

Enhanced degradation and decreased stability of eye lens α -crystallin upon methylglyoxal modification[☆]

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Abstract

Methylglyoxal (MGO), a potent glycating agent, forms advanced glycation end products (AGEs) with proteins. Several diabetic complications including cataract are thought to be the result of accumulation of these protein-AGEs. α -Crystallin, molecular chaperone of the eye lens, plays an important role in maintaining the transparency of the lens by preventing the aggregation/inactivation of several proteins/enzymes in addition to its structural role. Binding of adenosine-5-triphosphate (ATP) to α -crystallin has been shown to enhance its chaperone-like function and protection against proteolytic degradation. In the earlier study, we have shown that modification of α -crystallin by MGO caused altered chaperone-like activity along with structural changes, cross-linking, coloration and subsequent insolubilization leading to scattering of light [Biochem. J. 379 (2004) 273]. In the present study, we have investigated ATP binding, stability and degradation of MGO-modified α -crystallin. Proteolytic digestion with trypsin and chymotrypsin showed that MGO-modified α -crystallin is more susceptible to degradation compared to native α -crystallin. Furthermore, ATP was able to protect native α -crystallin against proteolytic cleavage but not MGO-modified α -crystallin. Interestingly, binding studies indicate decreased ATP binding to MGO-modified α -crystallin and support the decreased protection by ATP against proteolysis. In addition, differential scanning calorimetric and denaturant-induced unfolding studies indicate that modification of α -crystallin by MGO leads to decreased stability. These results indicate that MGO-modification of α -crystallin causes partial unfolding and decreased stability leading to enhanced proteolysis. Cross-linking of these degraded products could result in aggregation and subsequent insolubilization as observed in senile and diabetic cataract lenses.

Keywords: α -crystallin; lens; methylglyoxal; trypsin; chymotrypsin; ATP; stability; degradation; differential scanning calorimetry

Cataract results from loss of transparency of the normal crystalline eye lens and it is the major cause of blindness. Though, many risk factors are associated with the pathogenesis of cataract, aging and diabetes are dominant risk factors (Baynes et al., 1989; Harding, 1991; Congdon et al., 2003). The eye lens contains high concentrations of proteins

(35% of wet weight), largely crystallins. Three major classes of crystallins, α -, β -, and γ -crystallins constitute about 90% of the total soluble lens protein and are considered to determine the refractive properties of the lens (Delaye and Tardieu, 1983; Harding, 1991). Being long-lived proteins with very little turnover, lens crystallins are susceptible to various post-translational modifications such as truncation at N and C termini (Thampi et al., 2002a,b), glycation (Lyons et al., 1991; Tessier et al., 1999), phosphorylation (Kantorow and Piatigorsky, 1998), racemization, deamidation, disulfide formation and methionine oxidation (Hanson et al., 2000; Ueda et al., 2002). Among these, non-enzymatic glycation is a predominant post-translational modification involved in aging and diabetic cataracts (Chiou et al., 1981; Perry et al., 1987; Baynes et al., 1989; Harding, 1991; Lyons et al., 1991). Glycated protein levels were shown to be increased in lens

Abbreviations: AGE, advanced glycation end products; DSC, differential scanning calorimetry; MGO, methylglyoxal; sHSP, small heat shock proteins.

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with age and in diabetes (Chiou et al., 1981; Perry et al., 1987). In non-enzymatic glycation (Maillard reaction) protein amino groups react with glyating agents such as sugars and reactive dicarbonyls, initially forming a Schiff base, which further forms advanced glycation end products (AGEs) by several subsequent reactions. Formation of these protein-AGEs is likely to affect the protein conformation and function, which may in turn lead to changes in its interaction with other molecules (Beswick and Harding, 1987; Liang and Rossi, 1990; Kumar et al., 2004).

Among the lens proteins, α -crystallin is a major constituent reaching up to 50% of total soluble protein (Groenen et al., 1994; Ganea, 2001; Horwitz, 2003). In the lens, α -crystallin has been considered to play an important role in maintaining the transparency because of its molecular chaperone-like function in preventing the aggregation of other proteins and also being a key structural element (Groenen et al., 1994; Reddy et al., 2000; Ganea, 2001; Brady et al., 2001; Horwitz, 2003). Methylglyoxal (MGO), a major dicarbonyl compound, is present in high concentrations in lens compared to plasma or any other tissue and its levels increase several fold during diabetes (Thornalley, 1993; Ahmed et al., 2003). Compared to other potential glyating agents, MGO is highly reactive and forms AGEs with proteins. Moreover, the chaperone-like activity of α -crystallin was shown to be compromised in diabetic human and rat lenses (Thampi et al., 2002a). Thus, studies are initiated to investigate the effect of MGO on α -crystallin structure and function (Derham and Harding, 2002; Nagaraj et al., 2003; Kumar et al., 2004). MGO modification of α -crystallin led to cross-linking, coloration and subsequent insolubilization of lens proteins causing scattering of light (Derham and Harding, 2002; Kumar et al., 2004). However, contradicting results were reported for chaperone-like activity of MGO-modified α -crystallin (Derham and Harding, 2002; Nagaraj et al., 2003; Kumar et al., 2004). Previously, we have shown that MGO can induce opacification in lens organ culture and accumulation of substantial quantities of AGE- α -crystallin in the insoluble fraction (Kumar et al., 2004). Several reports suggest that pathogenesis of age-related nuclear cataracts are associated with insolubilization of α -crystallin (Takemoto and Boyle, 1998; Truscott et al., 1998). Increased susceptibility of lens proteins to proteolysis was reported in human cataracts (Harding, 1972). Furthermore, biochemical analysis of the insoluble fraction from nuclear cataract lenses showed coloured, cross-linked proteins and highly modified protein fragments largely derived from α -crystallin (Truscott et al., 1998).

In the lens, adenosine-5-triphosphate (ATP) has been found to be present in significant concentrations (~ 3.5 mM) and its levels were found to decrease in cataract lenses (Hothersall et al., 1988; Deussen and Pau, 1989). Although, binding of ATP to α -crystallin has been reported (Palmisano et al., 1995), the significance of ATP interaction with α -crystallin remained unclear. ATP binding was known to cause the release of denatured target proteins bound to

α -crystallin, which might be further refolded with the involvement of other co-chaperones or may be targeted to degradation machinery (Wang and Spector, 2001). Studies have shown that binding of ATP to α -crystallin not only enhances its chaperone-like function, but also protects it from degradation by proteases such as trypsin and chymotrypsin (Muchowski and Clark, 1998; Muchowski et al., 1999). Although, N- and C-terminal degradation of lens proteins including α -crystallin was observed in diabetic and age-related cataracts, the mechanism of degradation is not understood. Furthermore, effects of non-enzymatic browning by MGO on α -crystallin susceptibility to proteolysis and interaction with other important small molecules such as ATP have remained unknown.

In the present study, we have investigated the effect of non-enzymatic browning of α -crystallin by MGO on its susceptibility to degradation, ATP mediated protection against proteolysis and stability to get more insights into dicarbonyl-induced modifications of eye lens α -crystallin. Digestion studies by trypsin and chymotrypsin suggest that MGO-modified α -crystallin is more susceptible to degradation compared to native α -crystallin. Moreover, presence of ATP could not prevent the degradation of glyated α -crystallin by proteases as observed with native α -crystallin. Furthermore, binding studies showed a decrease in ATP binding sites for MGO-modified α -crystallin. In addition, differential scanning calorimetric and denaturant-induced unfolding studies suggest decreased stability for MGO-modified α -crystallin.

1. Materials and methods

1.1. Materials

ATP, acrylamide, bis-acrylamide, guanidine hydrochloride (Gdm-Cl), β -mercaptoethanol, MGO (40% v/v), trypsin and chymotrypsin were purchased from Sigma Chemical Co (St Louis, MO). SDS-PAGE markers were procured from Bio-Rad Inc. (California, USA). Sephacryl S-300HR was from Amersham Biosciences (Uppsala, Sweden).

1.2. Purification of α -crystallin

α -Crystallin was purified from calf lenses obtained from local slaughterhouse. Lenses were homogenized in 0.05 M Tris buffer, pH 8.0 containing 0.1 M NaCl and 0.02% sodium azide. The homogenate was centrifuged at 15 000g for 30 min at 4°C to remove insoluble material and the supernatant was applied onto a Sephacryl S-300HR size exclusion column (100 \times 2.5 cm) equilibrated with the above buffer. The proteins were also eluted using the same buffer. Fractions corresponding to α_L -crystallin were pooled and dialysed. The purity of α -crystallin was assessed by SDS-PAGE and protein concentration determined by a modified Lowry method.

1.3. Incubation of α -L-crystallin with methylglyoxal

Stock of 1 M MGO was prepared in 100 mM sodium phosphate buffer, pH 7.5 and the pH was adjusted to neutral if required. α -Crystallin was incubated with different concentrations of MGO in 100 mM sodium phosphate buffer, pH 7.5 at 37°C in dark under sterile conditions for various time periods as mentioned in figure legends. At the end of incubation period, unbound MGO was removed by dialysis against the same buffer and protein was estimated as mentioned above.

1.4. Proteolytic digestion studies

Unmodified and MGO-modified α -crystallin digestion was studied, using the proteases trypsin and chymotrypsin. For each reaction, 200 μ g of α -crystallin was taken in a final volume of 150 μ l containing 100 mM Tris, pH 7.4, 3.5 mM $MgCl_2$, 10 mM KCl and 0.01% Tween-20. Proteolytic cleavage in the presence of ATP was studied by adding ATP to the above reaction mixture at a final concentration of 3.5 mM. Incubation was started by adding 10 μ l of trypsin or chymotrypsin (0.17 mg ml⁻¹) and samples were incubated at 37°C. At each time period, 20 μ l aliquots were withdrawn and the reaction was quenched with 2 μ l of 100 mM PMSF and placed on ice. Samples were resolved on a 15% polyacrylamide gel and stained with Coomassie blue R-250.

1.5. ATP binding studies

Binding of ATP to native and MGO-modified α -crystallin was measured by fluorescence spectroscopy. Fluorescence measurements were performed on a Cary Eclipse spectrofluorometer. The spectral bandwidths were 2 and 5 nm, respectively, for excitation and emission. To determine ATP binding constants for native and MGO-modified α -crystallin, small aliquots of ATP stock (7.5 mM) solution were successively added to α -crystallin (3.75 μ M in 20 mM sodium phosphate buffer pH 7.5). After each addition of the ATP, the contents were thoroughly mixed and equilibrated for 5 min. Decrease in fluorescence intensity at 335 nm (ex.: 280 nm) was then measured and the readings were corrected for buffer blanks and dilutions. Binding constant and number of binding sites for ATP were calculated from Scatchard plot. Concentration of ATP was calculated by using the extinction coefficient value, $\epsilon_{259} = 15.4$.

1.6. Differential scanning calorimetry

Calorimetric experiments were carried out using a VP-DSC (Microcal Inc) instrument. Samples were exhaustively dialysed at 4°C against 0.02 M potassium phosphate buffer pH 7.4, containing 0.1 M potassium chloride and 1 mM DTT. Before introduction into the calorimetric cells, both dialysate and protein solutions were thoroughly

degassed under vacuum at room temperature. The protein concentration used for calorimetric studies was 1 mg ml⁻¹ for both native and MGO-modified α -crystallin. Differential scanning calorimetry (DSC) scans were recorded from 10 to 90°C at a heating rate of 60°C hr⁻¹.

1.7. Unfolding studies

Denaturant-induced unfolding experiments were performed by incubating protein solutions (0.1 mg ml⁻¹) overnight at room temperature with varying molarities of Gdm-Cl in 0.1 M phosphate buffer to ensure that equilibrium was attained before measurements were made. The wavelength of maximum emission and fluorescence intensity at 335 nm was monitored by excitation at 280 nm.

2. Results and discussion

Dicarbonyl compounds such as MGO and glyoxal (GO) have been identified as predominant source for the formation of AGE in various tissues including the lens. Recently, we have demonstrated that non-enzymatic browning of α -crystallin by MGO/GO produced protein-AGEs which resulted in the formation of covalent cross-links leading to high-molecular weight (HMW) aggregates (Kumar et al., 2004). However, in vitro modification of α -crystallin with MGO resulted in either increased or decreased chaperone-like activity depending upon the type of assay and conditions employed to measure chaperone activity (Derham and Harding, 2002; Nagaraj et al., 2003; Kumar et al., 2004). Several modifications such as hydrogen peroxide mediated oxidative damage, which lead to increased carbonyl content and cross-linking, are known to enhance the degradation of modified proteins (Huang et al., 1995). Nonetheless, the effect of non-enzymatic glycation of lens proteins on their susceptibility to degradation has not been studied. Therefore, in this study, we have investigated the effect of MGO on α -crystallin with respect to its susceptibility to proteases, ATP binding, stability and unfolding for a greater understanding of MGO-induced modifications to lens proteins with regard to cataractogenesis.

The proteolytic susceptibility of α -crystallin modified with 5 mM MGO for 24 hr was analysed by SDS-PAGE. Compared to native α -crystallin, MGO-modified α -crystallin was rapidly digested by trypsin (Fig. 1A). Trypsin is a serine protease that cleaves at the C-terminal of the basic amino acids Lysine and Arginine. At 0 time, native bovine α -crystallin showed two bands at around 20 kDa (corresponding to α A and α B), but MGO-modified α -crystallin shows HMW bands. These HMW-aggregates are apparently related to the formation of covalent cross-links due to MGO-modification (Riley and Harding, 1995; Kumar et al., 2004). Despite the cross-linking of MGO-modified α -crystallin it was digested completely in contrast to its

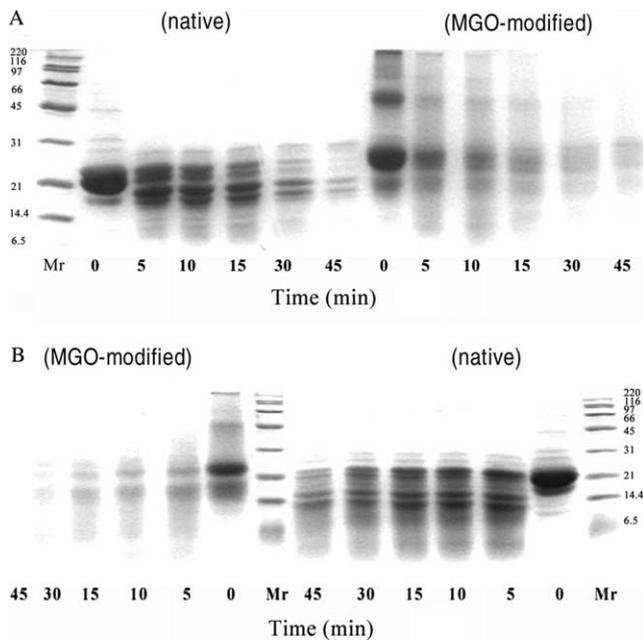


Fig. 1. Proteolysis of native and modified α -crystallin by trypsin (A) and chymotrypsin (B) at 37°C. α -Crystallin was modified by incubating with 5 mM MGO for 24 hr. Samples were analysed by SDS-PAGE on 15% gels. The time course for the proteolysis is indicated at the bottom of the gel. Molecular weight standards in kDa are marked alongside.

native counterpart. Similarly, MGO-modified α -crystallin showed more susceptibility to digestion by another protease chymotrypsin, which cleaves at amino acids with bulky side chains, such as Phe, Trp or Tyr (Fig. 1B). Thus, SDS-PAGE analysis of trypsin and chymotrypsin digested native/modified α -crystallin demonstrates that MGO-modification enhances the susceptibility to degradation by proteases. Such enhanced degradation of MGO-modified α -crystallin may result from the exposure of buried target sites for proteases as a consequence of its unfolding. Our previous studies which show conformational changes at secondary and tertiary structure level of MGO-modified α -crystallin (Kumar et al., 2004) do suggest a possibility for exposure of proteolytic cleavage sites. Recent studies which show increased degradation of α -crystallins, particularly at C-terminus in diabetic lenses (Thampi et al., 2002a,b), further support the possibility that glycation may increase the susceptibility to proteolytic cleavage.

It should be noted that the presence of ATP has been shown to enhance chaperone activity and protect α B-crystallin against degradation by serine proteases, trypsin and chymotrypsin (Muchowski and Clark, 1998; Muchowski et al., 1999). The cleavage sites that were masked in the presence of ATP were shown to be in the region of the core α -crystallin domain between Glu67 and Ile161. In the present study, we have analysed the shielding effect of ATP against the proteolytic digestion of MGO-modified α -crystallin. As shown in Figs. 2A and 3A, digestion of native α -crystallin by trypsin and chymotrypsin was significantly protected in the presence of ATP similar to

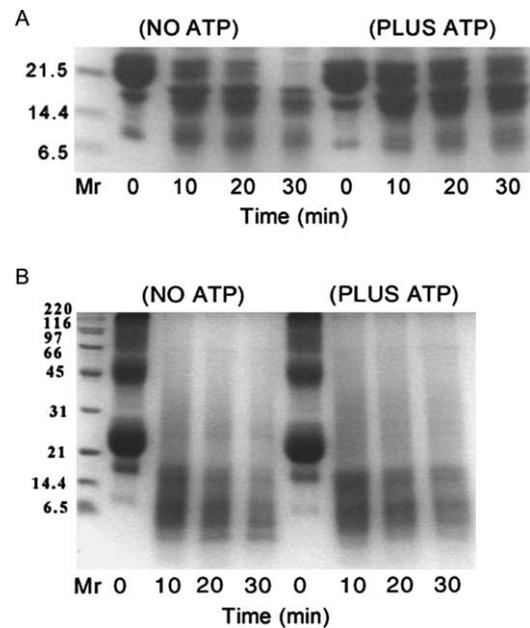


Fig. 2. Trypsin digestion of native (A) and MGO-modified (B) α -crystallin in the absence and presence of ATP at 37°C as analysed by SDS-PAGE. α -Crystallin was modified by incubating with 5 mM MGO for 24 hr. The time course for the proteolysis was indicated at the bottom of the gel. Molecular weight standards in kDa are marked alongside.

the earlier observation with α B-crystallin (Muchowski et al., 1999). However, the protection by ATP against chymotrypsin cleavage was not as prominent that of with trypsin. Interestingly, ATP could not afford protection to

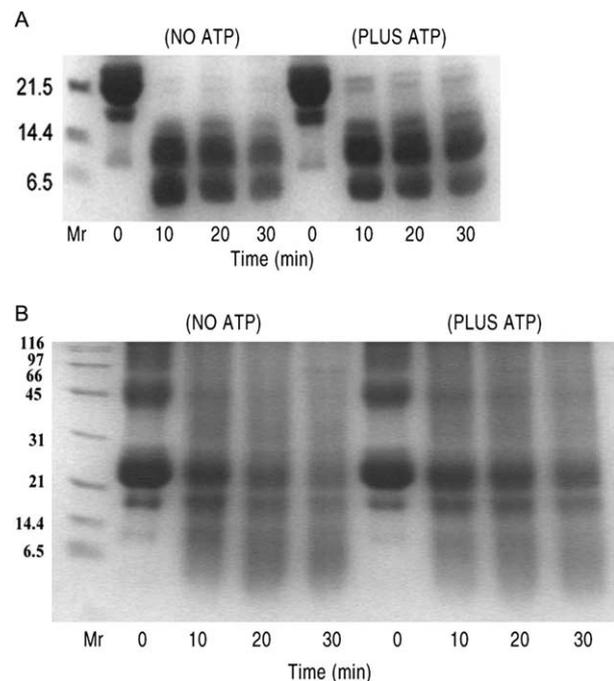


Fig. 3. Chymotrypsin digestion of native (A) and MGO-modified (B) α -crystallin in the absence and presence of ATP at 37°C as analysed by SDS-PAGE. α -Crystallin was modified by incubating with 5 mM MGO for 24 hr. The time course for the proteolysis was indicated at the bottom of the gel. Molecular weight standards in kDa are marked alongside.

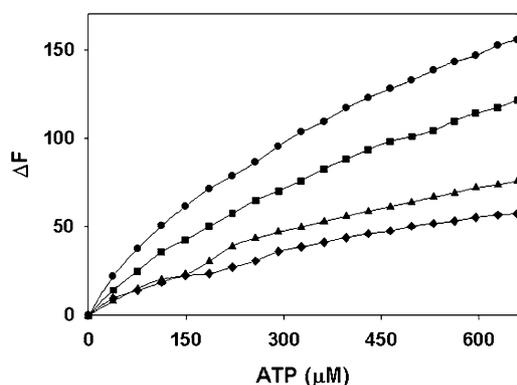


Fig. 4. Stern-Volmer plot for quenching of tryptophan fluorescence of native and MGO-modified α -crystallin by ATP. α -Crystallin was modified by incubating in the absence (circles) and presence of 1 (squares), 5 (triangles) and 10 mM MGO (diamonds) for 72 hr.

MGO-modified α -crystallin against trypsin/chymotrypsin digestion (Figs. 2B and 3B). This could be due to conformational changes resulting from modification and/or modification of amino acid residues by MGO that constitute ATP binding sites. Therefore, we have also investigated the binding of ATP to native and MGO-modified α -crystallin.

Quenching of the tryptophan fluorescence for native and MGO-modified α -crystallin in the presence of ATP was shown as a Stern-Volmer plot (Fig. 4), which indicates a decrease in ATP binding for MGO-modified α -crystallin. The data for the binding of ATP to native and MGO-modified α -crystallin is presented in the form of Scatchard plot for quantification (Fig. 5), where intercept on X-axis gives the number of binding sites per subunit while slope represents K_d (dissociation constant). Linear regression analysis of the experimental data yielded similar slopes for both the native and α -crystallin modified with different concentrations of MGO (Fig. 5 and Table 1), suggesting no significant change in ATP binding affinity (K_d) upon MGO modification. Interestingly, however, MGO-modified α -crystallin has fewer ATP binding sites compared to unmodified protein (Fig. 5 and Table 1). These data indicate that non-enzymatic browning of α -crystallin by MGO leads to decreased ATP binding sites thereby compromising the protection afforded by ATP against the proteolytic cleavage. Although, fluorescence emission studies may not provide accurate data to quantitate ATP binding to α -crystallin, these results could be used for a simple comparison between native and modified protein. In the context of decreased levels of ATP in different types of cataract (Harding and Crabbe, 1984; Hothersall et al., 1988), the observations that reduced ATP binding and decreased protection of MGO-modified α -crystallin by ATP attains a greater significance.

We have also analysed the stability of MGO-modified α -crystallin by differential scanning calorimetry and denaturant-induced unfolding to characterize further the fate of MGO-modified protein. In a DSC experiment,

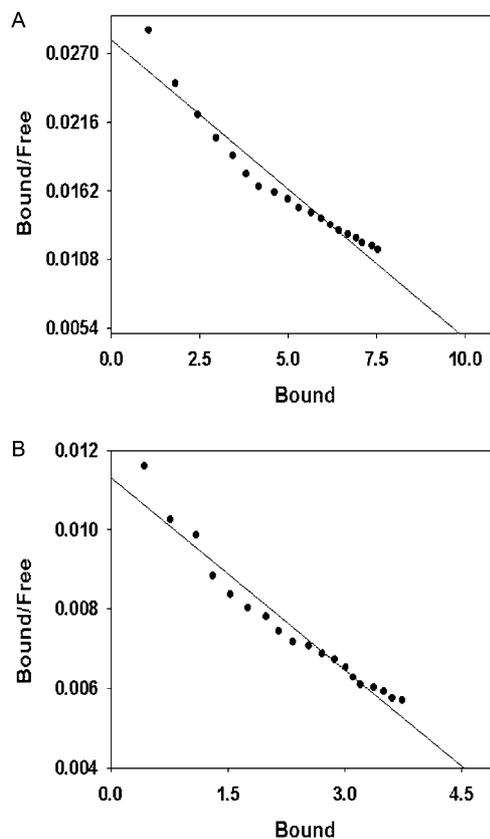


Fig. 5. Scatchard plots for the binding of ATP to native (A) and MGO-modified α -crystallin (B). α -Crystallin was modified by incubating in the absence and presence of 10 mM MGO for 72 hr. The concentration of protein bound ATP was determined by reverse titration method.

unfolding of native structure accompanies an endothermic transition in the thermogram. Native α -crystallin exhibited a positive endotherm at 65°C, similar to previous observations (Steadman et al., 1989; Gesierich and Pfeil, 1996). MGO-modified α -crystallin showed decreased stability as is evident from a decrease in the value of T_p , the temperature maximum heat capacity in a DSC thermogram; T_p was shifted to 56 and 53°C, respectively, for α -crystallin modified with 1 and 10 mM MGO compared to 65°C for the unmodified protein (Fig. 6A). However, the strong tendency of α -crystallin to undergo aggregation during thermal denaturation affects unfolding

Table 1
ATP binding studies of native and MGO-modified α -crystallin

Group	Binding constant (K_d , nM)	Binding sites (N)
Native α -crystallin	3.130	7.9
<i>Modified α-crystallin</i>		
1 mM	1.611	4.5
5 mM	1.27	0.14
10 mM	2.296	0.10

α -Crystallin was incubated with various concentrations of MGO for 72 hr and dialysed before titration. Values are average of three experiments.

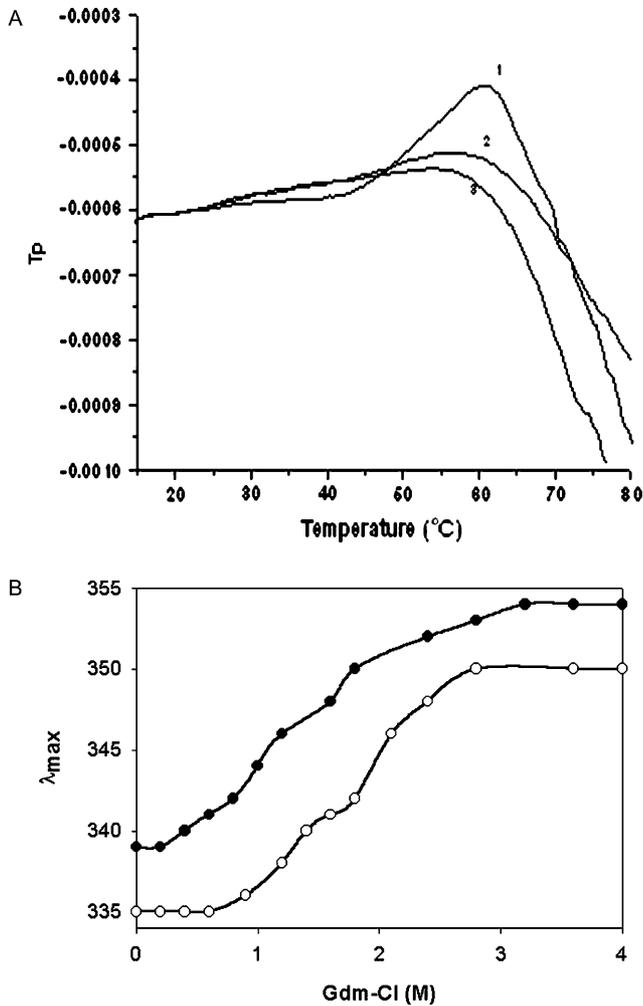


Fig. 6. Effect of MGO modification on the stability of α -crystallin. *Panel A:* Tracings of typical differential scanning calorimeter thermograms (heat capacity versus temperature) of native and modified α -crystallin. *Trace 1*—native α -crystallin, *Trace 2 and 3*— α -crystallin modified, respectively, by 1 and 10 mM MGO for 72 hr. *Panel B:* Gdmcl-induced unfolding of native (open circles) and modified α -crystallin with 10 mM MGO for 72 hr (closed circles) as a plot of λ_{\max} versus denaturant concentration.

baseline and prevents quantitative determination of thermodynamic parameters by DSC (Steadman et al., 1989; Gesierich and Pfeil, 1996), which is observed much more markedly for the MGO-modified α -crystallin in this study. Because of these limitations, we have described the thermal unfolding of native and MGO-modified α -crystallin only in qualitative terms. Further, denaturant-induced unfolding studies support the decreased stability for MGO-modified α -crystallin. As shown in Fig. 6B, MGO-modified α -crystallin showed higher λ_{\max} than native α -crystallin indicating partial unfolding of α -crystallin upon modification by MGO. Moreover, modified α -crystallin unfolds at lower concentrations of denaturant than native α -crystallin (Fig. 6B).

In conclusion, our studies show that non-enzymatic browning of α -crystallin by MGO leads to unfolding and a decrease in its stability, which in turn leads to the exposure of buried proteolytic sites causing enhanced proteolytic degradation. ATP could not protect the glycated α -crystallin from proteolytic degradation as was observed with native α -crystallin. Furthermore, MGO-modified α -crystallin has reduced ATP binding sites. These results thus support our earlier concerns that enhanced chaperone-like activity of MGO-modified α -crystallin needs to be understood with respect to its physiological relevance (Kumar et al., 2004). As depicted in Fig. 7, unfolding and conformational changes due to MGO modification increases susceptibility to degradation and subsequent light scattering due to cross-linking/insolubilization. Hence, it is quite possible that post-translational modification imposed by dicarbonyls may have unfavourable effects on the ability of α -crystallin to inhibit protein aggregation/enzyme inactivation in vivo. Results of the present study along with the previous data (Kumar et al., 2004) provide the basis for the role of non-enzymatic glycation in age-related brunescence and diabetic cataracts.

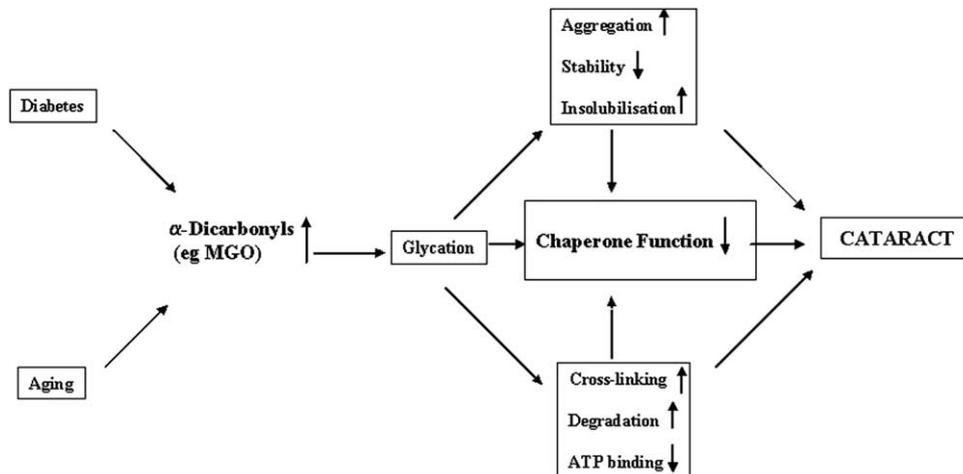


Fig. 7. Schematic representation of possible molecular effects of methylglyoxal on α -crystallin vis a vis lens opacification.

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