Effect of dicarbonyl-induced browning on α-crystallin chaperone-like activity: physiological significance and caveats of *in vitro* aggregation assays

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α-Crystallin is a member of the small heat-shock protein family and functions like a molecular chaperone, and may thus help in maintaining the transparency of the eye lens by protecting the lens proteins from various stress conditions. Non-enzymic glycation of long-lived proteins has been implicated in several age- and diabetes-related complications, including cataract. Dicarbonyl compounds such as methylglyoxal and glyoxal have been identified as the predominant source for the formation of advanced glycation end-products in various tissues including the lens. We have investigated the effect of non-enzymic browning of α-crystallin by reactive dicarbonyls on its molecular chaperone-like function. Non-enzymic browning of bovine α-crystallin *in vitro* caused, along with altered secondary and tertiary structures, cross-linking and high-molecular-mass aggregation. Notwithstanding these structural changes, methylglyoxal- and glyoxal-modified α-crystallin showed enhanced anti-aggregation activity in various *in vitro* aggregation assays. Paradoxically, increased chaperone-like activity of modified α-crystallin was not associated with increased surface hydrophobicity and rather showed less 8-anilinonaphthalene-l-sulphonic acid binding. In contrast, the chaperone-like function of modified α-crystallin was found to be reduced in assays that monitor the prevention of enzyme inactivation by UV-B and heat. Moreover, incubation of bovine lens with methylglyoxal in organ culture resulted in cataract formation with accumulation of advanced glycation end-products and recovery of α-crystallin in high proportions in the insoluble fraction. Furthermore, soluble α-crystallin from methylglyoxal-treated lenses showed decreased chaperone-like activity. Thus, in addition to describing the effects of methylglyoxal and glyoxal on structure and chaperone-like activity, our studies also bring out an important caveat of aggregation assays in the context of the chaperone function of α-crystallin.

Key words: advanced glycation end-products (AGE), aggregation, cataract, α-crystallin, methylglyoxal, molecular chaperone, non-enzymic glycation.

INTRODUCTION

Non-enzymic glycation (Maillard reaction) is a complex series of reactions between reducing sugars and amino groups of proteins, which leads to browning, fluorescence and cross-linking of the proteins. The reaction is initiated by the reversible formation of a Schiff base, which undergoes a rearrangement to form a relatively stable Amadori product. The Amadori product further undergoes a series of reactions through dicarbonyl intermediates to form AGE (advanced glycation end-products) [1]. It has been shown that the formation of AGE *in vivo* contributes to several pathophysiologies associated with aging and diabetes mellitus, such as chronic renal insufficiency, Alzheimer's disease, nephropathy, neuropathy and cataract [2–4]. Cataract is the leading cause of blindness world wide, where diabetes and aging are the major risk factors that accelerate the development of cataract [5]. Glycation of lens proteins has been considered to be one of the mechanisms responsible for both age-related and diabetic cataracts [5–7], since formation of AGE owing to glycation may alter the surface charge of the protein, leading to a conformational change that in turn may effect protein–protein and protein–water interactions and ultimately lead to decreased transparency of the eye lens [8–10].

A number of aldehydes and ketones, in addition to sugars, are known to form AGE. Methylglyoxal and glyoxal have been identified as a major source for AGE formation in various tissues including the lens [11–13]. High concentrations of methylglyoxal were detected in lens compared with plasma or any other tissue and methylglyoxal levels increase severalfold during diabetes [14]. Thus the role of methylglyoxal and other α-dicarbonyls in the pathogenesis of diabetic cataract has received considerable attention. Methylglyoxal is known to be formed non-enzymically by amine-catalysed sugar fragmentation reactions and by spontaneous decomposition of triose phosphate intermediates in glycolysis [14]. It is also a product of the metabolism of acetol, an intermediate in the catabolism of both threonine and the ketone body acetone [13]. It has been reported that methylglyoxal binds and modifies a number of proteins, including BSA, RNase A, collagen, lysozyme and lens crystallins [12,13,15,16]. Methylglyoxal is known to induce formation of heterogeneous AGE and protein cross-linking. Moreover, the nature of AGE structures and extent of modifications depend largely on the glycating reagents and conditions employed for the *in vitro* glycation reaction. Although products of these reactions have not been well characterized, few AGE have been identified, which include argpyrimidine, imidazolysines and *N*-ε-carboxyethyllysine or *N*-ε-carboxymethyllysine [17–21]. Some of these AGEs were detected in human lens, particularly brunescent cataractous lenses [18]. Although arginine is considered to be the main target in these modifications, other basic amino acids such as lysine, and to a lesser extent histidine and cysteine, are known to be modified in reactions of proteins with methylglyoxal [12,13,22].

α-Crystallin, an sHSP (small heat-shock protein) of the eye lens with conserved α-crystallin domain, has been shown to function

Abbreviations used: AGE, advanced glycation end-products; ANS, 8-anilinonaphthalene-l-sulphonic acid; DTT, dithiothreitol; G6PD, glucose-6-phosphate dehydrogenase; HMW, high molecular mass ('weight'); sHSP, small heat-shock protein.

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like a molecular chaperone [23–26]. Several studies indicated that the molecular chaperone function of α-crystallin may be instrumental in the maintenance of lens transparency vis-à-vis prevention of cataract [27–29]. α-Crystallin is a heteropolymer of 800 kDa with two subunits, αA and αB. Each subunit is of approx. 20 kDa and both exhibit 57 % similarity [24,26]. Despite the large amount of information available on the pathological significance of AGE protein in cataractogenesis, relatively little is known about the effect of AGE formation on the changes in structure and chaperone-like function of α-crystallin, particularly dicarbonyl modification on chaperone function. Recently, Nagaraj et al. [30] have demonstrated that methylglyoxal-modified α-crystallin shows increased chaperone-like activity in aggregation assays. However, one has to realize that glycation (AGE formation) has been implicated in the pathogenesis of cataract. Thus the significance of increased chaperone activity due to methylglyoxal modification with regard to lens transparency needs further investigation.

In the present study, we have assessed the effect of dicarbonyl-induced modification (AGE formation) on structure and function of α-crystallin. Our results indicate that on *in vitro* glycation by methylglyoxal/glyoxal, α-crystallin exhibited increased chaperone-like activity in model aggregation assays despite the adverse structural changes such as altered secondary and tertiary structures and decreased hydrophobicity. Moreover, glycation of α-crystallin has also resulted in massive aggregation due to cross-linking similar to molecular changes observed in diabetic cataract lens. However, in contrast with aggregation assays, methylglyoxal/glyoxal-modified α-crystallin showed a decreased chaperone-like activity in functional assays (enzyme inactivation). Further, methylglyoxal caused lens opacification (cataract) in organ culture, and chaperone activity of α-crystallin isolated from these cataract lenses was found to be decreased. Therefore these results also highlight the limitations of *in vitro* aggregation assays in the study of chaperone-like activity of α-crystallin under different circumstances.

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MATERIALS AND METHODS

Materials

BSA, thyroglobulin, citrate synthase, lysozyme, ferritin, G6PD (glucose-6-phosphate dehydrogenase; from *Leuconstoc mesenteroides*), 2,4-dinitrophenylhydrazine, ANS (8-anilinonaphthalene-l-sulphonic acid), NADPH, insulin, 2-mercaptoethanol, trichloroacetic acid, acrylamide, bisacrylamide, methylglyoxal [aq. 40 % (v/v)], glyoxal and TC-199 medium (M-3769) were purchased from Sigma Chemical Co. SDS/PAGE markers were from Bio-Rad Laboratories (Hercules, CA, U.S.A.), Sephacryl S-300HR was from Amersham Biosciences (Uppsala, Sweden) and restriction enzyme (*Sma*I) was from New England Biolabs (Beverly, MA, U.S.A.). α-Crystallin antibody was a gift from Dr J. S. Zigler (National Eye Institute, National Institutes of Health, Bethesda, MD, U.S.A.). Antibodies to methylglyoxal-AGE were raised against methylglyoxal-modified BSA in the New Zealand White rabbit (M. S. Kumar and G. B. Reddy, unpublished work).

Purification of α-crystallin

α-Crystallin was purified from calf lenses using a Sephacryl S-300HR size-exclusion column (100 cm × 2.5 cm) as described earlier [31]. The purity of α-crystallin was assessed by SDS/PAGE and protein concentration was determined by modified Lowry method.

Incubation of proteins with α-dicarbonyls

Stocks of 1 M methylglyoxal or glyoxal were prepared in 100 mM sodium phosphate buffer (pH 7.5) and the pH value was adjusted to neutral if required. α-Crystallin or other proteins were incubated with methylglyoxal and glyoxal (concentrations as mentioned in the Figure legends) in 100 mM sodium phosphate buffer (pH 7.5) at 37 °C in the dark under sterile conditions for different time periods up to 3 days. At the end of incubation, unbound dicarbonyls were removed by dialysis against the same buffer and protein content was estimated as mentioned above.

Assay of carbonyl groups

Protein carbonyl groups were estimated by the method of Uchida et al. [32].

Fluorescence measurements

Fluorescence measurements were performed using a Jasco spectrofluorimeter (FP-750). For all the measurements, 0.15 mg/ml protein in 20 mM sodium phosphate buffer (pH 7.4) was used. AGE-related non-tryptophan fluorescence of modified and unmodified α-crystallin was monitored by exciting at 370 nm and emission was recorded between 400 and 490 nm. Intrinsic tryptophan fluorescence of the protein samples was obtained by exciting at 280 nm and following the emission from 300 to 390 nm. Synchronous fluorescence scanning was performed between 260 and 500 nm (Hitachi F-4500 model) by interlocking the excitation and emission monochromators at a fixed wavelength difference (40 nm). Fluorescence of ANS in the presence of native and methylglyoxal- and glyoxal-modified α-crystallin was measured by exciting at 390 nm and following the emission between 450 and 550 nm. α-Crystallin (0.1 mg/ml) was incubated with 50 μM ANS for 30 min at room temperature (23 °C), and fluorescence of protein-bound dye was measured. The spectra were corrected with appropriate protein and buffer blanks.

Size-exclusion chromatography

The molecular masses of control and AGE-modified α-crystallin and other proteins were studied by gel-filtration chromatography on a 600 mm × 7.5 mm TSK-G4000SW column (Tosoh, Japan) using the Shimadzu HPLC system as described previously [33].

CD studies

CD spectra were recorded at room temperature using a Jasco J-715 spectropolarimeter. All spectra are the average of five accumulations. Far- and near-UV CD spectra were recorded at room temperature using cells of 0.01 and 0.02 cm path length respectively. All spectra were corrected for the respective blanks. Protein concentrations used for far- and near-UV were 0.15 and 1.5 mg/ml respectively. The CD results were expressed as molar ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$).

Chaperone activity assays

Chaperone activity of unmodified and methylglyoxal/glyoxal-modified α-crystallin or the other proteins was probed by

measuring the ability of these proteins to prevent the aggregation of substrate proteins denatured by reduction of disulphide bonds (insulin and abrin) or heat (citrate synthase and β_L-crystallin). The aggregation of proteins on denaturation was monitored by measuring the apparent absorbance at 360 nm as a function of time in the absence and presence of α-crystallin using a Cary100 spectrophotometer. Aggregation assays with abrin, insulin, citrate synthase and β_L-crystallin were performed essentially as described previously [33,34].

The potential of native and modified α-crystallin to prevent the inactivation of enzymes (G6PD and *Sma*I) due to heat or UV-B irradiation was assessed. The inactivation of G6PD on heat and UV treatment was monitored by measuring the residual activity in the absence or presence of α-crystallin. UV-B-induced inactivation of G6PD was performed essentially as described by Reddy et al. [35]. Thermal inactivation was performed by incubating G6PD (0.5 unit/ml in 100 mM Tris/HCl buffer, pH 7.4) at 45 °C in the presence and absence of modified or unmodified α-crystallin. *Sma*I inactivation in the absence and presence of α-crystallin was monitored by assessing the cleavage of plasmid DNA (pUC18). For inactivation, 2 μl (20 units/10 μl) of working enzyme was added to 1 μl of 10 × restriction enzyme buffer and 2 μl of native α-crystallin or 10 mM methylglyoxal-modified α-crystallin (5 mg/ml) and water to a final volume of 10 μl and incubated at 37 °C for 60 min. After inactivation, 2 μl (150 ng) of plasmid DNA was added and incubated for 60 min at 25 °C for digestion. The digested mixtures were run on 1 % agarose gel.

Lens organ culture

Calf eyes were obtained from a local slaughterhouse. Lenses were dissected from the eyes by anterior approach. Each isolated lens was incubated in 6 ml of modified TC-199 medium with antibiotics (filtered through 0.2 μm Millipore disc filters) at 37 °C under 95 % air and 5 % CO_2 by the method of Zigler and Hess [36]. Damaged lenses were identified by determining the protein content of an aliquot of the medium after an equilibration period of 2 h and were terminated. Undamaged lenses were incubated with and without 1 mM methylglyoxal for different time periods. At the end of incubation, lenses were homogenized in 10 vol. of 0.05 M Tris buffer (pH 8.0), containing 0.1 M NaCl and 0.02 % sodium azide. The homogenate was centrifuged at 15 000 ***g*** for 30 min at 4 °C and the resulting supernatant was collected (soluble fraction). The pellet was washed twice and constituted in the same buffer (insoluble fraction). α-Crystallin was isolated as described above from the soluble fraction.

Immunoblotting

Water-soluble and -insoluble proteins (5 μg) from control and methylglyoxal-incubated lenses were resolved by SDS/PAGE (10 % gel) and the proteins were transferred on to a nitrocellulose membrane. The membrane was blocked with 5 % (w/v) skimmed milk powder, reacted with respective primary antibody (1:1000) and later with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000) for subsequent detection, by using diaminobenzidine and hydrogen peroxide.

RESULTS AND DISCUSSION

Although there are numerous studies with regard to the pathological significance of AGE proteins in cataractogenesis, relatively little is known about the effect of AGE formation on α-crystallin, particularly dicarbonyl modification on chaperone function. Nevertheless, the chaperone-like activity of α-crystallin was shown to be compromised in diabetic human and rat lenses [37]. Some studies have tried to understand the molecular effects of glycation on α-crystallin, but have yielded contradictory results. On the one hand, *N*-ε-carboxymethyllysine adducts (produced on incubation with glyoxylic acid and $NaBH_3CN$) increased the chaperone-like activity of both αA- and αB-crystallins using insulin aggregation assay, although no major structural changes were noted [21]. On the other, some of the *in vitro*-induced post-translational modifications, similar to those occurring in diabetes, including glycation, were shown to result in loss of α-crystallin chaperone activity [38]. Similarly, two reports that described the effect of methylglyoxal modification on α-crystallin chaperone activity have produced opposite results [30,31]. Keeping aside the implications of these two studies (discussed later), the contradictory results appear to be mostly due to the different types of assays and conditions employed in these studies. In the present study, attempts were made to investigate in greater detail the molecular chaperone-like function of α-crystallin due to methylglyoxal- and glyoxal-induced browning by employing various complimentary assays to understand the effect of methylglyoxal on α-crystallin in a comprehensive manner. In addition, we have assessed the molecular changes associated with non-enzymic browning of α-crystallin to understand the effects of methylglyoxal on chaperone-like activity in relation to cataract.

Modification of α-crystallin by dicarbonyls

Accumulation of protein carbonyls in proteins by glyco-oxidative damage may contribute to protein dysfunction as well as many of the age-related pathologies characterized by protein cross-linking [39]. Hence, we measured protein carbonyls to assess the extent of protein glycation. Compared with unmodified α-crystallin, α-crystallin incubated with 10 and 100 mM methylglyoxal produced 21 and 70 nmol of carbonyl/mg of protein respectively. In parallel assays, 10 and 100 mM glyoxal produced 16 and 59 nmol of carbonyl/mg of protein respectively. Furthermore, AGE-related non-tryptophan fluorescence, which represents cumulative AGE fluorescence in a non-specific manner, was monitored to assess the extent of modification of α-crystallin by dicarbonyls. Non-tryptophan fluorescence due to protein-bound fluorescent AGE increased significantly after incubation with methylglyoxal and glyoxal (Figure 1A). In addition, synchronous fluorescence spectra of methylglyoxal/glyoxal-treated α-crystallin indicated the presence of chromophores with excitation maxima at higher wavelength (> 300 nm) and decrease in tryptophan absorption (Figure 1B). Increased carbonyl content together with increased AGE and synchronous fluorescence spectra provided an empirical measure of damage/modification of α-crystallin due to non-enzymic browning by α-dicarbonyls. Glycated α-crystallin possessed yellow to dark yellow–brown colour similar to proteins isolated from pigmented brunescent cataractous lenses (results not shown).

Chaperone-like function by aggregation assays

A commonly used test for chaperone activity of sHSP is the estimation of their capacity to suppress the aggregation of other proteins induced by various treatments such as DTT (dithiothreitol), heat and UV irradiation. Non-specific aggregation of proteins produces an increase in light scattering, which can be measured with a spectrophotometer or fluorimeter. The protective

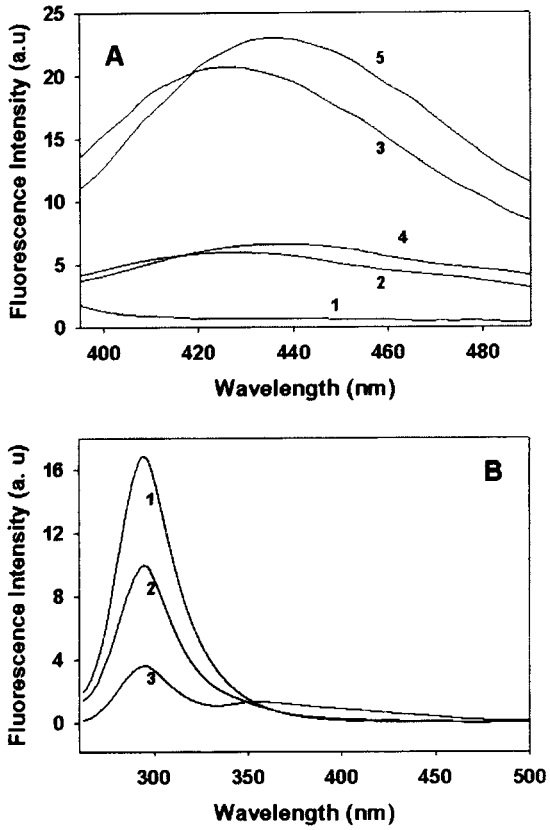

Figure 1 Modification of α-crystallin due to methylglyoxal and glyoxal treatment

(**A**) Non-tryptophan (AGE) fluorescence of native and modified α-crystallin; trace 1, native α-crystallin; traces 2 and 3, 10 and 100 mM methylglyoxal-treated α-crystallin respectively; traces 4 and 5, 10 and 100 mM glyoxal-treated α-crystallin respectively. **(B)** Synchronous fluorescence scan of native (trace 1), 10 mM (trace 2) and 100 mM (trace 3) methylglyoxal-treated α-crystallin.

ability of an sHSP can be estimated by decrease in the light scattering.

Chaperone-like activity of α-crystallin after modification was studied by assessing its ability to suppress the aggregation of substrates denatured by various complementary methods. We have used DTT-induced aggregation of insulin and abrin and heat-induced aggregation of citrate synthase and β_L-crystallin to monitor the chaperone-like activity. As shown in Figure 2(A), *in vitro* modification of α-crystallin by methylglyoxal/glyoxal resulted in increased chaperone-like activity in insulin aggregation assay at room temperature when compared with unmodified α-crystallin. Similarly, in citrate synthase aggregation assay (at 43 °C), modified α-crystallin exhibited increased chaperone-like activity as a function of increased concentration of methylglyoxal (Figure 2B) and further suggests that increased chaperone activity can be observed at methylglyoxal concentrations as low as 0.1 mM. Results with insulin and citrate synthase were in conformity with the observations of Nagaraj et al. [30]. To confirm these results, we employed two more aggregation assays, DTT-induced aggregation of abrin and heat-induced aggregation of β_L-crystallin. Recently, we have shown that DTT-induced aggregation of abrin, a plant protein, can be suppressed by α-crystallin and this assay also can be employed to study the chaperone-like function of sHSP [33]. In abrin aggregation assay as well, methylglyoxal-modified α-crystallin showed increased

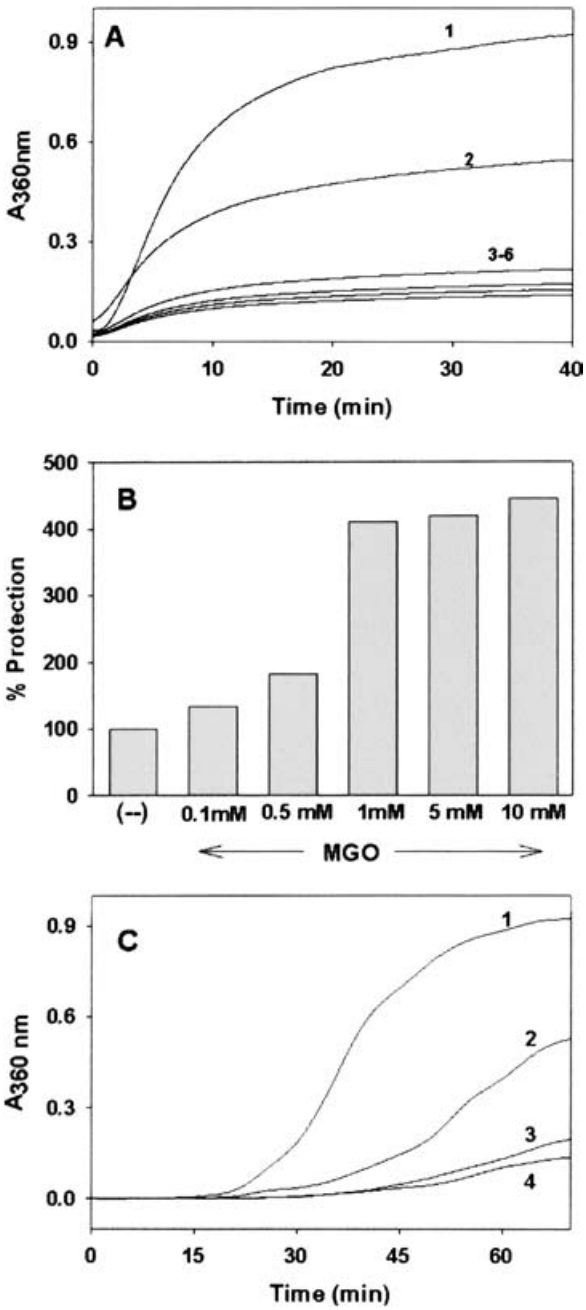

Figure 2 Chaperone-like activity of native and modified α-crystallin

(**A**) DTT-induced aggregation of insulin at room temperature; trace 1, insulin alone; trace 2, insulin with native α-crystallin; traces 3 and 4, insulin with 10 and 100 mM methylglyoxal-modified α-crystallin respectively; traces 5 and 6, insulin with 10 and 100 mM glyoxal-modified α-crystallin respectively. (**B**) Heat-induced aggregation of citrate synthase at 43 °C. Prevention of CS (citrate synthase) aggregation by native α-crystallin was taken as 100 % (bar 1); bars 2–6, α-crystallin modified with 0.1, 0.5, 1, 5 and 10 mM methylglyoxal respectively. (**C**) DTT-induced aggregation of abrin at room temperature; trace 1, abrin alone; trace 2, abrin with native α-crystallin; traces 3 and 4, abrin with 10 and 100 mM methylglyoxal-modified α-crystallin respectively. Equal concentrations of native and modified α-crystallin were used in 0.7 mg/ml insulin, 0.5 mg/ml abrin and 0.05 mg/ml CS aggregation assays.

chaperone-like activity (Figure 2C), suggesting that modified α-crystallin shows increased chaperone-like function in aggregation assays. However, there were some discrepancies in β_L-aggregation assay; chaperone activity increased for α-crystallin

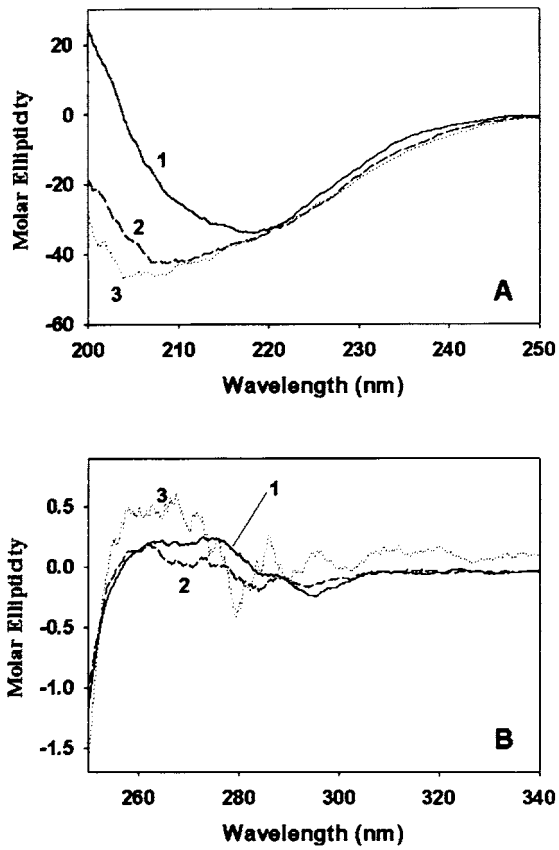

Figure 3 Far- (A) and near-UV (B) CD spectra of native and methylglyoxal-modified α-crystallin at room temperature

Trace 1, native α-crystallin; traces 2 and 3, 10 and 100 mM methylglyoxal-modified α-crystallin respectively.

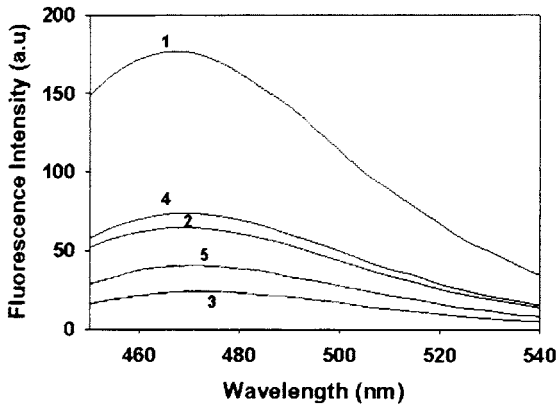

Figure 4 ANS fluorescence of α-crystallin after glycation

Trace 1, native α-crystallin; traces 2 and 3, 10 mM and 100 mM methylglyoxal-modified α-crystallin respectively; traces 4 and 5, 10 and 100 mM glyoxal-modified α-crystallin respectively.

that was modified with lower concentrations of methylglyoxal (methylglyoxal) but decreased with higher concentrations (> 30 mM; results not shown). Results with higher concentrations of methylglyoxal in β_L-aggregation were in conformity with an earlier report, wherein crystallin was modified with 30 mM and assayed against β_L-aggregation [31].

Secondary and tertiary structures of AGE-modified α-crystallin

To understand the mechanism for the enhanced chaperone-like function of α-crystallin on dicarbonyl-induced non-enzymic glycation, we have monitored secondary- and tertiary-structural states by CD and fluorescence spectroscopy. As shown in Figure 3(A), native α-crystallin has a maximum negative ellipticity at approx. 217 nm, typical of β-sheet structure as reported previously by many authors [21,23,25]. However, far-UV CD signal for methylglyoxal-modified α-crystallin increased and shifted to lower wavelengths, indicating altered secondary structure. Furthermore, near-UV CD spectra of methylglyoxal-modified α-crystallin also demonstrated altered signal, particularly in the aromatic region (Figure 3B), suggesting conformational changes at a tertiary-structural level due to glycation. However, it is possible that different oligomeric species of the modified crystallin (shown later) could also be responsible for the altered CD signal. Nevertheless, a loss of intensity and red shift (334–342 nm) of tryptophan fluorescence further confirm the altered tertiary structure of α-crystallin by dicarbonyls (results not shown). Moreover, decreased absorption in the aromatic region, from the synchronous fluorescence data (Figure 1C), substantiates the CD and fluorescence emission data for the altered structure of α-crystallin on modification with methylglyoxal/glyoxal. In addition, the above findings are further substantiated by the observations that methylglyoxal-modified α-crystallin is more susceptible to proteolytic digestion and its stability decreased as assessed by differential scanning calorimetry and denaturant-induced unfolding (M. S. Kumar and G. B. Reddy, unpublished work).

Surface hydrophobicity

A number of studies suggest that the chaperone-like activity of α-crystallin is mediated by the presence of surface-exposed hydrophobic patches, since binding of partially denatured proteins to α-crystallin is supposed to be driven largely by hydrophobic interactions [25,34,40,41]. Therefore we measured ANS binding to glycated α-crystallin for determining the role of increased surface hydrophobicity in enhancing chaperone activity. Figure 4 depicts the ANS-binding profiles for native and methylglyoxal/glyoxal-modified α-crystallin. At a saturating ANS concentration, the fluorescence intensity of ANS associated with modified α-crystallin is lower when compared with native α-crystallin and follows a concentration-dependent decrease for increased methylglyoxal/glyoxal concentration. A similar trend was observed when hydrophobicity was measured using a related high-affinity dye, bis-ANS (results not shown). Despite the apparent decrease in hydrophobicity, glycated α-crystallin does exhibit increased chaperone-like function in aggregation assays. Nevertheless, some studies suggest that there is no direct correlation between hydrophobicity and chaperone activity of α-crystallin [30,34,42].

Oligomer size and subunit profile

Oligomer size has been shown to influence the chaperone-like function of α-crystallin and other sHSP. Hence, we analysed the molecular masses of native and glycated α-crystallins by gel filtration on a TSK-G4000SW column. Figure 5(A) shows gel-filtration profiles of native- and methylglyoxal-modified α-crystallin. As expected, native α-crystallin eluted as an

Figure 5 Oligomeric size (A) and subunit profile (B) of α-crystallin after modification by dicarbonyls

HPLC chromatograms of native (trace 1), 10 mM (trace 2) and 100 mM (trace 3) methylglyoxal-modified α-crystallin. Elution profiles 669 kDa thyroglobulin, 440 kDa ferritin, 80 kDa γ-globulin and 67 kDa BSA are indicated. SDS/PAGE profiles of native and modified α-crystallin; lane 4, molecular-mass standards; lanes 3 and 5, native α-crystallin; lanes 2 and 1, 10 and 100 mM methylglyoxal respectively; lanes 6 and 7, 10 and 100 mM glyoxal-modified α-crystallin respectively.

oligomer of 800 kDa with a trace of HMW [high molecular mass (weight)] peak. Modification of α-crystallin with 10 mM methylglyoxal resulted in three major peaks: (i) an HMW peak (approx. 2000 kDa), (ii) 1000 kDa peak and (iii) a low-molecular-mass peak (approx. 100 kDa). On the other hand, α-crystallin modified with 100 mM methylglyoxal led to formation of a predominantly large soluble aggregate of the weight >2000 kDa. Similarly, glyoxal-induced modification resulted in the formation of large soluble aggregate (results not shown). We have further analysed the subunit profile of modified and native α-crystallin by SDS/PAGE. As shown in Figure 5(B), unmodified α-crystallin showed two bands at approx. 20 kDa (corresponding to αA and αB), but methylglyoxal/glyoxal-modified α-crystallin showed HMW bands, some of which could not even enter the 10% gel. These results indicate that there is a high cross-linking between the subunits on non-enzymic glycation with methylglyoxal and glyoxal. This may be the predominant mechanism for the formation of HMW aggregates. Formation of such a large soluble aggregate partially explains the increased chaperone-like activity of modified α-crystallin, as the large soluble aggregate may simply be binding the partially denaturing substrate proteins in different *in vitro* aggregation assays. Furthermore, sHSP chaperone function is hypothesized to provide a reservoir of partially unfolded proteins, which could be refolded by co-chaperones [43]. Such non-specific large aggregates (such as methylglyoxal/glyoxal-modified α-crystallin) bind the denatured target proteins (used in aggregation assays) resulting in decreased light scattering, thus giving an impression of increased chaperone-like activity. Moreover, it is highly unlikely that such large soluble aggregates exist *in vivo*, as they would go into insoluble protein fraction sooner or later (shown later). It should be noted that earlier studies that reported contradictory results on the effect of methylglyoxal on α-crystallin chaperone function did not analyse the molecular mass of α-crystallin on modification [30,31]. Although it has been suggested that structural perturbations can lead to enhanced chaperone-like activity [25,34,44,45], drastic alterations such as high cross-linking and formation of very HMW aggregates have not been linked to increased chaperone activity. Nevertheless, in many instances, if not all, increased oligomeric size or formation of HMW aggregate of α-crystallin or HSP25 (due to mutations or other treatments) resulted in increased chaperone-like activity [44,46,47].

To rule out the possibility of any large aggregate providing non-specific protection in aggregation assays, we have modified different proteins of various molecular masses, which are unrelated to sHSP (with respect to sequence, structure and function) with methylglyoxal and studied their chaperone-like activity. As can be seen from Figure 6(A), 66 kDa native BSA, 80 kDa γ-globulin and 669 kDa thyroglobulin did not suppress the aggregation of insulin. Similar was the effect of methylglyoxal-modified γ-globulin and BSA. Remarkably, methylglyoxal-modified thyroglobulin did suppress insulin aggregation completely at the same molar concentration of α-crystallin. In an attempt to correlate this observation with the molecular mass, we have analysed these modified proteins by gel filtration. BSA appears to have dimerized as its mass increased to 110 kDa after incubation with 10 mM methylglyoxal for 3 days (Figure 6B). Similarly, γ-globulin mass increased to 146 kDa after modification with methylglyoxal. Interestingly, modification of thyroglobulin with methylglyoxal led to formation of a large aggregate of 2000 kDa similar to that of glycated α-crystallin (Figure 6B). These results, for the first time, provides a clue that protein aggregate of sufficiently large mass or any protein which can be modified to form a large aggregate may provide chaperone-like protection in aggregation assays employed to assess chaperone-like activity of sHSP and similar proteins. In this context, it should be noted that α_s-casein, a protein with no well-defined secondary and tertiary structures but which exists as a micellar aggregate, was shown to behave like a molecular chaperone [48].

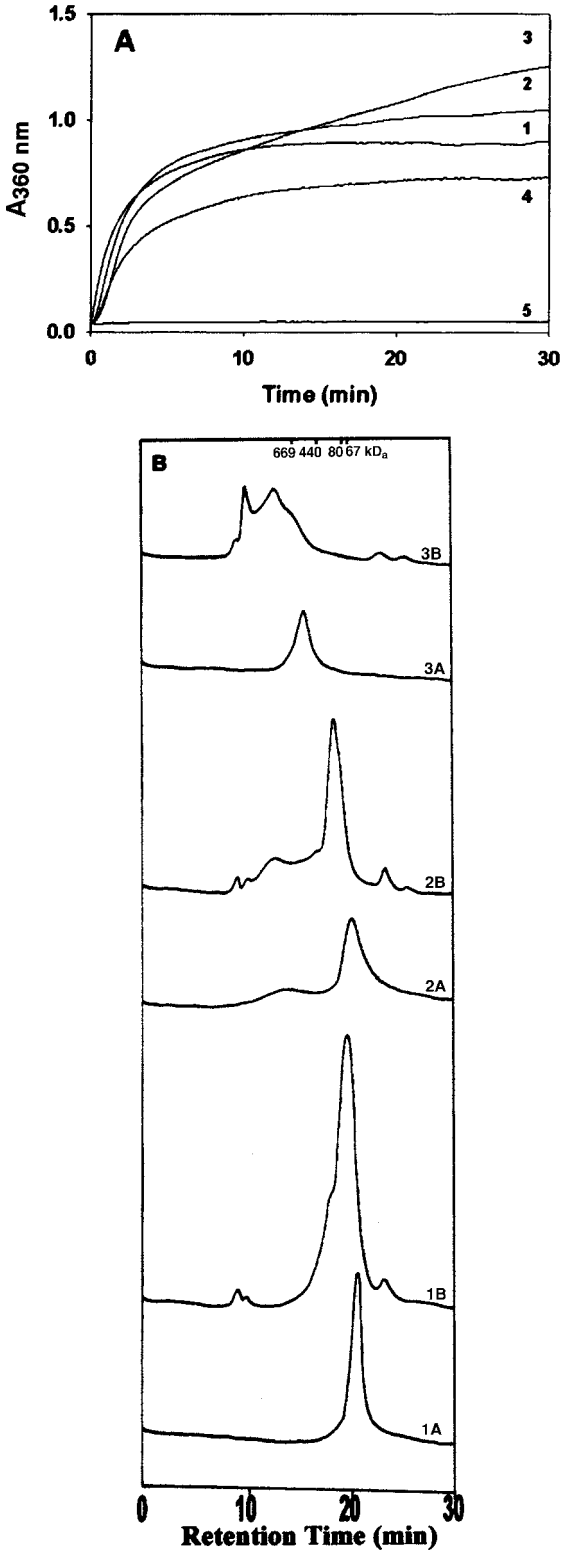

Figure 6 Chaperone-like activity and molecular masses of TG (thyroglobulin), γ-globulin and BSA

(**A**) Chaperone-like activity of TG and BSA in insulin aggregation assay. Trace 1, insulin alone; traces 2 and 4, insulin with native BSA and TG respectively; traces 3 and 5, insulin with BSA and TG modified with 10 mM methylglyoxal respectively. (**B**) Effect of methylglyoxal modification on molecular masses of TG, BSA and γ-globulin. HPLC chromatograms of native (traces 1A, 2A and 3A) and 10 mM methylglyoxal-modified BSA, γ-globulin and TG (traces 1B, 2B and 3B) respectively.

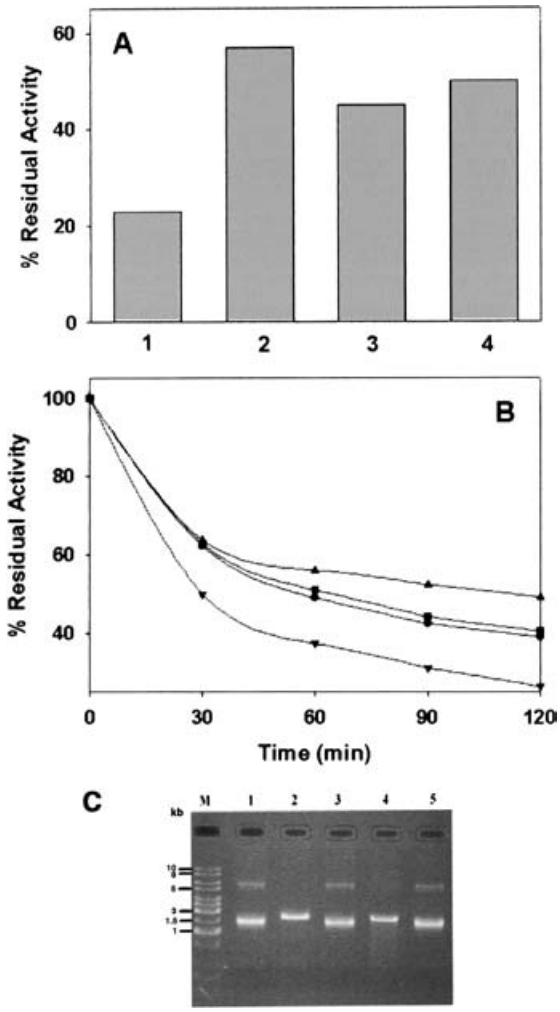

Figure 7 Chaperone-like activity of native and modified α-crystallin in enzyme inactivation assays

(**A**) Protection of UV-B-induced inactivation of G6PD by native and modified α-crystallin after 3 h at 25 °C. Bar 1, G6PD alone; bars 2–4, G6PD along with native, 10 mM methylglyoxal- and glyoxal-modified α-crystallin respectively. (**B**) Heat-induced inactivation of G6PD as a function of time in the absence (▼) or in the presence of native (▲), 10 mM (■) and 100 mM (●) methylglyoxal-modified α-crystallin. Data for (**A**, **B**) are average of three independent experiments. (**C**) Protection of *Sma*I from heat inactivation by native and modified α-crystallin. Lane M, DNA standards; lane 1, uncut plasmid; lanes 2–5, plasmid digested with native *Sma*I, heat-inactivated *Sma*I in the presence of native and methylglyoxal-modified α-crystallin respectively. Profile in (**C**) represents one of the three individual experiments.

Chaperone-like activity of modified α-crystallin in non-aggregation assays

To substantiate the possibility that the aggregate of α-crystallin (also thyroglobulin) formed due to methylglyoxal modification possess chaperone-like activity, we have investigated the chaperone-like activity using enzyme inactivation assays. Although the efficiency of α-crystallin to protect enzyme activity and/or renaturation of enzymes has been considered to be low [26], some studies have clearly demonstrated that α-crystallin can protect enzymes against inactivation [35,49–52]. Earlier we have shown that UV-B- and heat-induced inactivation of G6PD can be prevented by αA and αB crystallins [35]. As shown in Figure 7(A), native α-crystallin protected G6PD against UV-B inactivation. However, unlike in aggregation assays, modified α-crystallin showed lesser protection against UV-B inactivation of the

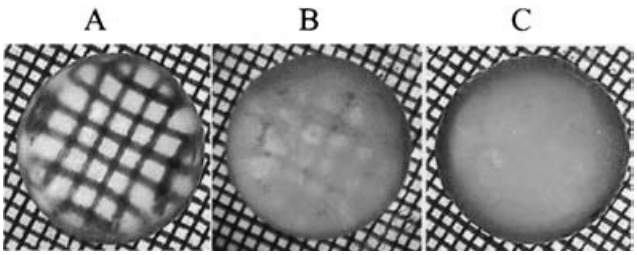

Figure 8 Methylglyoxal-induced opacification of lens in organ culture

Calf lenses were cultured in modified TC-199 as described in the Materials and methods section in the presence of 1 mM methylglyoxal for 0 day (**A**), 2 days (**B**) and 4 days (**C**). Transparency of the lens that was cultured for 4 days in the absence of methylglyoxal was similar to 0 day lens.

G6PD. Similarly, the ability of methylglyoxal/glyoxal-modified α-crystallin in preventing the heat-induced inactivation of G6PD was significantly lower when compared with that of unmodified α-crystallin (Figure 7B). Some studies have shown that α-crystallin can protect restriction enzymes from heat-induced inactivation [49,50]. Thus we have also assessed the ability of methylglyoxal-modified α-crystallin in preventing the heat-induced inactivation of *Sma*I. As shown in Figure 7(C), cleavage of plasmid DNA (pUC18) by *Sma*I resulted in a single band (lane 3). However, when *Sma*I was incubated at 37 °C for 1 h, it could not cleave the plasmid DNA due to inactivation (lane 4). Whereas native α-crystallin protected *Sma*I activity from heat inactivation, methylglyoxal-modified α-crystallin did not (lanes 5 and 6). It should be noted that the amount of G6PD and *Sma*I proteins used in these inactivation assays was so low that there cannot be a measurable aggregation.

Implications of dicarbonyl modification of α-crystallin

The observation that modified α-crystallin showed enhanced chaperone-like activity in aggregation assays but not in functional assays (where it showed decreased chaperone-like activity) suggest two possibilities. Either the mechanism of chaperone-like function may be different in different assays or the aggregation assays may not be an appropriate measure of chaperone activity in different circumstances (i.e. advanced glycation in the present study). Nevertheless, based on the results obtained with aggregation assays, Nagaraj et al. [30] propose that enhanced chaperone activity of α-crystallin by dicarbonyls may be beneficial to lens in terms of chaperone-like activity. However, one has to realize that glycation (AGE formation) has been implicated in the pathogenesis of various pathological complications including cataract. Furthermore, if the enhanced chaperone-like activity of α-crystallin due to glycation is considered to be a true observation, it would be difficult to reconcile the fact that glycation-induced cataract could be a result of increased chaperone-like activity of α-crystallin. Hence, we investigated the effect of methylglyoxal on lens transparency and chaperone activity in lens organ culture.

Effect of methylglyoxal in lens organ culture

Very interestingly, but not surprisingly, incubation of lens with methylglyoxal resulted in cataract formation in a time-dependent manner as assessed by the transmission of the light from the grid through the lens (Figure 8). Although increased levels of

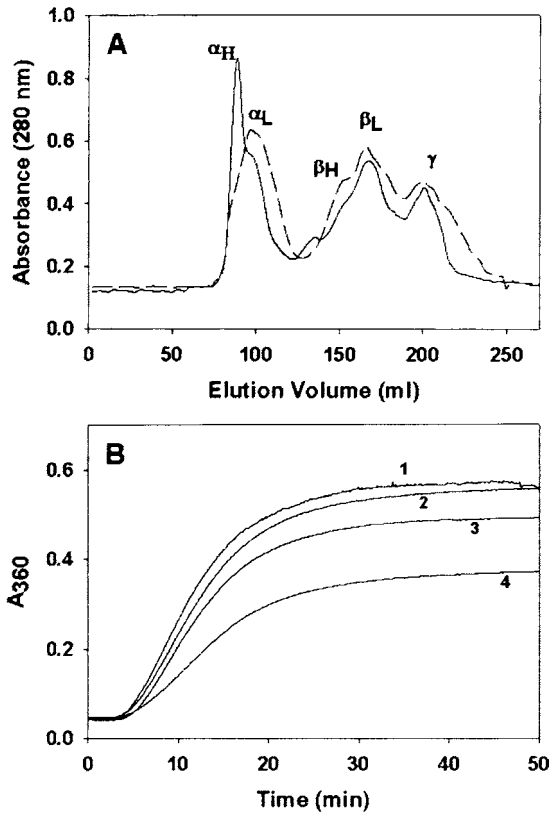

Figure 9 Gel-filtration profile of soluble fraction (A) and chaperone-like activity of α-crystallin (B) from the lenses incubated with methylglyoxal

(**A**) Gel-filtration profile (on a Sephacryl S-300 column) of soluble fraction of untreated (- - -) and methylglyoxal-treated lens for 4 days (—). (**B**) Chaperone-like activity of α-crystallin isolated from the lenses cultured with and without methylglyoxal, monitored against heat-induced aggregation of CS (citrate synthase) at 43 °C; trace 1, CS alone; traces 2 and 3, CS with α_H- and α_L-crystallin respectively from methylglyoxal-treated lens; trace 4, CS with α-crystallin from control lens.

methylglyoxal-modified proteins were implicated in cataractogenesis in the past, for the first time we show that methylglyoxal can induce cataract formation in organ culture. Methylglyoxal-induced loss of lens transparency is associated with a decrease in soluble protein content and a concomitant increase in the insoluble fraction compared with untreated lens (total protein, 251 and 233 mg/g lens; soluble protein, 237 and 195 mg/g lens; insoluble protein, 9.7 and 20.4 mg/g lens respectively for control and methylglyoxal-treated lens). As shown in Figure 9(A), α-crystallin from methylglyoxal-treated lens appeared in two peaks, α_H- and α_L-crystallin, whereas α-crystallin from untreated lens eluted as a single peak by gel filtration of the soluble fraction on a Sephacryl S-300 column and suggests that α-crystallin is modified to HMW form. However, the chaperone activity of both α_H- and α_L-peaks decreased when compared with α-crystallin peak of untreated lens in citrate synthase aggregation assay (Figure 9B) and also in insulin and β_L-crystallin aggregation assays (results not shown). The decreased chaperone activity of α_H-crystallin of methylglyoxal-treated lens might be due to the fact that α_H is inherently bound to substrate proteins in the lens. Furthermore, a fraction of α-crystallin, which was highly modified, has appeared in insoluble fraction as shown by immunoblotting with both methylglyoxal-AGE and α-crystallin antibodies (Figure 10). In addition, detection of increased AGE in insoluble fraction of methylglyoxal-treated lens indicates that

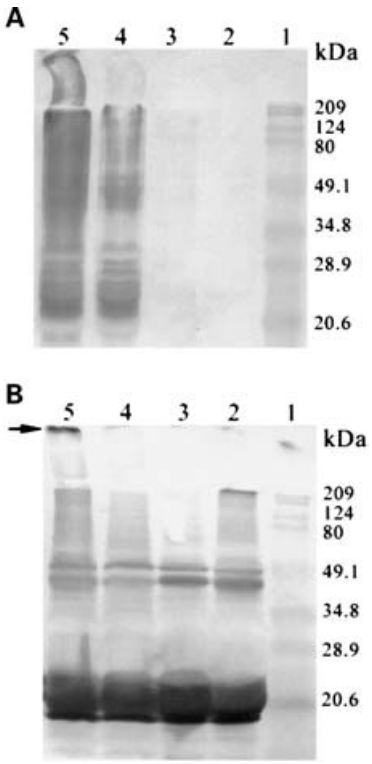

Figure 10 Immunoblot of soluble and insoluble fractions of control and methylglyoxal-treated lenses with anti-AGE (A) and anti-α-crystallin (B) antibodies

Lane 1, molecular-mass standards; lanes 2 and 3, water-soluble fraction of control and methylglyoxal-treated lenses respectively; lanes 4 and 5, water-insoluble fraction of control and methylglyoxal-treated lenses respectively. Arrow in (**B**) indicates HMW α-crystallin detected in water-insoluble fraction of methylglyoxal-cultured lenses.

formation of AGE due to methylglyoxal modification of lens proteins may lead to aggregation and insolubilization, finally resulting in cataract formation. Hence, the results on chaperone-like activity of α-crystallin obtained by aggregation assays need to be assessed critically under different circumstances.

Although methylglyoxal is known to modify arginine, lysine, histidine and cysteine residues, increased chaperone activity of methylglyoxal-treated α-crystallin has been attributed to exclusive modification of Arg^{21}, Arg^{49} and Arg^{50} [30]. Furthermore, the same authors report that only αA-crystallin shows enhanced chaperone activity due to methylglyoxal modification but not αB-crystallin [30], which is contradictory as αB-crystallin has an almost equal number of arginine residues and they are conserved in both the subunits. Derham and Harding [31], who have observed, like us, a decreased chaperone-like activity for methylglyoxal-modified α-crystallin with β_L-aggregation, suggest that arginine modification itself may not be responsible for the altered chaperone function. In addition, recently, Ahmed et al. [22] have detected increased hydroimidazolones as the major methylglyoxal-derived AGE in human cataractous lenses other than argpyrimidine. Therefore it can be logically reasoned that arginine modification alone may not be responsible for the altered chaperone function. Thus, irrespective of specific modifications, results showing enhanced chaperone-like activity of *in vitro* dicarbonyl-modified α-crystallin using aggregation assays should be considered as method-based results rather than of actual significance to the lens physiology.

In summary, we report in the present study that non-enzymic browning of α-crystallin by reactive carbonyls leads to alterations in secondary and tertiary structures and results in formation of very HMW aggregates due to cross-linking of subunits. Nonetheless, *in vitro* modified α-crystallin displayed increased chaperone-like activity in aggregation assays, although its hydrophobicity was reduced. In contrast, modified α-crystallin was found to exhibit decreased chaperone function in enzyme inactivation assays. However, α-crystallin isolated from methylglyoxal-treated lenses showed reduced chaperone-like activity even in aggregation assays. Based on the results, we bring out an important caveat of *in vitro* aggregation assays used for assessing chaperone-like activity of sHSPs, that they may provide conflicting results, depending on the conditions employed and the status of chaperone protein. Keeping apart the chaperone function, methylglyoxal-induced changes to α-crystallin are relevant to the molecular changes that might occur in the diabetic eye lens as the greater tendency of reactive carbonyls to induce intermolecular cross-linked protein aggregates, which could increase the scattering of light.

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