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Effect of Methylglyoxal Modification of Human α-Crystallin on the Structure, Stability and Chaperone Function

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Abstract α-Crystallin functions as a molecular chaperone and maintains transparency of eye lens by protecting other lens-proteins. Non-enzymatic glycation of α-crystallin by methylglyoxal, plays a crucial role on its chaperone function and structural stability. Our studies showed that methylglyoxal modification even in lower concentration caused significant decrease in chaperone function of α-crystallin as reflected both in thermal aggregation assay and enzyme refolding assay. Thermal denaturation studies showed drastic reduction of denaturation temperature with increase in the degree of modification. Thermodynamic stability studies by urea denaturation assay reflected a decrease of transition midpoint. Quantitatively we found that ΔG° of native α-crystallin decreased from 21.6 kJ/mol to 10.4 kJ/mol due to 72 h modification by 10 mM methylglyoxal. The surface hydrophobicity of α-crystallin after MG modification, was found to be decreased. Circular dichroism spectroscopy revealed conversion of β-sheet structure to random coil structure. Significant cross-linking was also observed due to methylglyoxal modification of human α-crystallin.

Keywords Methylglyoxal modification · α-crystallin · Chaperone function · Thermodynamic stability · Surface hydrophobicity

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1 Introduction

α-Crystallin is the primary protein constituent of vertebrate eye lens [4, 11]. It has two highly homologous chains αA- and αB-crystallin 20 kDa each. It exists as a large oligomer of 30–40 subunits having the A:B chain ratio as 3:1 in human lens [4, 11, 20]. Along with β- and γ-crystallin, lens crystallin accounts for about 90% of total soluble protein in a highly concentrated form and constitutes the refractive medium [45]. This protein has also been found in some non-lenticular tissues in small amounts [41] and has been linked to neurodegenerative diseases [31]. It is now well known that α-crystallin has molecular chaperone-like function which plays a crucial role in the maintenance of the lens transparency [36]. As a molecular chaperone, it prevents the aggregation of its substrate proteins caused by partial unfolding due to thermal or non-thermal stress conditions. There have been a large number of studies to understand the nature of interaction between α-crystallin and its substrate and many of the factors that influence the chaperone function of α-crystallin have been identified [6].

Eye lens is a highly dense mass of proteins. The protein turnover in the lens is very low and thus lens does not have a mechanism to dispose damaged proteins [23, 36]. Lens proteins are constantly subjected to UV light, oxidative stress and other external insults. With age its chaperone-like function is diminished and its ability to prevent protein aggregation is lost. The major disease, which is related to the loss of functionality of α-crystallin, is cataract, the leading cause of blindness worldwide. The mechanism by which the chaperone-like activity of α-crystallin gets lost is poorly understood.

Among many factors that are believed to be responsible for the loss of functionality of α-crystallin, post-translational modification is one of the most important ones [14].

Lens crystallins are known to undergo various post-translational modifications such as phosphorylation [24], glycation [22, 32, 37], carbamylation [53], methionine oxidation [50], deamidation [22], disulphide formation [22], N- and C-terminal truncation [46, 47] etc. Among these modifications, non-enzymatic glycation of α-crystallin has been reported to be one of the most significant causes leading to age related cataract, particularly for diabetic patients [22, 32, 37]. A number of aldehydes, ketones and sugars are known to form Schiff base adducts [16, 26] which subsequently produce advanced glycation end products (AGE) and some of which have been detected in human cataractous lens. Malondialdehyde, an end product of lipid peroxidation and a side product of thromboxane biosynthesis has been reported to produce AGE and thus produce cataract in experimental animals [5].

Methylglyoxal (MG) has been identified as one of the major sources of AGE in lens [16, 25, 26, 48]. MG, a dicarbonyl body metabolite is synthesized as a glycolysis by product and is a very reactive glycating agent [2, 34]. MG can glycate proteins at a faster rate than glucose and other glycating agents. MG is present in high concentration in lens compared to plasma or any other tissue and its level increases several folds during diabetes [2, 21, 33, 38] and other oxidative stress mediated diseases like osteoarthritis [51] and uremia [29, 52] as well. There have been scattered reports on the effect of MG on the chaperone function of α-crystallin [10, 16, 21, 26], but the reports are often contradictory. For example, Biswas et al. [10] reported increase of chaperone function of α-crystallin on MG modification although the reverse was reported by Kumar et al. [26]. Again, chemical cross-linking was reported due to MG modification but yet gross structural destabilization was reported [25, 26]. No quantitative measure of stability of MG modified α-crystallin is however known till date. More study is needed to clarify the issues.

In this paper we have studied the effect of MG modification of human α-crystallin on some spectroscopic properties such as tryptophan fluorescence, fluorescence quenching, secondary structure, surface hydrophobicity as well as thermal and thermodynamic stability.

2 Materials and Methods

Eye lenses from a 8 years old and a 13 years old boys, after eye ball surgery (cases of congenital disease and accidental damage), were obtained from the Department of Opthalmology, NRS Medical College and the Regional Eye Centre of Medical College, Kolkata and stored at −70 °C. Methylglyoxal (MG), DTT (Dithiothreitol), urea, Sephacryl S-300 HR, DEAE anion exchange and Sephadex G-25 were obtained all from Sigma, USA. Bis-ANS (1, 1' bi (4-anilino) naphthalene sulfonic acid) was from Molecular Probes, USA. Lactate dehydrogenase, NADH, Na-pyruvate were from Sisco Research Laboratories, India. Other commonly used materials required for this study were all of analytical grade.

2.1 Isolation and Purification of Human α-Crystallin

Human α-crystallin was purified through chromatographic procedures [8]. Human eye lenses were homogenized on ice in 10 mM tris–HCl buffer, pH 7.2 containing 100 mM NaCl, 1 mM EDTA, 0.02% sodium azide and 0.2 mM PMSF, then centrifuged at 4 °C at 10,000 r.p.m for 30 min. The supernatant fraction was loaded onto a Sephacryl S-300 HR column (95 × 1.5 cm). Second peak contained α-crystallin [6]. Further purification was done using a Sephacryl S-400 HR column. SDS–PAGE of purified α-crystallin showed a single band corresponding to 20 kDa. The proteins were dialysed in micro dialysis tubing against the buffer 50 mM phosphate buffer, pH 7.5 for four changes over 24 h and then stored in −20 °C until used.

2.2 Modificaion of α-Crystallin by MG

Human α-crystallin, 1 mg/ml (≈50 μM) was incubated with 100, 250, 500 μM and 10 mM MG at 37 °C in a sterile shaker incubator for different time intervals, up to 120 h. MG (Sigma) was purified to remove impurities, such as formic acid and formaldehyde, before use. The buffer, used, was 50 mM phosphate buffer, pH 7.5. At different time intervals samples were removed and dialyzed in micro-dialysis tubing against four changes of same buffer over 24 h. The samples were then ready for immediate experiments. All experiments were done in triplicate.

2.3 UV Spectral Study of Native & MG Modified α-Crystallin

The protein spectra of native and 100, 250, 500 and 10 mM MG modified α-crystallin (0.05 mg/ml) of different time intervals (24–120 h) were taken at UV 2401PC spectrophotometer (Shimadzu) in the wavelength range 400 to 230 nm using 1 cm path length cell.

2.4 Fluorescence Spectra of Native & MG Modified α-Crystallin

The fluorescence emission spectra of α-crystallin (0.05 mg/ml) in presence of 0, 100, 250, 500 μM and 10 mM MG for different time intervals (4–120 h) were taken after excitation at 295 nm using a Hitachi F-4500 spectrofluorimeter.

Protein concentration used was 0.05 mg/ml in 50 mM phosphate buffer, pH 7.5.

In order to calculate the approximate loss of arginine due to modification, experiments were carried out to determine the intensity of fluorescence at 385 nm for α-crystallin (0.05 mg/ml) incubated with 10 mM MG in 50 mM phosphate buffer, pH 7.5 using 320 nm as the excitation wavelength. Different concentration of L-arginine (Sigma) in the range 10–200 μM incubated with 10 mM MG at 37 °C for 24 h served as standards.

2.5 Acrylamide Quenching of Tryptophan Fluorescence of MG Modified α-Crystallin

Tryptophan fluorescence quenching experiment was done using neutral quencher acrylamide. The protein concentration used in the cuvette was 0.1 mg/ml and the stock quencher concentration was 5 M for acrylamide. Study was done with unmodified and 250 μM and 10 mM MG modified α-crystallin (0.05 mg/ml) of different time intervals (24–72 h). α-Crystallin solution (600 μl) in 50 mM phosphate buffer, pH 7.5 was taken in a 1 ml quartz fluorescence cuvette having 1 cm path length. Acrylamide was added in small aliquot. After each addition the solution was mixed well and then readings of fluorescence emission were taken at 340 nm after 2 min with the excitation wavelength set at 295 nm. A control titration was performed in a similar way replacing the protein with buffer and keeping other things constant.

2.6 Surface Hydrophobicity of Native and MG Modified α- Crystallin

The hydrophobicity of native and MG modified proteins were evaluated using Bis-ANS as hydrophobic probe [15]. Bis-ANS (10 μM) was incubated with 0.05 mg/ml native and MG (100 μM–10 mM) modified proteins of different time periods (7–120 h). The incubation period was 1 h at room temperature and the fluorescence spectra of incubated proteins were taken in the range of 450–550 nm after exciting at 390 nm. The spectra were corrected for buffer blanks and control proteins (not incubated with bis-ANS).

2.7 Bis-ANS Titration of Native and MG Modified α-Crystallin

The hydrophobic binding sites in native and 10 mM MG modified α-crystallin were evaluated using bis-ANS as a hydrophobic probe [42]. All the proteins of 0.05 mg/ml (2 ml) in 50 mM phosphate buffer, pH 7.5 were taken in a 3 ml fluorimeter cuvette placed inside a Hitachi-4500 spectrofluorimeter maintained at the incubation temperature 25 °C using a water bath. The solution was titrated with concentrated bis-ANS solution (316 μM) adding a small aliquot at a time. After each addition the solution was stirred magnetically for 1 min and fluorescence data was recorded at 490 nm using 390 nm as the excitation wavelength [12, 27]. Both the excitation and emission band passes were 5 nm each. Fluorescence intensity at 490 nm was measured from each spectrum and readings were corrected for buffer blanks and dilution. In order to analyze the data according to Scatchard equation, a reverse titration of 0.1 μM bis-ANS by modified proteins were performed [8]. Here also the fluorescence intensity at 490 nm was recorded and a plot of 1/fluorescence intensity versus 1/protein concentration was generated [15]. These reverse titration data were used to obtain the quantitative relationship between fluorescence intensity change and bound bis-ANS [39].

2.8 Circular Dichroism Spectroscopy

Far-UV CD measurements: *Far*-UV CD spectra of native and MG (100 μM–100 mM) modified human α-crystallin (for 24–120 h) were taken at room temperature using Jasco J-600 spectropolarimeter flashed with dry nitrogen. Spectra were collected from 250 to 200 nm at 1 nm slit width and 20 nm/min scan speed using a cylindrical quartz cell of 1 mm path length. Proteins (0.5 mg/ml) were dissolved separately in 50 mM phosphate buffer, pH 7.5. The CD data were expressed as molar ellipticity in deg.cm^2.d mol^{-1}. Five scans were acquired and averaged for each sample.

$$\text{Molar Ellipticity} = \theta_{\text{deg}} * 100 * \text{M}_{\text{mean residue}}/\text{c} * \text{d}$$

where θ_{deg} is the measured ellipticity, $\text{M}_{\text{mean residue}}$ is the ratio between molecular weight of the protein/total number of residues, c is the protein concentration in mg/ml and d is the path length in cm.

2.9 SDS–PAGE

It was performed on a vertical Biorad mini gel electrophoresis unit using 12% (w/v) poly acrylamide gel under reducing condition according to Laemmli. In each lane 10 μg of protein was loaded. Protein bands were visualized by Coomassie blue staining. Unmodified as well as different concentration of MG (100 μM–10 mM) modified human α-crystallins for different time periods (4–120 h) were run in the gel.

2.10 Urea Denaturation Study of Native and MG Modified α-Crystallin

To investigate the thermodynamic stability of human unmodified and MG (250 μM–10 mM) modified α-crystallins (for 7–72 h) we measured the urea denaturation

profiles by tryptophan fluorescence spectroscopy as described elsewhere [8, 9]. For unfolding, solutions of 0.05 mg/ml α-crystallin (unmodified and MG modified) were incubated for 18 h with urea ranging from 0–8 M in 50 mM sodium phosphate buffer, pH 7.5. The intrinsic tryptophan fluorescence emission was scanned through 310–400 nm, with 295 nm as the excitation wavelength. Each spectrum was corrected for blank by subtracting the buffer spectrum. The intrinsic tryptophan fluorescence intensities at 337 nm and 350 nm were measured and the denaturation profile was constructed by plotting I_{337}/I_{350} against the concentration of urea [9].

2.11 Thermal Denaturation Study of Native and MG Modified α-Crystallin

Human unmodified and 10 mM MG modified (24, 48 and 72 h) α-crystallin (0.1 mg/ml in 50 mM phosphate buffer, pH 7.5) was taken in fluorescent cuvettes, which were placed in a thermostatic cell holder in the spectrofluorimeter (Hitachi F-4500). The cell holder was maintained at constant temperature by circulating water from a constant temperature water bath. After a set temperature was reached, the sample was equilibrated for at least 5–6 min to attain the temperature before emission spectra was collected. The temperature was started from 25 °C and samples were heated up to 80 °C with an interval of 2 °C. Each sample was excited at 295 nm wavelength and emission spectra of tryptophan fluorescence were taken from 310–450 nm. Each spectrum was corrected by buffer subtraction and taken as triplicate. Emission maximum was determined from these spectrums by derivative analysis.

2.12 Assay of Chaperone Function of Native and MG Modified α-Crystallin

Chaperone activity of human native and MG modified α-crystallin proteins was determined by a thermal aggregation assay using human β_L-crystallin as natural substrate [10]. The ability of α-crystallin to prevent the increase in turbidity upon heating solutions of substrate was used for measuring the chaperone activity [36]. A typical assay solution (600 µl) contained α-crystallin (0.015 mg/ml) and substrate (0.15 mg/ml) in 50 mM phosphate buffer, pH 7.5 and 100 mM NaCl. The assay solution was taken in a black masked quartz cuvette that was placed in the spectrophotometer cell holder maintained at 60 °C by a peltier device. The aggregation was recorded by light scattering at 400 nm in a kinetic mode using a UV-2401PC spectrophotometer [36]. Experiments were carried out at different ratio between α-crystallin and substrate, keeping the substrate concentration fixed at 0.15 mg/ml and varying the α-crystallin concentration. Assays were done with low range to high range (100 µM to 10 mM) of MG modified human α-crystallins for different time intervals (24–120 h). All assays were repeated in triplicate.

Chaperone activity was also assayed at physiological temperature by measuring the native and modified (by 250 µM and 10 mM MG for 24–72 h) α-crystallins mediated refolding of the enzyme lactate dehydrogenase (LDH) [8], from its fully unfolded state. Lactate dehydrogenase (LDH) was denatured in 6 M Gu-HCl containing denaturation buffer for 8 h at 25 °C at a concentration of 1 µM over night. Refolding of the enzyme was initiated by diluting the denatured LDH 100-fold in a refolding buffer of pH 7.5. The activity of refolded enzyme was assayed by adding 20 µl of refolding mixture to 580 µl refolding buffer mentioned above containing 0.1 mM NADH and 0.4 mM sodium pyruvate pre-incubated at 37 °C in presence of 30 µM unmodified and MG modified proteins and measuring the decrease in absorbance at 340 nm for 180 s.

3 Results

3.1 UV Spectral Analysis of Unmodified and MG Modified α-Crystallin

All our studies were carried out in two different ranges of MG concentration namely, low (100–500 µM) and high (10 mM). In order to monitor the progress of the chemical modification of α-crystallin by MG we took the UV spectra (400 to 230 nm) of α-crystallin (0.05 mg/ml) of human using a spectrophotometer. Results for three different concentrations of MG (250, 500 µM and 10 mM) mediated α-crystallins for different time intervals (24–120 h) have been shown in panel A, B and C (Fig. 1), respectively. Trace a of panel A, B and C in Fig. 1 shows the spectrum of unmodified human α-crystallin, which exhibits the typical aromatic peak at 280 nm. There was no additional peak of the unmodified α-crystallin in the spectral range. For 250 µM MG modified α-crystallin there is also no additional peak but with increase in modification time, the absorbance at 280 nm has been increased (Fig. 1 A). But in cases of 10 mM MG modified α-crystallin an additional peak at 336 nm appeared. With increase in modification time the intensity of the peak at 336 nm increased. A concomitant increase in the absorbance at 280 nm was also observed (Fig. 1C). In case of 500 µM MG modified α-crystallin, there is no distinctly visible peak at 336 nm although the absorbance at 336 nm is somewhat increased compared to that of unmodified protein (Fig. 1 B). The 336 nm peak represented the contribution from the chromophore introduced by MG modification and therefore increase in absorbance at 336 nm (ΔA_{336}) qualitatively indicated relative increase of modification by MG. It was

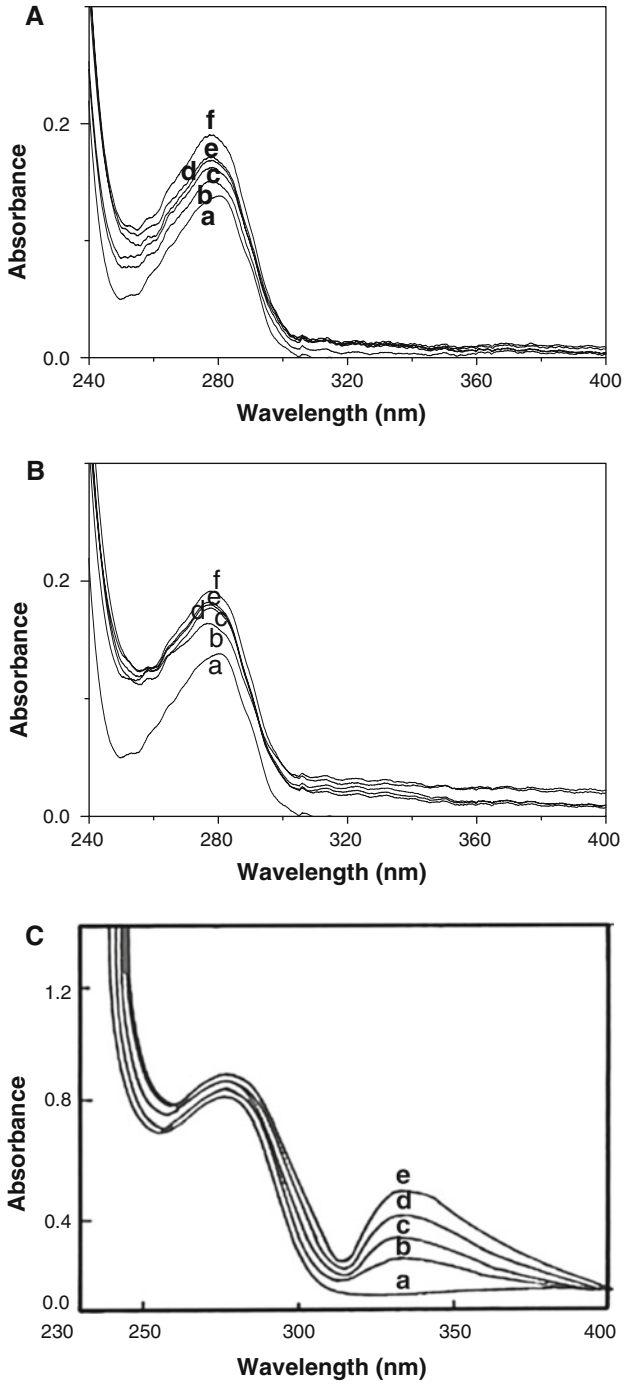

Fig. 1 UV spectroscopic properties of human unmodified and MG modified α-crystallins. **A** UV Spectra of *a* Unmodified, *b* 24 h, 250 μM MG, *c* 48 h, 250 μM MG, *d* 72 h, 250 μM MG *e* 96 h, 250 μM MG, and *f* 120 h, 250 μM MG. **B** UV Spectra of *a* Unmodified, *b* 24 h, 500 μM MG, *c* 48 h, 500 μM MG, *d* 72 h, 500 μM MG *e* 96 h, 500 μM MG and *f* 120 h, 500 μM MG. **C** UV Spectra of *a* Unmodified, *b* 24 h, 10 mM MG, *c* 48 h, 10 mM MG, *d* 72 h, 10 mM MG and *e* 72 h, 100 mM MG. Concentration of α–crystallin in each sample is 0.05 mg/ml

seen that such a modification was a slow process and even after 72 h (with 10 mM MG modification) complete modification did not take place. However, absorbance does not detect primary modification product such as hydroimidazolone MG-H1 [2].

3.2 Fluorescence of MG Modified α-Crystallin

Intrinsic fluorescence emission spectra of the tryptophan residues convey folding information of the globular protein. The fluorescence emission spectra of unmodified and MG modified α-crystallin were taken in the range 310–450 nm using 295 nm as the excitation wavelength. The spectra of different concentration of MG modified proteins (100, 250 and 500 μM) at a given time (24 and 96 h) have been shown in panel A and B in Fig. 2, respectively. The spectra at a fixed concentration (500 μM and 10 mM) of MG mediated proteins at different time intervals (24–120 h) have been shown in panel C and D in Fig. 2. The tryptophan spectra of unmodified α-crystallin exhibit maximum emission (λ_{max}) at 337 nm (Fig. 2 A, B, C, D trace a). In case of 24 and 96 h modification, the fluorescence intensity of the modified proteins has been decreased gradually depending on the concentration of MG modification (Fig. 2 A, B traces- b, c, d). In 96 h modification a slight development of another peak around 385 nm has been observed for the protein with 250 μM MG modification (Fig. 2 B trace- c) which is very prominent in case of 500 μM MG modification (Fig. 2 B trace- d). Even in case of 500 μM MG modification, the gradual increase of the other peak at 385 nm has been observed with the increase of time of MG incubation (Fig. 2 C peaks-c_2, d_2, e_2). 10 mM MG modified human α-crystallin (4 h) showed very little or negligible shift of λ_{max} with small decrease in fluorescence intensity at its λ_{max} (Fig. 2D). But the spectrum of 24 h, 10 mM MG modified protein (Fig. 2D) not only showed significant shifting of λ_{max} and decrease of fluorescence intensity; it also showed the initiation of development of another peak around 385 nm. Spectrum of 48 h, 10 mM MG modified protein (Fig. 2D) showed further red shift of emission maxima of the main peak to 343 nm and the second peak became more prominent with increase in fluorescence intensity. Till 96 h, the intensity of the second peak of 10 mM MG modified protein, kept on increasing with modification reaction time and that of the first peak concomitantly decreased (Fig. 2D traces e, f). The decrease in fluorescence intensity of the first peak (337–345 nm) with red shift of emission maxima is indicative of the loss of structural integrity of α-crystallin with high range of MG modification. The 385 nm peak was used to estimate the loss of arginine content. The intensity at 385 nm for the 10 mM MG modified protein was used in the standard curve to determine approximately the number of arginine residues lost in MG modification. It was found that at 24, 48 and 72 h approximately 2.8, 3.4 and 4.0

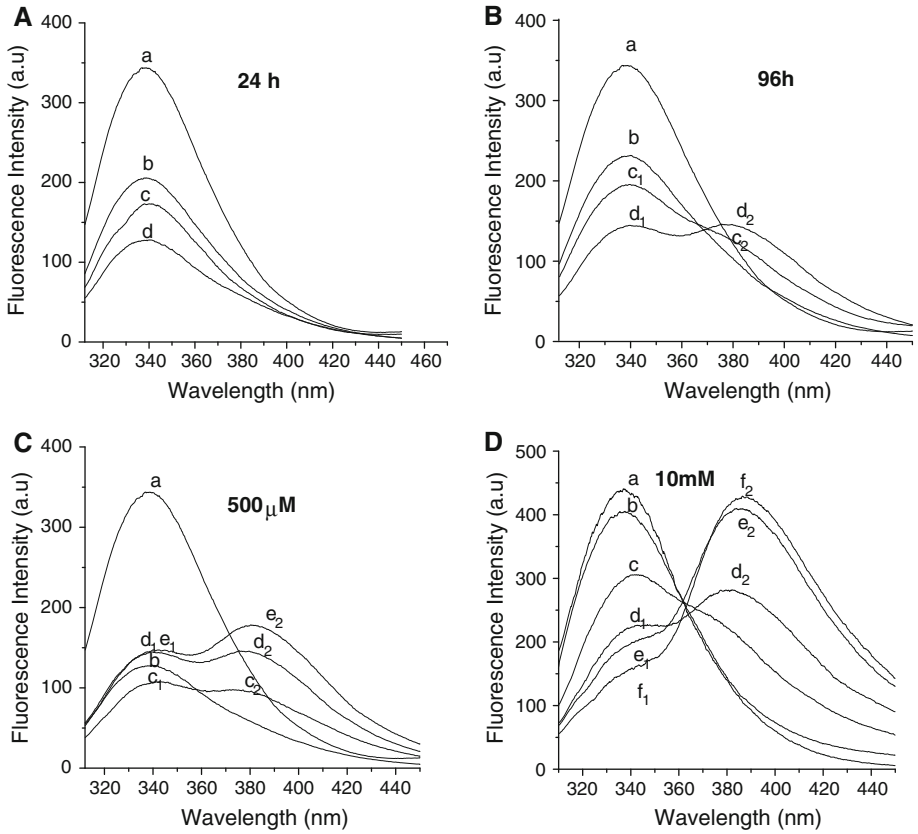

Fig. 2 Tryptophan fluorescence spectra of MG modified human α-crystallin. Protein concentration of 0.05 mg/ml in 50 mm phosphate buffer (pH 7.5), excitation wavelength of 295 nm, excitation and emission band passes of 5 nm each and scan speed of 240 nm/min were used. **A** *a* unmodified α-crystallin; *b* 100 μM MG modified α-crystallin; *c* 250 μM MG modified α-crystallin; *d* 500 μM MG modified α-crystallin. **B** *a* unmodified α-crystallin; *b* 100 μM MG modified α-crystallin; *c* 250 μM MG modified α-crystallin (c_1: 1st fluorophore, c_2: 2nd fluorophore); *d* 500 μM MG modified α-crystallin, (d_1: 1st fluorophore, d_2: 2nd fluorophore). **C** *a* unmodified α-crystallin; *b* 24 h modified α-crystallin; *c* 72 h modified α-crystallin; *d* 96 h modified α-crystallin; *e* 120 h modified α-crystallin. **D** *a* unmodified α-crystallin; *b* 4 h modified α-crystallin; *c* 24 h modified α-crystallin; *d* 48 h modified α-crystallin; *e* 72 h modified α-crystallin; *f* 96 h modified α-crystallin

number of arginine per molecule was lost due to modification by 10 mM MG under our experimental condition.

3.3 Study of Tryptophan Fluorescence Quenching by Acrylamide

Tryptophan fluorescence quenching experiments can provide information on the microenvironment of the tryptophan, which usually remains buried within the folded globular structure. Acrylamide is a non-ionic quencher, which can usually penetrate appreciably into the globular structure to quench a fluorophore [17]. The accessibility of the fluorophore is expressed by a constant K_{SV}, known as the Stern–Volmer Quenching constant [28]. Stern–Volmer equation for fluorescence quenching can be expressed as

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are fluorescence intensities of any fluorophore in absence and presence of the quencher, respectively and [Q] is the quencher concentration.

We have compared the accessibility of tryptophan of unmodified and different concentration of MG modified α-crystallins by acrylamide quenching. The Stern–Volmer plot for 250 μM MG modified protein for 24 and 72 h has been shown in panel A in Fig. 3. Panel B of Fig. 3 shows the Stern–Volmer plot for 10 mM MG modified protein for 24, 48 and 72 h. Unmodified protein shows the minimum K_{SV} value (1.9 M^{-1}) indicating the buried nature of tryptophan. However, on MG modification the K_{SV} increased with extent of modification. K_{SV} of 250 μM MG modified protein for 24 and 72 h are 2.21 and 2.9 M^{-1} where as it is 2.86 for 24 h, 3.34 for 48 h and 3.8 for 72 h, respectively for 10 mM MG modified proteins. These data clearly

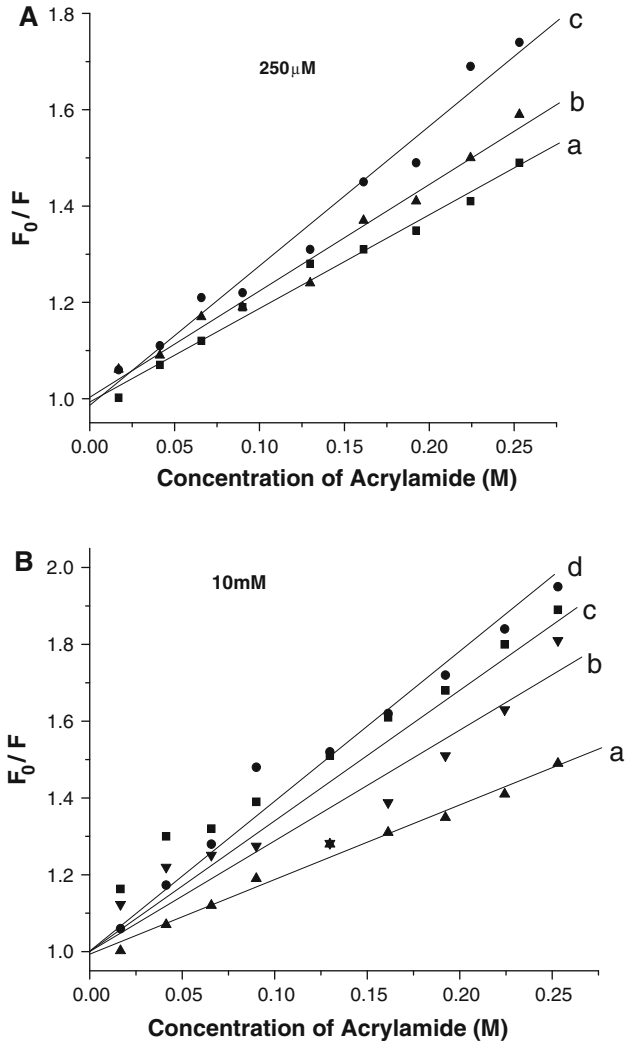

Fig. 3 Acrylamide quenching of tryptophan fluorescence of human α-crystallin before and after MG modification. F_o and F represent the fluorescence emission intensity at 340 nm without and with acrylamide addition, respectively. The concentration of α-crystallin was 0.1 mg/ml and the excitation wavelength was 295 nm. **A** *a* human unmodified α-crystallin; *b* 24 h modified human α-crystallin; *c* 72 h modified human α-crystallin; **B** *a* human unmodified α-crystallin; *b* 24 h modified human α-crystallin; *c* 48 h modified human α-crystallin; *d* 72 h modified human α-crystallin

indicate that MG modification led to enhanced exposure of tryptophan in α-crystallin.

3.4 Surface Hydrophobicity of Unmodified and MG Modified α-Crystallin

Bis-ANS is a hydrophobic fluorescence probe that binds to accessible hydrophobic pockets leading to substantial increase in its fluorescence intensity [13]. Thus Bis-ANS fluorescence study may provide information regarding surface hydrophobicity of a protein molecule. We measured the fluorescence spectra of Bis-ANS in presence of α-crystallin (0.1 mg/ml) that has been subjected to different concentration of MG treatment (100 μM to 10 mM) for various lengths of time (4 to 120 h). The λ_{max} practically remained unchanged for MG modified proteins with increase in reaction time but the Bis-ANS fluorescence intensity decreased gradually in case of 100, 250 and 500 μM MG modified proteins with the increased length of incubation time. The Bis ANS spectra of 250 μM MG modified proteins for different time intervals have been shown in Panel A in Fig. 4. The Bis-ANS fluorescence intensity of 10 mM MG modified proteins kept on decreasing with time of reaction (Fig. 4 B) and the degree of decrease of intensity is drastic and much higher in case of 10 mM MG modified protein when compared to 100–500 μM MG modified proteins. These data indicated

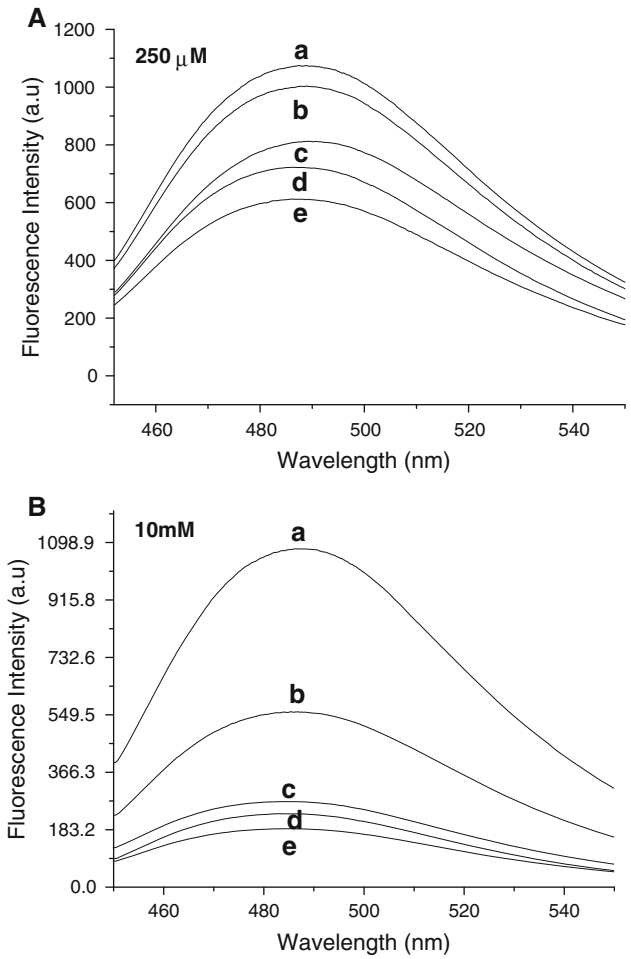

Fig. 4 Bis ANS fluorescence spectra in presence of MG modified α-crystallin. Unmodified and MG modified human α-crystallin (0.05 mg/ml) was incubated with 10 μM Bis-ANS for 1 h in room temperature in dark. Excitation wavelength was 390 nm. **A** Trace *a* unmodified, trace *b* 24 h, trace *c* 48 h, trace *d* 72 h, trace *e* 120 h. **B** Trace *a* unmodified, trace *b* 7 h, trace *c* 24 h, trace *d* 48 h, trace *e* 72 h

Table 1 Hydrophobic binding sites (*n*) and dissociation constants (K_d) of human unmodified and 10 mM MG modified α-crystallins

Type of α-crystallin	*n*	K_d
Unmodified	0.38 ± 0.003	1.21
24 h MG modified high affinity binding sites	0.13 ± 0.003	1.78
24 h MG modified low affinity binding sites	0.25 ± 0.002	6.4
48 h MG modified high affinity binding sites	0.14 ± 0.002	1.84
48 h MG modified low affinity binding sites	0.24 ± 0.001	5.0
72 h MG modified high affinity binding sites	0.12 ± 0.001	1.74
72 h MG modified low affinity binding sites	0.19 ± 0.001	4.8

that the number of hydrophobic pockets was reduced in MG modified proteins.

In order to quantify the number of hydrophobic pockets the binding data were quantitatively analyzed by the Scatchard equation [13]. Bis-ANS binding sites (*n*) were decreased from 0.38 to 0.12 with 72 h 10 mM MG modified protein (Table 1). Scatchard plots for 48 and 72 h, 10 mM MG modified proteins show two distinct slopes corresponding to one relatively high and low affinity-binding site. The affinity constant (K_d) and number of binding sites (*n*) have been summarized in Table 1. It is found that in addition to the original affinity constants of Bis-ANS to unmodified proteins, some additional binding sites having ~four-fold higher K_d (low affinity), are developing due to MG modification.

3.5 Far UV Circular Dichroic Spectra of MG Modified Human α-Crystallin

Far UV CD spectra reflect changes in the secondary structure in protein molecules. To investigate such changes during time (24–120 h) dependent modification of α-crystallin by different concentration of MG (100 μM–100 mM), far UV circular dichroic spectra were taken. The results are shown in Fig. 5 A and B. CD spectra of unmodified human α-crystallin (Fig. 5 A, B trace a) showed a minimum around 217 nm, which indicated the predominant presence of *β*-sheet conformation. This was also consistent with the published literature. However, the MG modified α-crystallins show different profiles. Panel A in Fig. 5 shows the profile of 250 μM MG modified protein for 24–120 h modification where the concomitant decrease in the negative ellipticity has been found and a tendency of slight left shift of minima is also observed. The minima of 120 h, 500 μM MG modified protein has shifted to 210 nm (Figure not shown here). The minima of 24, 48 and 72 h, 10 mM and 72 h, 100 mM MG modified α-crystallins were shifted to 205 nm. In case of 72 h, 100 mM MG modified α-crystallin the decrease in the negative ellipticity has been found (Fig. 5 B traces b, c, d, e). The circular dichroic

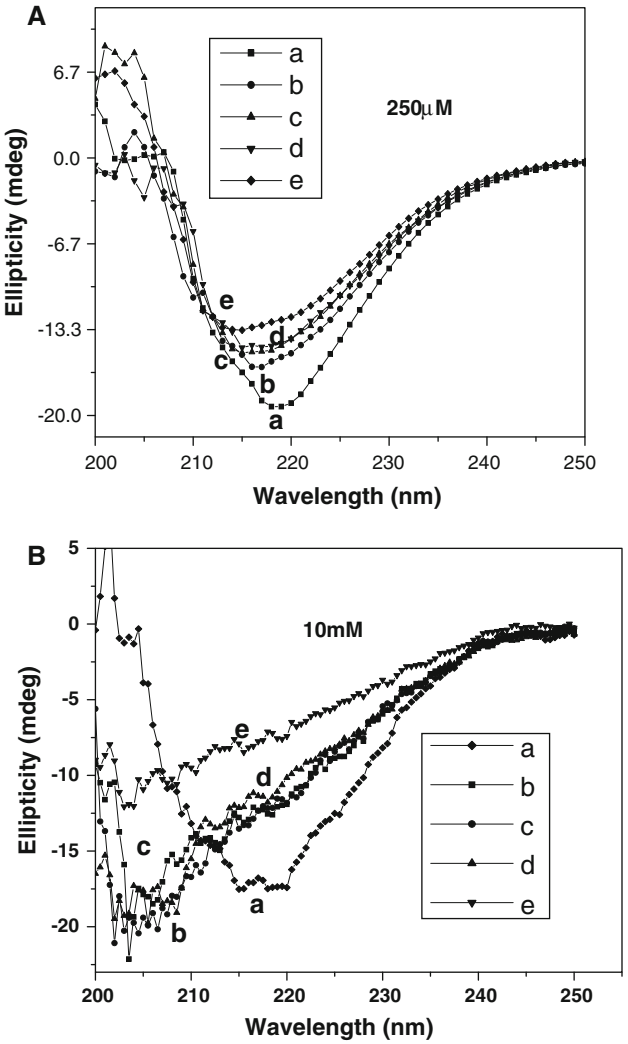

Fig. 5 Far UV circular dichroic spectra of human α-crystallin before and after time dependent MG modification. Protein concentration was 0.5 mg/ml in 50 mM phosphate buffer, pH 7.5 at 25 °C. A cylindrical quartz cell of 1 mm pathlength and scan speed of 20 nm/min were used. **A** trace *a* unmodified α-crystallin; trace *b* 24 h modified; *c* 48 h modified; *d* 96 h modified; *e* 120 h modified. **B** Trace *a* human unmodified α-crystallin; trace *b* 24 h modified; *c* 48 h modified; *d* 72 h modified; *e* 72 h MG (100 mM) modified α-crystallin

minimum at 205 nm represents unordered structure. These data were qualitatively consistent with the fluorescence data and indicated loss of *β*-sheet and other ordered structures with increase in random structure.

3.6 SDS–PAGE of MG Modified α-Crystallin

MG modification of proteins has been reported to form covalent cross-links [25, 26]. To identify covalent crosslink formation as a function of the time of reaction of α-crystallin with MG, the reaction products were analyzed on 12% SDS–PAGE. Results for α-crystallin modified by 250 and 500 μM MG for 24 to 120 h modification have been

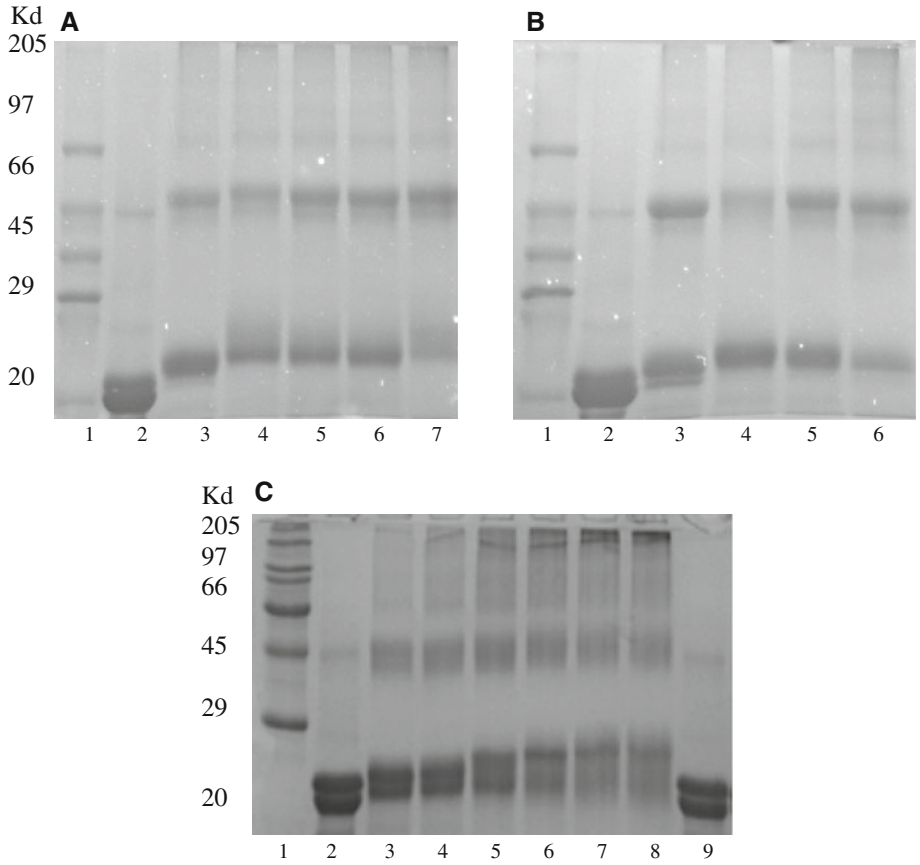

Fig. 6 SDS-PAGE (12%) of human α-crystallin before and after MG modification. **a** *Lane 1* Marker proteins; *lane 2* unmodified α–crystallin; *lane 3* 24 h, 250 μM MG modified; *lane 4* 48 h, 250 μM MG modified; *lane 5* 72 h, 250 μM MG modified; *lane 6* 96 h, 250 μM MG modified; *lane 7* 120 h, 250 μM MG modified and lane. **b** *Lane 1* Marker proteins; *lane 2* unmodified α–crystallin; *lane 3* 24 h, 500 μM MG modified h; *lane 4* 48 h, 500 μM MG modified; *lane 5* 72 h, 500 μM MG modified; *lane 6* 96 h, 500 μM MG modified; **c** *Lane 1* Marker proteins; *lanes 2 & 9* unmodified α–crystallin; *lane 3* 4 h, 10 mM MG modified; *lane 4*: 7 h, 10 mM MG modified; *lane 5* 14 h, 10 mM MG modified; *lane 6* 24 h, 10 mM MG modified; *lane 7* 48 h, 10 mM MG modified and *lane 8* 72 h, 10 mM MG modified α-crystallin

shown in Fig. 6A and B and same for 10 mM MG modified proteins for different time intervals have been shown in Fig. 6 C. Unmodified α-crystallin showed two bands at approximately 20 kDa corresponding to αA and αB-crystallin (lane 2 of Fig. 6 A, B, C and lane 9 of C). With the progress of MG modification, the high molecular weight bands started to appear. At a fixed concentration of MG the modified crystallins showed the increase of high molecular weight bands (Fig. 6 A, B, C lanes 3, 4, 5, 6, 7, 8) with the increase of time of modification. Similarly, at a given time the intensity of the high molecular weight bands increased depending on the concentration of MG also. Simultaneously the intensity of the band at 20 kDa was found to reduce gradually with time in case of high range of MG modification (Fig. 6 C). In case of 48 h and 72 h, 10 mM MG modified proteins, very faint bands were observed at 20 kD position where as the band around 200 kD became prominent (Fig. 6 C lanes 7 & 8).

3.7 Chaperone Activity of MG Modified α-Crystallin

Chaperone-like activity is the most important function of α-crystallin, and such function is known to be temperature dependent. We studied the chaperone-like activity of the human unmodified and the MG modified α-crystallin. We measured the percentage of protection offered by MG modified α-crystallin against thermal as well as non-thermal aggregation of different substrates. β_L-Crystallin was used as the substrate for thermal assay. The aggregation was measured at 60 °C by monitoring the apparent absorbance (scattering due to aggregation) at 400 nm [18] and the profile is shown as trace a (Fig. 7, A, B, C). The concentration of α-crystallin was chosen in such a way as to result in nearly 100% protection (Fig. 7 A, B, C trace b). The chaperone activity of 100 and 250 μM MG modified human α-crystallins (for 24–120 h of modification) has been displayed in panel A and B in Fig. 7. The same for 10 mM MG modified protein is shown in panel C in Fig. 7. In case of MG modified proteins decrease of chaperone activity has been found clearly according to the increase in concentration of MG and time of modification. Low concentration of MG (100 and 250 μM) mediated human α-crystallin proteins showed decreased chaperone activity (Fig. 7, A & B), which is further decreased in case of high concentration of MG (10 mM) modified proteins also (Fig. 7 C). α-Crystallin modified by 100, 250 μM and 10 mM MG for 24 h showed a decrease in the percentage of protection which remained at ~91, 89 and 66%, respectively against β_L-crystallin (Fig. 7, A, B, C- trace c). Chaperone activity was further reduced to 84, 73 and 32%

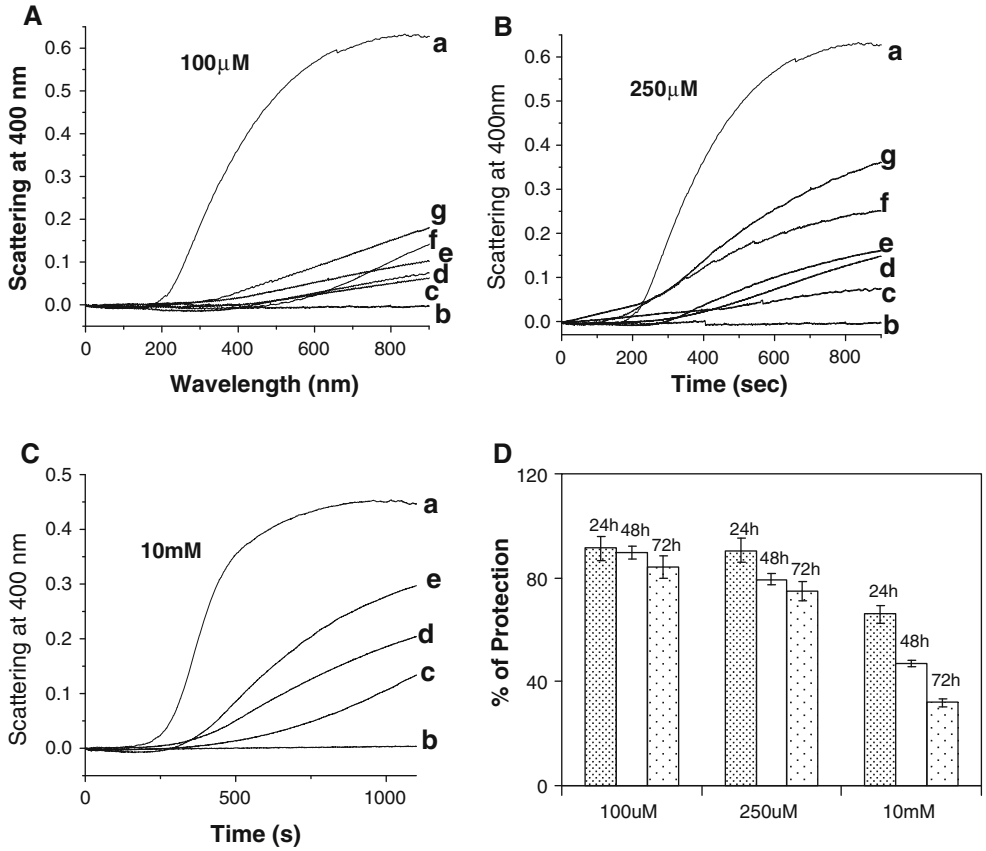

Fig. 7 Chaperone function of MG modified and unmodified α-crystallin. Prevention of thermal aggregation of β_L-crystallin by α-crystallin modified by 100 μM (**A**), 250 μM (**B**) and 10 mM (**C**) MG for different time at 60 °C. Assay mixture contained 0.15 mg/ml β_L-crystallin and 0.015 mg/ml α-crystallin in 50 mM phosphate buffer containing 100 mM NaCl, pH 7.5. **A** *a* β_L-crystallin; *b* β_L-crystallin + unmodified α-crystallin; *c* β_L-crystallin + MG modified α-crystallin, 24 h; *d* + MG modified α-crystallin, 48 h; *e* + MG modified α-crystallin, 72 h; *f* + MG modified α-crystallin, 96 h; *g* + modified α-crystallin, 120 h. **B** *a* β_L-crystallin; *b* β_L-crystallin + unmodified α-crystallin; *c* + MG modified α-crystallin, 24 h; *d* + MG modified human α-crystallin, 48 h; *e* + MG modified α-crystallin, 72 h; *f* + MG modified human α-crystallin, 96 h; *g* + MG modified α-crystallin, 120 h. **C** *a* β_L-crystallin alone; *b* β_L-crystallin + unmodified α-crystallin; *c* + MG modified α-crystallin, 24 h; *d* + MG modified α-crystallin, 48 h; *e* β_L-crystallin + MG modified α-crystallin, 72 h. **D** Comparative analysis of percentage of protection of β_L-crystallin by α-crystallin as a function of reaction time with different concentration of MG

when 72 h MG modified α-crystallins of 100, 250 μM and 10 mM were used as chaperone (Fig. 7 A, B, C- trace e). Percentage protection (chaperone activity) of unmodified and modified proteins as a function of the reaction time with different concentration of MG as calculated from the data shown in three panels (Fig. 7 A, B, C) is shown in panel D of Fig. 7.

Assay of chaperone activity by thermal aggregation at 60 °C, though used commonly, does not represent physiological condition; we have therefore also used ability to refold substrate enzymes at physiological temperature as alternative assay of chaperone activity. Like classical molecular chaperones in vitro, α-crystallin was known to refold denatured enzyme to a considerable extent [8]. The effectiveness of MG modified proteins in refolding of fully denatured LDH was determined in vitro at 37 °C and the profile of 250 μM and 10 mM MG modified α-crystallins both have been shown in Fig. 8A. In absence of any added chaperone, dilution of chemically denatured LDH into a refolding solution yielded only 4–5% reactivation (Fig. 8A trace a). The reactivation yield of LDH reached up to 30% in presence of 30 μM native or unmodified human α crystallin (Fig. 8 A trace b). When different MG modified proteins were used, the reactivation yield varied with the time and concentration of MG modification. In presence of 24 h 250 μM and 10 mM MG modified proteins the refolding yields were 22 and 18%, respectively (Fig. 8 A-traces f and c). MG modified α-crystallin for 72 h with 250 μM MG showed the refolding yield 16% where as 72 h, 10 mM MG modified α-crystallin failed to yield any significant activity (6.5%) (Fig. 8 A traces g and e), compared to the control. The percentage of refolding activity of

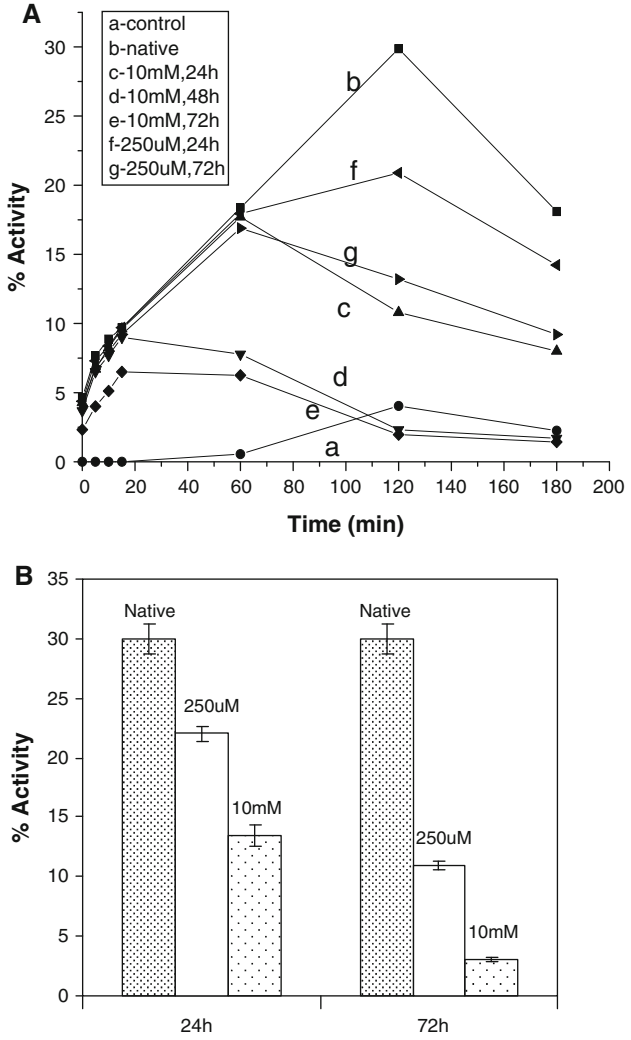

Fig. 8 LDH refolding assay by human unmodified and MG modified α-crystallin. Time course of reactivation of LDH (10 nM) in presence of 30 μM human unmodified and MG modified α-crystallin. Lactate dehydrogenase (LDH), was denatured in 6 M Gu-HCl containing denaturation buffer for 8 h at 25 °C at a concentration of 1 μM. Refolding of the enzyme was initiated by diluting the denatured LDH 100-fold in a refolding buffer of pH 7.5. The activity of refolded enzyme was assayed by adding 20 μl of refolding mixture to 580 μl refolding buffer containing 0.1 mM NADH and 0.4 mM sodium pyruvate in absence or presence of 30 μM α-crystallin preincubated at 37 °C and measuring the decrease in absorbance at 340 nm for 180 s. **A** Trace *a* control (no α-crystallin), trace *b* α-crystallin, no MG; trace *c* α-crystallin + 10 mM MG, 24 h; trace *d* α-crystallin + 10 mM MG, 48 h; trace *e* α-crystallin + 10 mM MG, 72 h; trace *f* α-crystallin + 250 μM MG, 24 h; trace *g* α-crystallin + 250 μM MG 72 h. **B** Comparative analysis of percentage of refolding activity of unmodified and MG (250 μM and 10 mM) modified human α-crystallins as a function of time

unmodified and different time dependent MG (with different concentration) modified proteins was shown in Fig. 8 B which showed the reduction of refolding activity according to the concentration of MG modification as well as the time of modification.

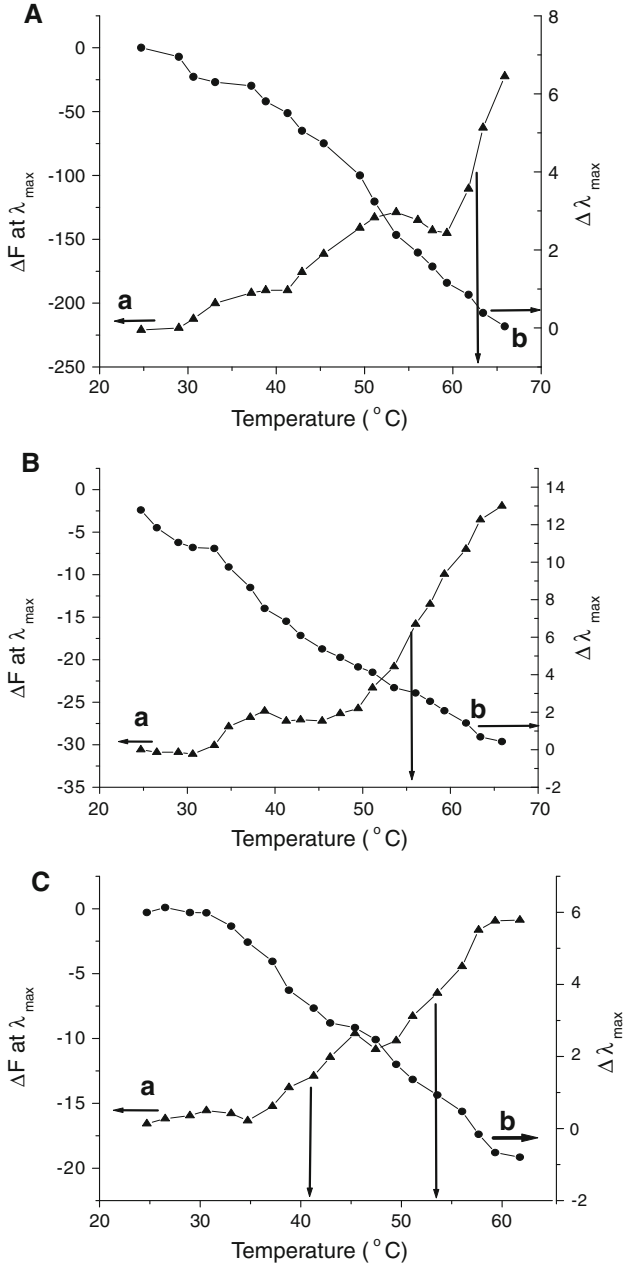

Fig. 9 Thermal stability of native and MG modified α-crystallin. **A** Change in tryptophan fluorescence emission intensity and emission maximum of human unmodified α-crystallin. **B** First fluorophore (peak e_1, Fig 2 D) of 72 h, 10 mM MG modified α-crystallin **C** Second fluorophore (peak e_2, Fig 2 D) of 72 h 10 mM MG modified α-crystallin as a function of gradual temperature increase. Protein conc. was 0.1 mg/ml in 50 mM phosphate buffer (pH 7.5). trace *a* ΔF at λmax; trace *b* Δ λmax

3.8 Thermal Denaturation Study of MG Modified α-Crystallin

We also studied the effect of the MG modification on the thermal stability of α-crystallin. Progress of denaturation was followed through fluorescence emission intensity and

emission maximum of intrinsic tryptophan residues of α-crystallin. The emission spectrum at 25 °C was considered as the native spectrum. The difference of fluorescence intensity as well as emission maximum between the native and the thermally treated α-crystallin has been plotted against temperature and a representative set of data has been shown in Fig. 9 A, B, C trace a and b respectively. Trace A in three panels in Fig. 9 corresponds to difference of fluorescence intensity where as trace b corresponds to difference of emission maxima. It may be recalled that the tryptophan fluorescence spectral pattern of 48, 72 and 96 h modified proteins show the existence of 2 fluorophores simultaneously (Fig. 2 D), one due to tryptophan and the other due to production of argpyrimidine and pentosidine crosslink [1, 3, 49]. Unmodified human α-crystallin shows the sharp transition between the temperature 60–66 °C (Fig. 9 A trace a). The midpoint of this transition is taken to be 63 °C. In Fig. 9 B, 10 mM MG modified protein for 72 h has been displayed where the transition is measured by tryptophan fluorescence (peak e_1 in Fig. 2 D) and it shows sharp transition between transition 50–60 °C, the midpoint being 55 °C. The transition measured from peak e_2 in Fig. 2 D, which is attributed to the chromophore of MG modification, occurs in two ranges namely 36–45 °C and 50–56 °C. The midpoints of this transition can be taken as 41 and 53 °C (Fig. 9 C). 41 °C may correspond to the melting of MG reacted domain where as 53 °C is close to be the melting of the tryptophan domain of the MG modified protein. All these data collectively showed that different domains of MG treated α-crystallin melted at different temperatures and on modification by MG, the thermal stability of α-crystallin is decreased considerably. The conclusions were also supported by data of other MG incubations (data not shown).

3.9 Thermodynamic Stability of MG Modified α-Crystallin

Thermodynamic stability of α-crystallin in solution was studied by chemical denaturation. A chaotropic agent urea was used in increasing concentration to vary the extent of denaturation, which was followed by measuring tryptophan fluorescence as a function of urea concentration. Native and fully unfolded human α-crystallin was known to have emission maxima at 337 nm over 350 nm, respectively [43]. We have therefore plotted the ratio of fluorescence intensities at 337 and 350 nm to construct the unfolding profile as a function of urea concentration. The profile of 250 μM MG modified 24 and 72 h α-crystallins have been shown in Fig. 10 B and C and the same of 10 mM MG modified proteins for 7 and 72 h have been shown in Fig. 10 D and E. The intensity ratio gradually decreased with increase of urea concentration. The profile showed low co-operativity indicating multistage denaturation process, as has been observed by others [7]. The profile has been analyzed initially by sigmoidal fitting, which yielded 2.77 M urea as the transition mid point in case of unmodified human α-crystallin (Fig. 10 A). This value is in good agreement with values reported in literature [7]. The $C_{1/2}$ values provide a relative measure of the stability of proteins as a lower $C_{1/2}$ value indicates a decline in stability. The $C_{1/2}$ values of 24 and 72 h modified proteins with 250 μM MG are 2.08 ± 0.09 and 1.89 ± 0.1 M. The $C_{1/2}$ values of 7 and 72 h modified α-crystallins with 10 mM MG are 2.13 ± 0.4 and 1.04 ± 0.82 M, respectively, which indicate drastic reduction of stability due to time dependent reaction with MG. We then proceeded to analyze the data more quantitatively.

Sun et al. [43] analyzed the unfolding profile of recombinant wild type αA-crystallin. They calculated the thermodynamic stability according to three state model Native ↔ Intermediate ↔ Unfolded using the following equation.

$$F(U) = \frac{I_0 + I_1(\exp(-\Delta G_1^0 + m_1[U])/RT) + I_\alpha(\exp(-\Delta G_2^0 + m_2[U])/RT)}{1 + (\exp((-\Delta G_1^0 + m_1[U])/RT) + (\exp((-\Delta G_2^0 + m_2[U])/RT)}$$

This model involved a stable intermediate (I) in the denaturation pathway where I_0, I_1 and I_∞ are the spectroscopic parameters for 100% native, intermediate and unfolded form, respectively. ΔG_1^0 refers to the standard free energy change between native and intermediate form, ΔG_2^0 refers to the standard free energy change between intermediate and unfolded form at zero urea concentration. ΔG^0 being the sum of ΔG_1^0 and ΔG_2^0 refers to the standard free energy change of unfolding at zero urea concentration. Parameters of equilibrium urea unfolding of unmodified as well as 250 μM 24 and 72 h and 10 mM MG modified (7 and 72 h) human α crystallins at 25 °C, obtained from the three-state model (N↔I↔U) fit have been listed in Table 2. ΔG^0 of native protein, 21.6 kJ/mol, obtained here is in close agreement with literature values [7]. ΔG^0 for 250 μM MG modified protein decreased to 17.3 kJ/mol in 24 h and 14.9 kJ/mol in 72 h. More drastic reduction in

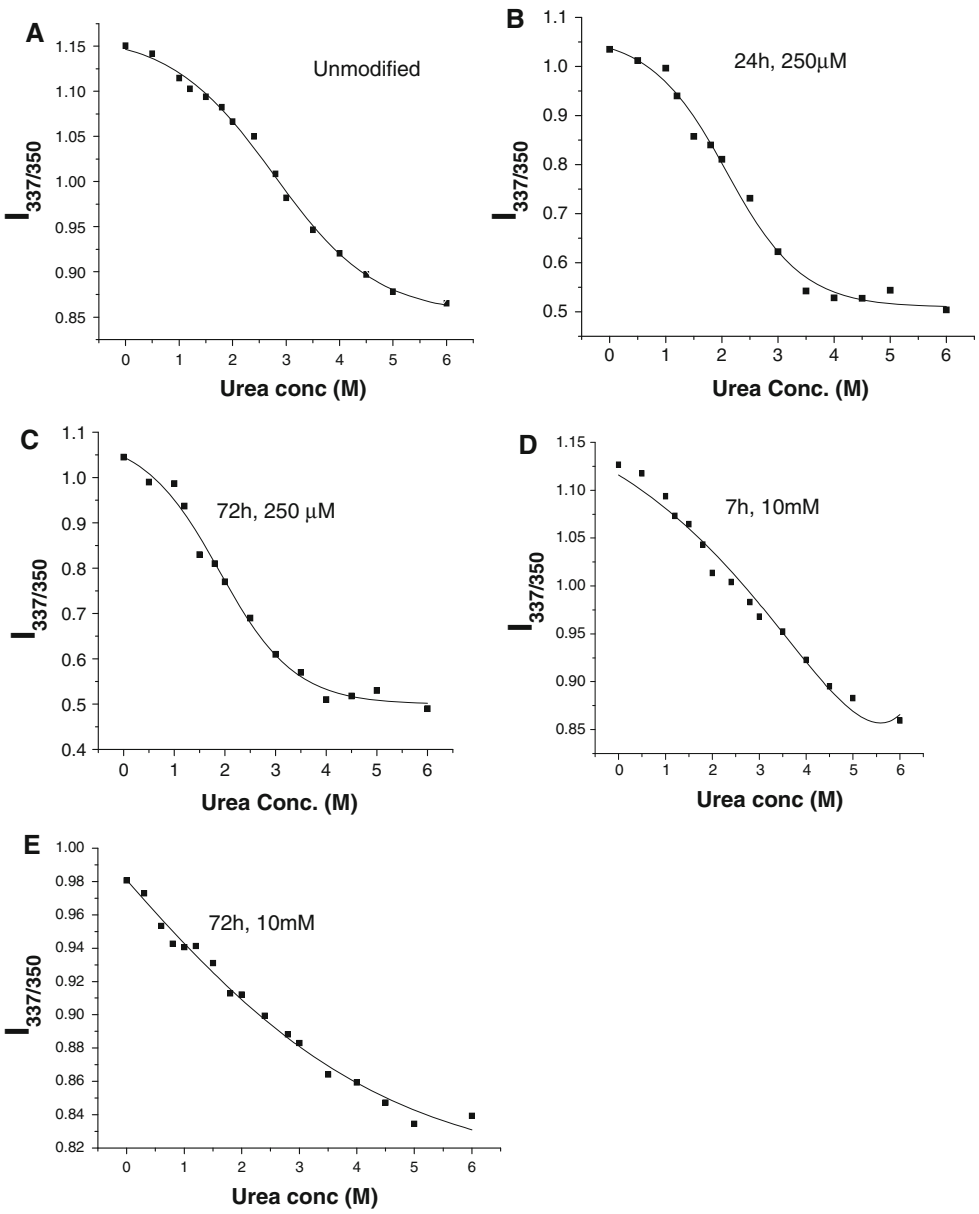

Fig. 10 Equilibrium urea denaturation profile of human α-crystallin before and after MG modification; Unmodified and 72 h MG modified human α-crystallin of 0.05 mg/ml concentration were incubated at 25 °C for 18 h with urea 0–8 M in 50 mM phosphate buffer, ph 7.5. $I_{337/350}$ represents the ratio of fluorescence emission intensity at 337 and 350 nm with 295 nm as excitation. **A** Human unmodified α-crystallin, **B** 24 h, 250 μM MG modified α-crystallin; **C** 72 h, 250 μM MG modified α-crystallin, **D** 7 h, 10 mM MG modified α-crystallin, **E** 72 h, 10 mM MG modified α-crystallin

Table 2 Parameters of equilibrium urea unfolding of unmodified and MG modified (7–72 h) human α-crystallin at 25 °C, obtained from the three-state model (N↔I↔U) fit

Systems studied	$\Delta G_1°$ (kJ/mol)	$\Delta G_2°$ (kJ/mol)	$\Delta G°$ (kJ/mol)	m_1 (kJ/mol.M)	m_2 (kJ/mol.M)
Unmodified	4.82 ± 3.74	16.79 ± 18.17	21.62 ± 21.9	1.32 ± 0.38	2.6 ± 2.3
24 h, 250 μM MG modified	3.62 ± 0.001	13.7 ± 8.76	17.3 ± 8.77	0.89 ± 0.22	1.87 ± 1.47
72 h, 250 μM MG modified	3.1 ± 0.0001	11.78 ± 10.67	14.88 ± 10.67	0.78 ± 0.3	1.53 ± 1.21
7 h, 10 mM MG modified	3.8 ± 0.0	13.23 ± 12.07	17.03 ± 12.07	0.84 ± 0.29	1.83 ± 1.38
72 h, 10 mM MG modified	2.1 ± 0.0001	8.27 ± 9.26	10.37 ± 9.26	0.43 ± 0.21	0.96 ± 0.71

ΔG^0 to 10.4 kJ/mol was observed in modification by 10 mM MG for 72 h. However, at 7 h for 10 mM MG, ΔG^0 was 17 kJ/mol. This data shows that lower concentration of MG at higher incubation of time could do same decrease in stability as observed in higher concentration of MG at lower incubation time.

4 Discussion

A unique feature of the eye lens is that its central part has very little protein turnover. Hence the major proteins of the eye lens, namely the crystallins must survive for the entire lifetime during which they are highly susceptible to various post-translational modifications such as phosphorylation, glycation, oxidation, deamidation, truncation etc. The major adducts formed by MG modification of proteins are MG-H (Hydroimidazolone). Among three isomers, MG-H1 is the most important product. Two fluorophores like arg-pyrimidine and pentosidine crosslinks are also formed as the glycation product but in lesser amount [1, 3, 49]. Many of these end products are now well characterized although some are yet to be fully characterized. Primary targets of MG in α-crystallin are arginine and lysine residues, which are major centers of positive charges. Disruption of charges of centers is likely to alter conformation of α-crystallin and hence its interaction with substrate proteins and thus chaperone function. Besides, α-crystallin has to be extremely stable to survive the lifetime of a person to function as a molecular chaperone. It is therefore important to study the effect of MG modification on the α-crystallin stability in addition to its chaperone function and other properties. Since various reports have shown that MG level can get elevated under various disease conditions, it's quite relevant to study structural and functional changes in α-crystallin due to MG modification.

We have chosen two different ranges of MG concentration namely, low (100–500 μM) and high (10 mM) for the modification of α-crystallin for different lengths of time in order to study the spectroscopic properties, chaperone activity and stability of MG modified proteins. We found no significant qualitative differences in the trends of the results obtained using two MG concentration ranges. The degree of changes in modified proteins has been increased according to the concentration of MG as well as the time of incubation.

The products of the reaction of MG with α-crystallin have not been fully chemically analyzed. Adduct formation of MG with protein may involve both lysine and arginine residues and can vary from protein to protein [30, 40]. Our UV spectroscopic data (Fig. 1C) shows that the MG modified α-crystallin has a peak at 336 nm. Since this peak has no contribution from aromatic residues or other known chromophores of α-crystallin, intensity of this peak qualitatively reflects relative progress of the chemical modification. Since the tryptophan emission of α-crystallin occurs around 337 nm, the emission is absorbed by the MG modified chromophore, which further emits at 385 nm (Fig. 2 A, B, C, D). Thus tryptophan and MG reaction product chromophore forms a fluorescence resonance energy transfer (FRET) donor–acceptor pair. Such data can be very useful to quantitatively characterize the extent of MG modification. Since MG modification occurs through positively charged centers like lysine or arginine [30], the resultant charge misbalance may lead to conformational changes in α-crystallin.

Indeed conformational changes are reflected by tryptophan fluorescence quenching data, which reflected significant changes in tryptophan environment indicated by enhanced accessibility of the tryptophan by the quencher acrylamide upon MG modification (Fig. 3 A, B). Conformational changes are also indicated by the bis-ANS fluorescence data, which indicated that number of hydrophobic clefts on the α-crystallin surface was decreased on MG modification (Fig. 4 A, B). Obviously destruction of the hydrophobic clefts is due to unfolding of the protein. MG modification, specially at 10 mM concentration, leads to unfolding of the α-crystallin as is clearly indicated by the CD data (Fig. 5 A, B). MG modification of α-crystallin was also reported by many workers [26] to form protein cross-link. Our data (Fig. 6 A, B, C) confirm this finding. Since hydrophobicity is directly linked to chaperone function, we decided to quantify the decrease in hydrophobicity by determining the number of bis-ANS binding sites as was done earlier [8]. The Scatchard plot of MG modified proteins revealed that MG modification not only reduced the number of binding sites, but it also generated some low affinity binding sites ($K_D \sim 5$ μM) and some high affinity sites ($K_D \sim 1$ μM). Low affinity binding sites lead to weaker interaction between chaperone-substrate. Therefore MG modification not only decreases its ability to capture substrate-molecule, the interaction gets weaker also.

Surface hydrophobicity and hydrophobic interactions are believed to play an important role in the molecular chaperone functions of α-crystallin [42]. Many workers reported that protein–protein interactions between chaperone and substrate are primarily hydrophobic in nature [35]. The decrease in surface hydrophobicity correlates well with chaperone function of α-crystallin, which shows a progressive decline in percentage protection in thermal aggregation of β_L-crystallin (Fig. 7). It is interesting to note that despite substantial unfolding, as observed by CD and fluorescence experiments (Figs. 5 and 2), residual hydrophobic clefts remain on α-crystallin surface and anti-aggregation activity, though low, is clearly noticeable even after 72 h of 10 mM MG modification. We checked that the ability of α-crystallin to refold the enzyme LDH also decreased with increasing extent of MG modification (Fig. 8). It is interesting to note that at the early stages of modification with 10 mM MG (24–48 h), the total number of high and low affinity sites remain unchanged, only after 72 h, some decrease in total number of hydrophobic sites are visible (Table 1). It is also interesting to note that about 30% of the original binding sites having K_d in the range

1.2–1.8 remain intact even after 72 h MG modification (with 10 mM MG). This reveals that despite substantial loss of original structure, about 30% of the native like structure is retained by the MG (10 mM) modified α-crystallin. These data can also explain the relatively large reduction in Bis-ANS fluorescence (Fig. 4 A, B) and chaperone activity even at 24 h MG (10 mM) modification (Fig. 7 and Table 1).

It may be the worth mentioning here that contrary to our results some workers reported that MG modification increased chaperone functions [19, 26, 34]. However, our detail investigations of surface hydrophobicity clearly indicated loss of substrate binding sites. Although partial unfolding has been reported by some workers to improve chaperone function [15]. Both fluorescence (Fig. 2 A, B, C, D) and far UV CD data (Fig. 5 A, B) indicated extensive unfolding and loss of folded structure in α-crystallin. Under these conditions our results seem to be quite justifiable. It is also worth mentioning that recent studies have emphasized the role of subunit dynamics on the chaperone function of α-crystallin. Since the subunits undergo chemical cross-linking by MG, the dynamics is somewhat lost. This may also explain the loss of chaperone function of α-crystallin due to MG modification.

Loss of structural stability of α-crystallin is indicated by the CD data. The loss of structural stability was determined both by thermal and chemical denaturation. α-Crystallin is known to undergo structural melting above 60 °C [44]. From intrinsic tryptophan fluorescence spectroscopy our T_m value of 63 °C for native α-crystallin is consistent with literature reports [44]. However, 10 mM MG modified proteins showed significant lowering of T_m indicating drastic reduction in stability. It is interesting to note that one of the domain of the 72 h MG (10 mM) modified α-crystallin melts at 41 °C while other domain melts between 53–55 °C. Our results of the chemical denaturation experiments are also consistent with thermal stability study. Significant reduction in stability becomes apparent only after 7 h MG (10 mM MG) modification (Table 2). The lack of co-operativity in the equilibrium unfolding profile of 72 h MG modified α-crystallin (Fig. 10 C trace e) indicates that a multiple step denaturation is taking place [7]. The ΔG^o values for 72 h MG (10 mM) modified α-crystallin shows only 50% reduction in free energy of denaturation. It may be possible that chemical cross-linking may have contributed somewhat against chemical denaturation.

In summary, it can be concluded that MG modification destroys the folded structure and hydrophobic clefts in α-crystallin. It also reduces its molecular chaperone function to a considerable extent due to reduction in hydrophobicity as well as due to cross-linking and loss of subunit exchange dynamics. Both thermal and thermodynamic stability are diminished significantly due to MG modification. All these may have a combined effect of accelerating cataract formation of eye lens.

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