not exclude the possibility that the two subunits (29–30K and 26K) identified as carrying the reactive γ-glutaminyl functionalities might also contribute ϵ-NH_2 lysines to cross-linking. The dimers generated by transglutaminase action might actually comprise a variety of cross-linked species with molecular weight values ranging approximately between 50K and 60K, and corresponding to diverse combinations of monomers. Proteolytic trimming occurred in the lens prior to or during purification of crystallins might also alter the exact molecular weight values obtained. Inasmuch as leupeptin was included in all reaction mixtures (Lorand et al., 1985), it is unlikely that Ca^{2+}-induced proteolysis could have given rise to heterogeneity after cross-

linking or following amine incorporation into protein components.

The βBp subunit is a principal component of β-crystallins in the bovine and perhaps in other species too. McFall-Ngai et al. (1986) found that an antiserum to βBp recognized a band with a molecular weight of about 50K on transblots from human lens SDS-PAGE profiles and that the amount of this band increased with the age of the propositus. Though there was no suggestion that this 50K band might have been a product of transglutaminase catalysis and that it might in this sense be similar to the ~55K cross-linked β-crystallin species described earlier (Lorand et al., 1985), we thought it was important to ask the question whether βBp participated in the transglutaminase-mediated generation of such β-crystallin dimers. The experimental results presented in Figure 6 (panel C, lane 2) are entirely clear in this regard. Not only did the antiserum to the bovine βBp react with the corresponding rabbit ~23K subunit but also it recognized the ~55K cross-linked dimeric product essentially with the same sensitivity. This important finding lends added support to the physiological significance of transglutaminase in the aging process in lens. The enzyme, present in a latent form, might become activated through redistribution of Ca^{2+} in the tissue, proteolytic cleavage, release from inhibition, or combination with positive effectors. Though the experiments presented in this paper focused on crystallin subunits as substrates, cross-linking in the aging lens might resemble the situation in Ca^{2+}-enriched human red cells (Lorand et al., 1976, 1978; Bjerrum et al., 1981) and could involve membrane constituents as well. Future efforts will be directed to examining these questions and to further delineate the role of transglutaminase in the irreversible processes associated with cataract formation.

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Photochemical Properties of *Escherichia coli* DNA Photolyase: Selective Photodecomposition of the Second Chromophore†

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ABSTRACT: *Escherichia coli* DNA photolyase contains a stable flavin radical and a second chromophore (SC) of unknown structure. The effects of flash (both conventional and laser) excitation of either the radical alone or both the radical and the second chromophore have been investigated by variation of the excitation wavelengths. Radical excitation leads to an electron abstraction by the lowest excited doublet state of the radical from an amino acid residue, probably a cysteine or tyrosine. On a longer time scale, a back-reaction occurs that can be prevented by the presence of certain electron donors, e.g., thiols, NADH, or tyrosine, but not pyrimidine dimers. Excitation of the second chromophore leads to electronic energy transfer from second chromophore excited states to the ground-state flavin radical doublet state, thus increasing the population of the lowest excited doublet state. Repetitive excitation of the enzyme with white light leads to photodecomposition of the second chromophore but not of the flavin adenine dinucleotide cofactor. Enzyme with photodecomposed SC retains full activity.

DNA photolyases (EC 4.1.99.3) catalyze the photochemical conversion of pyrimidine dimers in DNA into pyrimidines, thus reversing the effect of far-UV (200-300-nm) radiation. The enzyme from *Escherichia coli* contains the FAD[1] blue neutral

Table I: Activities of Various Forms of Photolyase

form of enzyme	rel $\epsilon_{366}\phi$
E-FADH^0	1
E-FADH$_2$	8
SC†-E-FADH$_2$	5

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