Transglutaminase-mediated cross-linking of α-crystallin: structural and functional consequences

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Abstract Aggregation and covalent cross-linking of the crystallins, the major structural proteins of the eye lens, increase light scattering by the lens leading to opacification and cataract. Disturbance of calcium homeostasis in the tissue is one of the factors implicated in cataractogenesis. Calcium-activated transglutaminase (TG)-catalyzed cross-linking of some lens proteins has been reported earlier. We show here that α-crystallin, a major structural protein in the lens and a member of the small heat shock protein family, is also a substrate for TG-mediated cross-linking, indicating the presence of donor Lys and acceptor Gln residues in the protein. Upon TG-catalyzed dimerization, the secondary and tertiary structures of the protein are altered, and its surface hydrophobicity reduced. The chaperone-like property of the protein, suspected to be one of its functions in situ, is substantially reduced upon such cross-linking. These results, taken together with earlier ones on lens β-crystallins and vimentin, suggest that TG-mediated events might compromise lens function. Also, since α-crystallin occurs not only in the lens but in other tissues as well, such TG-catalyzed cross-linking and the associated alterations in its structure and activity would be of general pathological interest.

Key words: Calcium; Transglutaminase-mediated cross-linking; Chaperone-like action; α-Crystallin; Cataract

1. Introduction

Calcium-activated, enzyme-mediated modifications of proteins induce changes in the protein profiles in various tissues. In tissues where the turnover of molecules is slow, such modifications can lead to compromised function. The eye lens, which is highly protein-rich, is one such tissue where protein turnover occurs over several decades in time. Modification of the proteins here is thought to lead to lens opacification or cataract. An increase in the intracellular level of free (unbound) calcium is seen in the lens during ageing and in cataract [1]; such increased calcium levels seem to trigger the activity of some otherwise latent enzymes. Important among them are calcium-dependent calpain and transglutaminase (TG). TG has attracted attention after the isolation of ε-(γ-glutamyl)-lysine isopeptide cross-linked products from cataract-bearing lenses [2]. TG catalyzes acyl-transfer reactions by which such isopeptide cross-links are formed between glutamine and lysine residues of proteins and polypeptides [3,4]. TG acts solely on glutamine residues, which function as acceptor substrates, but also displays high specificity for donor lysine substrates [5,6].

Of the three major families of proteins, namely α-, β-, and γ-crystallins, that constitute the mammalian lens, β-crystallins are primarily known to act as TG substrates, forming dimers and oligomers [2,7,8]. In their pioneering work, Lorand and coworkers showed that incubation of lens homogenate in the presence of calcium leads to the formation of 55 and 110 kDa protein products of β-crystallins. In bovine eye lenses, three β-crystallins (βB2-, βB3- and βA3-crystallins) have been identified as potential amine-acceptor substrates for TG [9,10]. The intermediate filament protein vimentin is also reported to be a substrate for TG in the lens [11].

Such cross-linking is expected to have physiological consequences. Formation of high molecular weight aggregates will increase the scattering of light by the lens and decrease light transmission through the tissue. While the physiological role of intermediate filaments is well known, that of the β-crystallins, other than as structural proteins, is yet to be delineated, though studies from our laboratories suggest that they may act as endogenous depots of free calcium. The TG reactions of the other crystallins, namely α and γ, are yet to be studied in detail. We now report on the reaction with these two crystallins as well.

α-Crystallin, a major structural protein of the vertebrate lens, is also expressed in several non-lenticular tissues like heart, brain and kidney. The expression of this protein is increased several-fold during stress and in diseases [12–15]. It is a member of the small heat shock protein family, and possesses a chaperone-like property, which might help to keep the other lenticular proteins from aggregating and precipitating. It occurs in the lens as a hetero-aggregate of two gene products called αA- and αB-crystallins. Though it has so far not been reported to be a TG substrate, the αB-crystallin subunit is known to act as an amine donor for TG reactions [6,10,16]. Two other members of the α-crystallin family of heat shock proteins, sHSP25 [17] and CotM of *Bacillus subtilis* [18], also act as substrates of tissue TG.

We show in this paper that α-crystallin forms TG-mediated dimers, involving both αA- as well as αB-crystallins as substrates. Such cross-linking leads to changes in the structure, conformation, and in the chaperone-like properties of α-crystallin. In contrast to α- and β-crystallins, the core proteins of the mammalian lens, namely γ-crystallins, do not readily form

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TG-mediated cross-links. These results are of interest to the tissue functions of these proteins.

2. Materials and methods

2.1. Lens homogenates

Fresh bovine lenses were obtained from the local abattoir. Human lenses were collected from the donated eyes from the Ramayamma International Eye Bank of the L.V. Prasad Eye Institute, Hyderabad, India. Rabbit lenses were obtained from the animal house of the Centre. The lenses were decapsulated and homogenized in 50 mM Tris–HCl buffer, pH 7.5, containing 20% glycerol, 2 mM leupeptin and either $CaCl_2$ (2–8 mM) or EDTA (2 mM) as described earlier [8]. After incubation at 37°C for set periods of time, the reaction was stopped by the addition of 10 mM EDTA to the incubation mixture containing calcium, and analyzed using polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS–PAGE), with added dithiothreitol (DTT).

2.2. Purification of αA- and αB-crystallins

Bovine lens α-crystallin was isolated and purified on a Bio-Gel A-1.5m column and used for TG-mediated reactions. In some experiments, αA- and αB-crystallins were used for TG-mediated cross-linking studies. For this purpose, the subunits of α-crystallin were separated on a C_4 reverse phase column using a water–acetonitrile gradient containing 0.1% trifluoroacetic acid [19]. The peaks corresponding to αA- and αB-crystallins were pooled and lyophilized. The lyophilized samples were dissolved in 50 mM Tris–HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA and 8 M deionized urea and left overnight at 4°C. αA- and αB-crystallins were generated from these samples by extensive dialysis against 50 mM Tris buffer without urea. The resultant protein fractions were concentrated using an Amicon ultrafiltration unit with a cut-off of 10 000 Da.

2.3. Purification of TG

TG was purified from guinea pig liver as described earlier [20]. Briefly, fresh guinea pig liver (75 g) was homogenized in 150 ml of cold 5 mM Tris–HCl, pH 7.5, 2 mM EDTA (homogenization buffer) containing 250 mM sucrose, and centrifuged at 30 000 rpm for 1 h. The supernatant was passed through several layers of cheese-cloth and loaded on a DEAE-cellulose (DE52, Whatman) column, which was equilibrated with the homogenization buffer. After loading, the column was washed thoroughly and the enzyme was eluted with a linear gradient of NaCl (0–1 M) in the homogenization buffer. Selected fractions were pooled and dialyzed exhaustively against the buffer. The dialysate was subjected to ammonium sulfate precipitation (50% saturation), clarified by centrifugation and the supernatant was made to 80% saturation in ammonium sulfate at 4°C, centrifuged and the precipitate was dissolved in the buffer, dialyzed, and stored as aliquots at −20°C.

2.4. Reactions of the purified crystallins with TG

The reaction was carried out at 37°C, in a total volume of 100 μl of 50 mM Tris–HCl, pH 7.5, containing 20% glycerol, 2 mM leupeptin, 0.5 mg of the chosen crystallin, 4 μg of TG and 8 mM $CaCl_2$ (or 2 mM EDTA). In case of reactions in the presence of dansylcadaverine (2 mM), and of histamine (75 mM), the reactions were performed in the presence of 8 mM calcium [11]. The reaction was arrested by the addition of 20 mM EDTA. Intrinsic TG cross-linking was done by adding Ca^{2+} without adding the enzyme in the lens homogenate.

2.5. Electrophoresis

Reaction mixtures were added to one volume of 50 mM Tris–HCl, pH 6.8, 9 M urea, 40 mM DTT and 2% SDS, incubated at 37°C for 1 h. SDS–PAGE was performed on 12% acrylamide, and the gels were stained with Coomassie brilliant blue R. Two-dimensional (2-D) gel electrophoresis was performed by NEPHGE using pH 3–10 and 5–8 ampholines and 1200 Vh [21]. For Western blotting, the protein was transferred on a nitrocellulose membrane by a semi-dry method, and immunoblotted using polyclonal α-crystallin antibody.

2.6. Purification of cross-linked proteins

TG cross-linked dimers of α-crystallin were separated from the uncross-linked fraction on a Sepharose 6B gel filtration column (115×1.2 cm) in the presence of 6 M urea. The column was equilibrated with 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA containing 6 M urea. 10 mg of protein reaction mixture was applied to the column. Proteins were eluted with a flow rate of 7 ml/h and monitored by measuring absorption at 280 nm. The minor peak eluting before the monomer fraction was pooled, concentrated using an Amicon ultrafiltration unit with a 10 000 Da cut-off, and dialyzed extensively against the column buffer without urea.

2.7. Spectral measurements

The steady-state emission spectra of α-crystallin and its dimers were recorded on a Hitachi F-4010 fluorescence spectrophotometer with excitation at 295 nm. The excitation and emission band passes were set at 5 and 3 nm, respectively. The emission monochromator was scanned from 310 to 400 nm in the correct spectrum mode, to measure the intrinsic fluorescence of the proteins. For fluorescence measurements involving the extrinsic probe 8-anilinonaphthalene-1-sulfonate (ANS), 10 μl of a 10 mM methanolic stock of ANS was added to 1 ml of 0.2 mg/ml protein solution and incubated for about 20 min. The excitation monochromator was set at 365 nm (3 nm band pass), and spectra were recorded from 400 to 530 nm (1.5 nm band pass) in the correct spectrum mode. Circular dichroism (CD) measurements were carried out on a JASCO J-715 spectropolarimeter. Sample concentrations were 0.6 mg/ml in 50 mM Tris–HCl buffer containing 100 mM NaCl. The near- and far-UV CD spectra were recorded using 1 cm and 0.05 cm path length cells, respectively, and ellipticities were represented as millidegrees.

2.8. Assay of chaperone-like activity

The thermal aggregation of βL-crystallin at 60°C was used to monitor the chaperone-like properties of α-crystallins as described earlier [22]. The buffer (10 mM phosphate, pH 7.5, containing 100 mM NaCl) containing α-crystallin (native or control) or its TG-mediated dimer (final concentration 0.1 mg/ml) was pre-incubated at 60°C for 10 min before the addition of 150 μl of βL-crystallin to a final concentration of 0.2 mg/ml. Aggregation was monitored by measuring light scattering using a Hitachi F-4000 fluorescence spectrophotometer, with both excitation and emission monochromators set at 465 nm and band passes at 3.0 and 1.5 nm, respectively. The results were expressed as percentage protection, calculated as $(I_t - I_{t+\alpha})/I_t$, where I_t is the intensity of scattered light for target protein βL and $I_{t+\alpha}$ is the intensity of scattered light in the presence of native or dimeric α-crystallin [19].

3. Results

3.1. Detection of endogenous TG activity in lens homogenates

We incubated human lens homogenates with various concentrations of $CaCl_2$ (2–8 mM) at 37°C and analyzed the products using electrophoresis. SDS–PAGE, in the presence of DTT and urea, showed the presence of bands around 40–45 kDa, 55 kDa and 110 kDa (see Fig. 1a). Similar bands were also seen when rabbit lens and bovine lens homogenates (data not shown) were treated with calcium likewise. Lorand et al. [8] had earlier shown this reaction to be due to the cross-linking of the lens proteins by endogenous TG activated by the added calcium, and the 55 and 110 kDa products to be due to the consequent dimerization and oligomerization of the β-crystallins. That the formation of some of these bands is inhibited by the Ca^{2+} chelator EDTA (Fig. 1) is consistent with this interpretation.

3.2. TG-mediated dimerization of α-crystallin

While the 55 and 110 kDa products have been identified as arising from β-crystallins, the product in the 40–45 kDa region (which had been ignored or not readily detected so far) needs to be identified. Based on molecular mass considerations, it appears to be due to the involvement of α- and/or γ-crystallins, either as homo- or hetero-dimers. Accordingly,

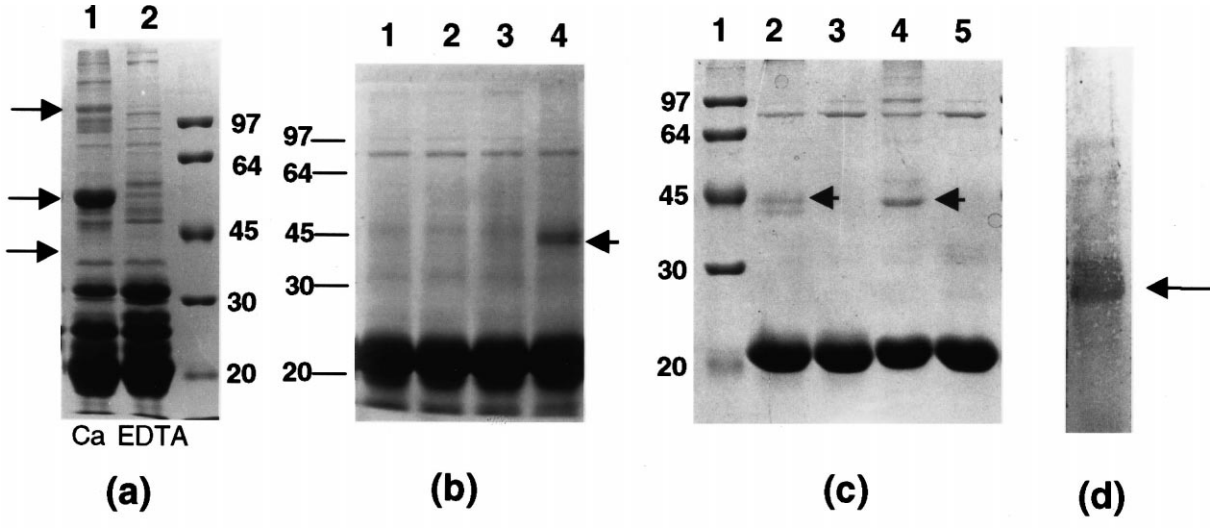

Fig. 1. Calcium-activated TG-induced cross-linking of lens proteins. a: SDS–PAGE profile of human lens homogenate with 8 mM Ca^{2+} (lane 1) or 2 mM EDTA (lane 2). Lane 3 is the molecular mass standards. Arrows indicate the cross-linked products. Human lens homogenates were incubated at 37°C in a total volume of 100 μl of 50 mM Tris–HCl, pH 7.5 (containing 20% glycerol and 2 mM leupeptin) plus either 8 mM $CaCl_2$ or 2 mM EDTA. The reaction was arrested by the addition of 20 mM EDTA. Electrophoresis was done by solubilizing the mixture in 50 mM Tris–HCl, pH 6.8, 9 M urea, 40 mM DTT and 2% SDS, incubated at 37°C for 1 h prior to running the gel. b: TG-mediated dimerization of α-crystallin. SDS–PAGE profile of α-crystallin treated with TG in the presence of 2 mM EDTA (lane 1), and in the presence of 2 mM $CaCl_2$+75 mM histamine (lane 2), 2 mM $CaCl_2$+2 mM dansylcadaverine (lane 3), and in the presence of 8 mM $CaCl_2$ (lane 4). Arrow indicates the dimer in lane 4. c: Dimerization occurs in αA- and in αB-crystallins. SDS–PAGE profile of αA-crystallin (lanes 2 and 3) and αB-crystallin (lanes 4 and 5) treated with TG in the presence of 8 mM Ca^{2+} (lanes 2 and 4), or 2 mM EDTA (lanes 3 and 5). Lane 1 has molecular mass markers. The arrows indicate the dimers formed. d: Antibody-based identification of α-crystallin in the cross-linked dimer. Western blotting of α-crystallin dimer using polyclonal antibody.

we treated isolated (bovine) α-, β-, and γ-crystallins with the enzyme TG in the presence of added Ca^{2+}. While β-crystallin yielded the expected cross-linked products, we found that α-crystallin too yielded cross-linked products, with a molecular mass in this molecular weight region (Fig. 1b). In contrast, when we tried the same experiments with γ-crystallins, we found that they did not generate any such products under these experimental conditions. Thus the 40–45 kDa protein seen in Fig. 1 appears to be Ca^{2+}-activated, TG-mediated dimer(s) of α-crystallin. (In addition, higher molecular weight oligomers are also formed, though in much lower yields). EDTA and the competitive inhibitors dansylcadaverine and histamine inhibit the formation of the product, confirming the involvement of the enzyme TG in the reaction. Fig. 1c shows that both αA- and αB-crystallins are capable of forming TG-mediated dimers. Since only a small fraction is cross-linked, we have confirmed the results by Western blotting using α-crystallin antibody as shown in Fig. 1d.

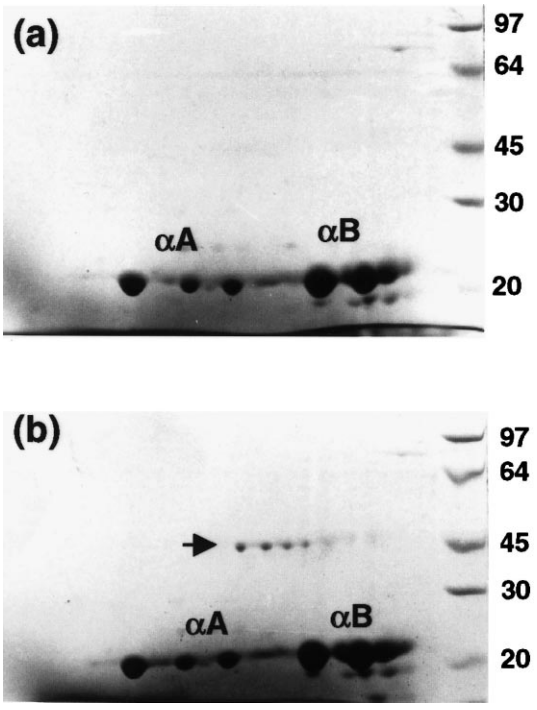

Fig. 2. 2-D electrophoresis reveals both αA and αB dimers. 2-D electrophoresis profile of α-crystallin treated with TG (a) in the presence of 2 mM EDTA, (b) in the presence of 8 mM Ca^{2+}. The arrow indicates the dimers formed.

α-Crystallin consists of two gene products, αA and αB, which can be resolved on 2-D gel electrophoresis. In addition, these molecules occur both in the free and phosphorylated forms (usually called αA1 (phosphorylated), A2 (free), and likewise αB1 and B2). 2-D gel electrophoresis patterns, shown in Fig. 2a, reveal this multiplicity of subpopulations of the protein. Incubation of the protein with TG produces no reaction, while the addition of calcium leads to several types of dimers. Of the 10 possible dimers arising out of A1, A2, B1 and B2, we can distinctly see at least four in Fig. 2b. From their relative positions in the 2-D gel, they appear to be hetero-dimers. Homo-dimers of αA- and αB-crystallins are not as readily seen in the 2-D gel as in the SDS–PAGE of Fig. 1c.

3.3. Analysis and study of the dimers

The dimeric fraction was separated from the unreacted fraction on a gel filtration chromatography on Sepharose 6B in the presence of 6 M urea. The purification profile is shown in Fig. 3, where the dimer elutes before the monomeric fractions

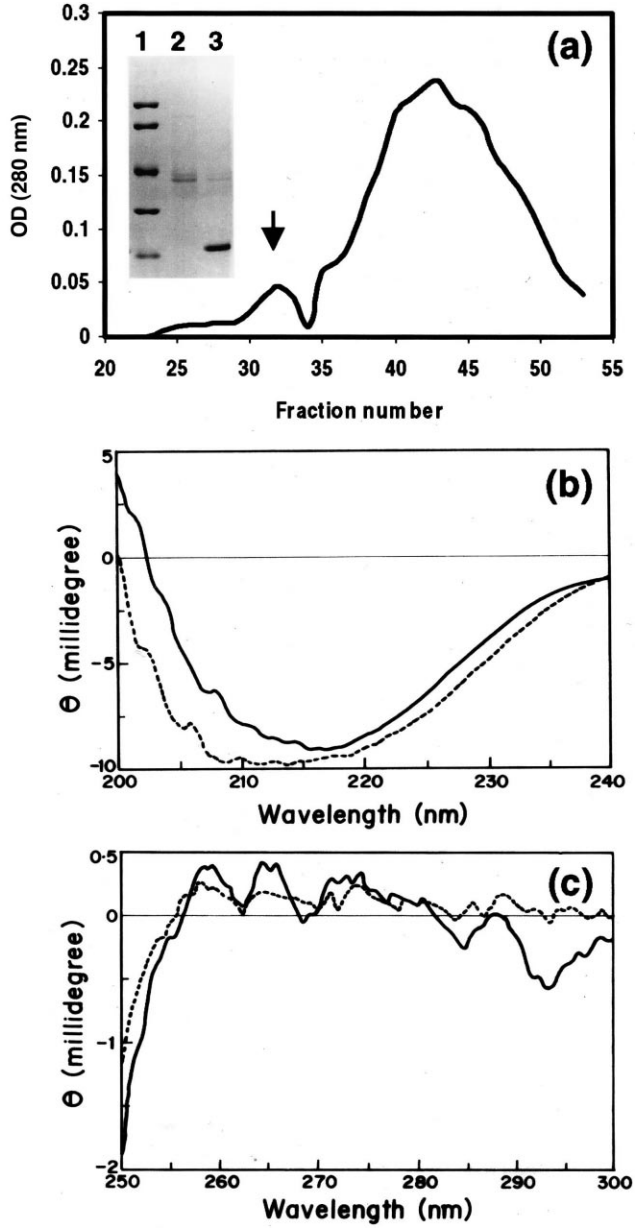

Fig. 3. (a) Purification of α-crystallin dimer. Purification of α-crystallin dimer was performed on a Sepharose 6B column (115×1.2 cm) in 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 6 M urea. Arrow indicates the dimer peak. Inset shows the SDS–PAGE pattern of the purified dimer (lane 2), unreacted α-crystallin (lane 3) with lane 1 as molecular weight standards. (b, c) CD spectra show alterations in the secondary and tertiary structures of α-crystallin upon TG-mediated dimerization. (b) Far-UV CD spectra of α-crystallin control (–) and its dimer (---). (c) Near-UV CD spectra of α-crystallin (–) and dimer (---). The concentration of protein used was 0.6 mg/ml in Tris–HCl, pH 7.4, containing 100 mM NaCl. Ellipticities are represented as millidegrees.

under dissociating conditions and is characterized by SDS–PAGE (Fig. 3a, inset). The purified dimer was refolded by extensive dialysis against 50 mM Tris–HCl, pH 7.5, containing 100 mM NaCl and was used for further spectral studies. For comparison, α-crystallin was treated similarly with 6 M urea and dialyzed against 50 mM Tris–HCl, pH 7.5, containing 100 mM NaCl and was used as a reference in all experiments. It is to be noted however that the 'dimer' we are working with is a mixture of homo- and hetero-dimers of αA- and αB-crystallins. The spectral and chaperone-like properties of this mixture of dimers are reported in Figs. 3 and 4.

3.4. Structural and conformational properties of the α-crystallin dimers

Fig. 3 compares the far- and near-UV CD spectra of α-crystallin dimers with those of native α-crystallin treated in the same way. The far-UV CD spectrum of the dimer showed alterations in the region of 215–205 nm and in the cross over point when compared with that of the native α-crystallin (Fig. 3b). The change in CD indicates alterations in the secondary structural elements upon dimerization. Such alterations in the secondary structure were also observed in the R116C mutant of α-crystallin, whose conformation was altered upon mutation [25]. The near-UV CD spectrum of the dimer shows a reduction in tertiary structure (Fig. 3c). The changes in the tertiary structure were also reflected in the intrinsic Trp emission fluorescence spectra. The intensity of Trp emission was decreased by 35% with a red shift of 2 nm and the appearance of a weak shoulder at 348 nm, indicating Trp to be in a more

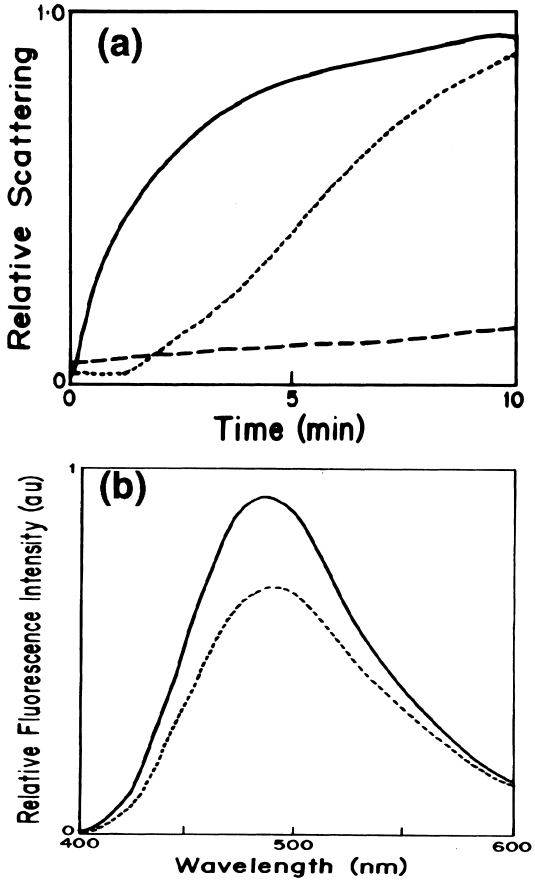

Fig. 4. a: Chaperone-like activity of α-crystallin decreases upon TG dimerization. Incubation at 60°C of βL-crystallin alone (–), and in the presence of α-crystallin (---) and in the presence of TG-mediated α-crystallin dimer (.....). βL-Crystallin 200 μg/ml in 10 mM phosphate buffer, pH 7.2, containing 100 mM NaCl, and α-crystallin or its dimer 100 μg/ml. b: Hydrophobic surface of α-crystallin is decreased upon TG dimerization. Emission spectra of the hydrophobic reporter fluorophore ANS (100 μM added to 0.1 mg/ml protein) bound to native monomeric α-crystallin (–), and its TG dimer (---).

flexible environment and exposed to solvent in the dimer (data not shown).

3.5. Chaperone-like properties of the α-crystallin dimers

The chaperone-like properties of α-crystallin and its variants have been studied extensively [22–25]. We have investigated the chaperone-like activity of the dimer and compared it with that of the parent native α-crystallin. The activity was assayed in terms of the ability to inhibit the heat-induced aggregation of βL-crystallin. Fig. 4 shows that native α-crystallin prevents the thermal aggregation of βL-crystallin almost completely; however, the protection ability of the dimer is only about 50% of that of the parent molecule, indicating a significant loss in the chaperone activity due to dimerization. Since the chaperone-like activity of α-crystallin has been shown to be associated with hydrophobicity [25], we monitored the surface hydrophobicity of the dimer and compared it with that of the parent native α-crystallin, using the hydrophobic probe ANS. The 20% decrease in the ANS emission spectrum of the dimer indicates a reduction in the surface hydrophobicity upon dimerization (Fig. 4b).

4. Discussion

While the crystallins had generally been thought to have only a structural role in the lens, there are growing indications that seem to suggest that they may have other physiological roles too. α-Crystallins show chaperone-like activity and help in vitro in inhibiting the aggregation and precipitation of other lens proteins. The role of β-crystallins is not clear, though work from our laboratory and elsewhere (Vrensen, personal communication) suggests that they may aid calcium homeostasis by acting as sequestering agents or depots of free Ca^{2+} ions. It is likely that TG-mediated cross-linking of β-crystallins might alter this ability and thus the homeostasis.

Our present results show that α-crystallins can also be TG cross-linked, and that their chaperone-like ability is thereby compromised. The third major class of proteins in the mammalian lens, namely γ-crystallins, has so far not been implicated in any non-structural, physiological role in the tissue. It is interesting that despite their high sequence homology with the β-crystallins, they do not form TG-mediated cross-links. It would seem that they do not have appropriately disposed Gln and Lys residues accessible to enable TG to catalyze the isopeptide bond formation. Interestingly, γ-crystallins are the ones that form the dityrosine cross-links most readily among the mammalian lens crystallins, presumably because of the availability of properly disposed Tyr residues to make the bond [26,27].

It is in this context that the present results are interesting. Our data show that TG cross-links α-crystallin to produce dimers (and higher oligomers). Both αA- and αB-crystallins act as TG substrates, indicating that both proteins have the requisite acceptor glutamine and donor lysine residues needed for the cross-linking. TG-mediated dimerization is seen to lead to a noticeable loss of the tertiary structure, and to an alteration in the secondary structure that is consistent with the suggestion that it might be due to changes in the packing of secondary structural elements [28]. The chaperone-like property is weakened significantly, which appears to be due to changes in the surface hydrophobicity. Interestingly, such differences in secondary and tertiary structure, decrease in hydrophobicity with a concomitant reduction in the chaperone-like activity have also been observed in a R116C mutant of αA-crystallin [29]. Earlier studies have indicated that the chaperone-like activity of α-crystallin decreases upon in vitro treatment with calpain II, in senile and induced cataract in vivo, and in old human lenses [30,31].

α-Crystallin belongs to the small heat shock protein family. Other members of this family, sHSP25 and CotM, are reported to be amine donor substrates for TG cross-linking [17,18]. Non-lenticular expression of α-crystallin is increased several-fold during stress and in diseases such as Alzheimer's disease [12–15]. It would be interesting to study the implications of possible cross-linking of non-lenticular α-crystallins by TG, particularly when its role is implicated in certain pathological conditions related with protein cross-linking and aggregations such as in Alzheimer's disease and in neuronal ageing. TG-catalyzed cross-linking might thus influence several pathological conditions.

It is also possible that the age- and disease-related post-translational modifications to these proteins might render the lens more reactive towards TG cross-linking. In fact, Weinreb and Dovrat [32] have shown that TG activity in the lens is affected by UV-A radiation and proteins become more reactive towards TG-mediated cross-linking, thus further implicating the reaction during ageing and in cataract.

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