Effects of modifications of α-crystallin on its chaperone and other properties

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The role of α-crystallin, a small heat-shock protein and chaperone, may explain how the lens stays transparent for so long. α-Crystallin prevents the aggregation of other lens crystallins and proteins that have become unfolded by 'trapping' the protein in a high-molecular-mass complex. However, during aging, the chaperone function of α-crystallin becomes compromised, allowing the formation of light-scattering aggregates that can proceed to form cataracts. Within the central part of the lens there is no turnover of damaged protein, and therefore post-translational modifications of α-crystallin accumulate that can reduce chaperone function; this is compounded in cataract lenses. Extensive *in vitro* glycation, carbamylation and oxidation all decrease chaperone ability. In the present study, we report the effect of the modifiers malondialdehyde, acetaldehyde and methylglyoxal, all of which are pertinent to cataract. Also modification by aspirin, which is known to delay cataract and other diseases, has been investigated. Recently, two point mutations of arginine residues were shown to cause congenital cataract. 1,2-Cyclohexanedione modifies arginine residues, and the extent of modification needed for a change in chaperone function was investigated. Only methylglyoxal and extensive modification by 1,2-cyclohexanedione caused a decrease in chaperone function. This highlights the robust nature of α-crystallin.

Key words: acetaldehyde, aspirin, cyclohexanedione, malondialdehyde, methylglyoxal.

INTRODUCTION

The manner of lens development is unique; because of the continuous differentiation of lens cells, younger cells crowd around older cells. There is no removal of lens protein with age or modification as new layers of fibre cells build up on top of existing cells. The lens proteins remain in an environment containing many reactive small molecules, such as sugars and cyanate, which can attack proteins *in vivo* without enzymes [1].

α-Crystallin (as well the other structural lens proteins β- and γ-crystallins) is intrinsically stable; however, this does not explain how enzymes important for cell metabolism found in the nucleus of aged lens in up to their eighth decade can still possess activity. This may be a testament to the ability of α-crystallin to serve as a molecular chaperone. α-Crystallin has been shown to protect enzymes against unfolding and thermal aggregation [2], inactivation by glycation [3] and thermal inactivation [4].

There is 55% sequence identity between small heat-shock proteins from *Drosophila melanogaster* and bovine α-crystallin [5]. This was the first indication that α-crystallins were related to molecular chaperones. The major lens protein α-crystallin was regarded previously as fulfilling a purely structural role. α-Crystallin is abundant in the ocular lenses of most vertebrate species, reaching levels of 50% of the water-soluble fraction in certain mammalian species [6]. The α-crystallin molecule has an average molecular mass of 800 kDa, consisting of 35–40 subunits each of 20 kDa. The aggregate consists of two gene products, αA- and αB-crystallin, comprised of 173 and 175 amino acid residues respectively and similar sequences (57% identity).

Many common changes are seen in human cataracts of different morphologies and also in experimental cataracts induced by different agents [1]. Post-translational modifications of the long-lived lens proteins play a major role in cataractogenesis. The effect of these cataract-inducing agents on the protective chaperone qualities of α-crystallin is of interest, especially as chaperone function decreases with age and cataract [7]. Modifications that are pertinent to cataract that have caused a decrease in chaperone function include sugars reactive enough to cause cross-linking of α-crystallin [8–10], extensive carbamylation [10] and the effects of oxidative species, including hydroxyl radicals and superoxide ions [8,9]. In the present study, we describe the effects of modification by malondialdehyde (MDA), methylglyoxal, aspirin, acetaldehyde and 1,2-cyclohexanedione (CHD) on the chaperone function of α-crystallin.

The cellular plasma membrane is a major site of oxidant-induced injury, due to its content of oxidizable lipid and protein. MDA is produced in mammalian tissues as an end-product of unsaturated lipid peroxidation and as a side product of thromboxane biosynthesis [11]. MDA increases in human cataract [12]. When oxidized phospholipids containing MDA were injected into the vitreous body of rabbits, posterior subcapsular cataract was induced within 24 h [13]. Aldehydes are relatively long-lived, and can diffuse from their site of origin and attack intracellular or extracellular targets.

Methylglyoxal, a dicarbonyl, is a biological metabolite formed from the triose phosphates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, both essential for the energy requirements of the glycolysis-dependent lens, and from oxidation of hydroxyacetone and aminoacetone [14]. It has been shown to cross-link proteins during glycation or Maillard reactions, resulting in protein-bound fluorescent molecules or advanced glycation end-products ('AGE'). Methylglyoxal and other dicarbonyls have been implicated in cataract and diabetic complications [14].

Acetaldehyde, a breakdown product from ethanol oxidation in the liver, has been shown to bind to proteins to form unstable and stable adducts that may play a role in the mechanism of toxicity *in vivo* [15]. Heavy alcohol consumption is a risk factor for cataract [16,17].

Many epidemiological studies have indicated that aspirin decreases the risk of cataract and other disorders, including heart

Abbreviations used: CHD, 1,2-cyclohexanedione; MDA, malondialdehyde.

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disease [1,18]. The acetylation of ϵ-amino groups of the lysine residue has been proposed as a protective measure to inhibit a variety of post-translational modifications. However, cyanate, for example, also reacts with lysine residues with opposing effects to aspirin upon cataract and chaperone function [10]. Both cyanate and aspirin cause a reduction in protein charge and would be expected to cause changes in protein conformation.

Point mutations of highly conserved arginine residues in αA-(Arg116Cys) and αB-crystallin (Arg120Gly) have been shown to cause autosomal-dominant congenital cataract and desmin-related myopathy respectively, in humans [19,20]. α-Crystallin mutants with these point mutations were shown to have significantly reduced chaperone function [21–23]. Methylglyoxal reacts with arginine and lysine residues in proteins, whereas CHD can, under mild conditions, react more specifically with arginine residues and therefore was used to investigate whether arginine residues are essential for chaperone function. The chemical modification of arginine residues with CHD may indicate whether αA-Arg^{116} and αB-Arg^{120} are essential for chaperone function.

MATERIALS AND METHODS

Materials

Lenses were from 3-month New Zealand rabbits and were supplied by an abattoir in Bicester, Oxfordshire, U.K. Lenses were dissected out, frozen and stored at −20 °C until use. Sephacryl G-200HR was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). CHD was from Fluka (Gillingham, Dorset, U.K.). Buffer material and acetylsalicylic acid were from BDH (Lutterworth, Leics., U.K.). Acetaldehyde, methylglyoxal and 1,1,3,3-tetraethoxypropane were from Sigma (Poole, Dorset, U.K.).

Isolation and purification of crystallins

Lenses were thawed, decapsulated, weighed and gently machine-homogenized in 0.05 M sodium phosphate buffer (pH 6.7) (degassed to minimize oxidation). The suspensions were centrifuged at 22440 ***g*** for 10 min at 4 °C. The supernatant was applied on to a Sephacryl S-300HR size-exclusion column (100 cm × 4 cm). The column was eluted using the above buffer at a constant flow rate of 45 ml/h [24]. Eluted fractions were collected, the absorbances were determined at 280 nm and an elution profile was drawn. Fractions corresponding to the high-mass fraction, α-low, β-high, β-low, λ- and γ-crystallin were pooled separately and dialysed against four changes of distilled water for 24 h at 4 °C. The pooled fractions were freeze-dried separately. The purity of the crystallins was checked by SDS/PAGE. They were stored at −20 °C until required.

SDS/PAGE

SDS/PAGE was performed using 12.5 % (w/v) polyacrylamide gels, as described by Laemmli [25], under reducing conditions on an LKB Bromma 2052 Midget Electrophoresis Unit (Pharmacia, Milton Keynes, U.K.). Protein bands were visualized by Coomassie Blue staining [1.25 % (w/v) in methanol/acetic acid/water (50:10:40, by vol.], then destained.

Preparation of modifying agents

MDA is unstable and undergoes an aldol-type self-condensation reaction to produce a mixture of polymers [26]. Therefore it was freshly prepared as described by Riley and Harding [27]. 1,1,3,3-Tetraethoxypropane was hydrolysed in acid (0.125 M in 0.125 M HCl in a total volume of 80 ml) for 24 h in a tightly stoppered flask at room temperature while stirring. The pH of the solution was adjusted with 2 M NaOH to 6.9 and buffered up to 100 ml with 100 mM sodium phosphate buffer (pH 6.9). The final concentration of MDA was 100 mM. Stock solutions of methylglyoxal, aspirin and acetaldehyde were prepared in degassed 0.05 M sodium phosphate buffer, adjusted to pH 6.7 and used immediately. CHD was prepared as described by Patthy and Smith [28] in degassed 0.2 M sodium borate buffer (pH 9). Stock solutions of 100 mM were prepared and used immediately.

Incubation of α-low crystallin

α-Crystallin (30 mg) was dissolved in 15 ml of modifying solution at 0, 1, 10, 30, 50, 70 and 100 mM. The solutions were then filtered through a sterilized Millipore filter (0.2 μm) into a sterilized 30 ml glass vial and incubated at 37 °C in a shaking water bath. At time 0 and after 24, 48 and 72 h, samples (3 ml) were removed from each of the solutions and dialysed in microdialysis tubing against four changes of distilled water over 24 h (except incubations using CHD which were allowed to continue for 2 weeks). The sample was then freeze-dried and analysed by SDS/PAGE. All incubations were repeated a total of three times.

β-Low crystallin heat-induced aggregation assay

The ability of α-crystallin to prevent the increase in turbidity upon heating solutions of β-low crystallin was assessed by a modification of the method described previously [2]. Final concentrations of 250 μg/ml β-low crystallin and 100 μg/ml α-crystallin gave a final mass/mass ratio of 1:2.5 α-crystallin to β-low crystallin in 1 ml plastic cuvettes. Two controls of β-low crystallin and the α-crystallin under observation were examined in every experiment. The cuvette solutions were stirred and placed in the six-cell holder of the Kontron (Chichester, West Sussex, U.K.) 930 spectrophotometer connected to a heated water bath set to 55 °C within the cuvettes. Light scattering resulting from heat-induced aggregation was monitored continuously at 360 nm, for 60 min [2]. Chaperone function was represented as the percentage of protection afforded by α-crystallin against the scattering produced by the β-low crystallin control aggregation after 60 min. All assays were repeated in triplicate.

Amino acid analysis

Amino acid analysis was carried out using an ABI 420a amino acid analyser (wavelength of 254 nm, run time of 20 min and 1 mg/ml; Heidelberg, Germany) and analysed with Dionex Chromeleon v4.30 software (Camberley, Surrey, U.K.).

Fluorescence studies

Fluorescence measurements were performed on a PerkinElmer (Beaconsfield, Bucks., U.K.) LB50B spectrofluorimeter. The tryptophan fluorescence spectra were obtained with excitation wavelength at 280 nm, and non-tryptophan fluorescence spectra after excitation at 340 nm. Crystallin solutions (0.3 mg/ml) in 0.05 M sodium phosphate buffer (pH 6.7) were used.

RESULTS

Incubation of α-crystallin with MDA

Incubation of MDA (1–100 mM) with α-crystallin at 37 °C over 72 h did not cause any gross modification, which would lead to

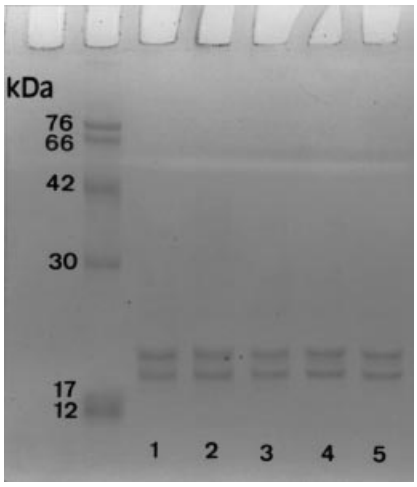

Figure 1 Effect of modifiers on α-crystallin

SDS/PAGE of α-crystallin after incubation for 72 h in the absence (lane 1) or presence of 100 mM each of MDA (lane 2), aspirin (lane 3), acetaldehyde (lane 4) and CHD (lane 5). Molecular-mass markers (in kDa) are indicated on the left.

truncation, cross-linkage or aggregation of the two α-crystallin subunit bands, as shown by SDS/PAGE (Figure 1; results at lower concentrations not shown). The protein became brown as the incubation period increased. There was no change in chaperone function of α-crystallin after 72 h of incubation with 100 mM MDA (results not shown), although 95% of the lysine residues were modified (Table 1). Tryptophan fluorescence was lost, possibly due to the absorbance by the brown solution (Figure 2A); however, tryptophan fluorescence was lost at low concentrations of MDA that did not become brown. Non-tryptophan fluorescence was increased with a shift to longer wavelengths, indicating formation of new fluorochromes (Figure 2B).

Incubation of α-crystallin with acetaldehyde

Incubation of acetaldehyde (1–100 mM) with α-crystallin at 37 °C over 72 h did not cause any gross change in the two α-crystallin subunit bands (Figure 1; results at lower concentrations are not shown). No colour change was observed. The α-crystallin preparations did not show any decrease in chaperone function after 72 h of incubation with 100 mM acetaldehyde (results not shown). No significant changes in amino acid composition were observed by amino acid analysis, but adduct formation may reverse in the acid conditions used for hydrolysis. Tryptophan fluorescence decreased slightly in intensity without the development of non-tryptophan fluorescence (Figures 2A and 2B).

Incubation of α-crystallin with methylglyoxal

α-Crystallin was incubated with different concentrations of methylglyoxal for up to 72 h. At time 0, there was no modification of α-crystallin preparations and no change in chaperone ability (results not shown). Within 24 h there was cross-linking and dimer formation of α-crystallin incubated with methylglyoxal at concentrations between 30–100 mM, with diffuse patterns at approx. 20 kDa (Figure 3A). The consequence of the cross-linking upon chaperone ability was a concentration-dependent decrease in function (Figure 3B). At 48 h, a similar pattern was observed to that at 24 h (results not shown). There was little chaperone ability with α-crystallin incubated with 70 and 100 mM methylglyoxal. Incubation of methylglyoxal at concentrations of 30–100 mM with α-crystallin at 37 °C over 72 h caused extensive concentration-dependent non-disulphide cross-linking. A concentration- and time-dependent decrease in chaperone function of methylglyoxal-modified α-crystallin was observed from 30 mM; there was no chaperone function of α-crystallin incubated with 70 and 100 mM methylglyoxal after 72 h (results not shown).

Table 1 Amino acid analysis of native and modified α-crystallin

α-Crystallin was treated in the absence or presence of the indicated modifiers for 72 h at 37 °C, except where stated otherwise, and amino acid analysis was performed as described in the Materials and methods section. Values are the percentages of amino acids present, with those for arginine and lysine in **bold**. AA, acetaldehyde.

Amino acid	Modifier…	None	+ AA (100 mM)	+ Aspirin (100 mM)	+ MDA (100 mM)	+ Methylglyoxal 1 mM	+ Methylglyoxal 50 mM	+ Methylglyoxal 100 mM	+ CHD 1 mM	+ CHD 50 mM	+ CHD 100 mM	+ CHD (100 mM) 7 days	+ CHD (100 mM) 14 days
Asp		8.86	8.81	9.08	10.11	9.29	9.83	9.78	9.01	9.48	9.55	9.74	10.12
Glu		9.72	9.87	9.85	11.23	10.56	11.21	11.42	9.59	10.33	10.38	10.36	10.79
Ser		10.08	10.34	9.93	9.69	10.56	11.48	11.88	9.88	10.61	10.72	11.56	11.72
Gly		5.66	5.79	5.73	6.32	6.14	6.43	6.49	5.84	6.29	6.43	6.66	6.63
His		3.88	3.72	3.86	3.85	3.91	4.17	4.20	3.59	3.87	3.66	3.24	3.57
Arg		**7.76**	**7.52**	**7.46**	**7.03**	**4.29**	**1.85**	**1.75**	**6.08**	**2.02**	**2.26**	**0.98**	**0.81**
Thr		3.60	3.53	3.70	2.80	3.63	3.93	3.89	2.68	2.99	2.91	3.27	3.38
Ala		2.78	2.94	2.44	4.63	3.07	3.87	4.05	3.65	3.98	4.14	4.36	4.42
Pro		6.85	6.97	6.61	8.05	7.22	8.11	8.17	7.18	7.76	7.97	8.64	8.72
Tyr		3.43	3.31	3.53	3.80	3.55	3.86	3.81	3.38	3.42	3.46	3.31	3.47
Val		6.92	7.41	7.19	8.04	8.35	8.64	8.99	6.95	7.24	6.51	5.91	6.07
Met		1.10	1.11	0.91	1.26	1.13	1.06	0.85	1.45	1.28	1.12	0.53	0.98
Cys		0.07	0.10	0.13	0.06	0.23	0.12	0.22	0.06	0.22	0.09	0.23	0.23
Ile		4.31	4.18	4.37	4.42	4.43	4.80	4.83	5.26	5.41	5.44	5.57	5.49
Leu		9.08	8.88	9.45	9.97	9.06	9.92	9.60	8.84	9.12	9.10	9.82	9.76
Phe		7.59	7.64	8.28	8.33	7.80	8.51	8.27	7.65	7.68	7.98	8.87	8.89
Lys		**7.97**	**7.87**	**7.48**	**0.39**	**6.77**	**2.19**	**1.75**	**8.88**	**8.29**	**8.26**	**6.95**	**4.92**

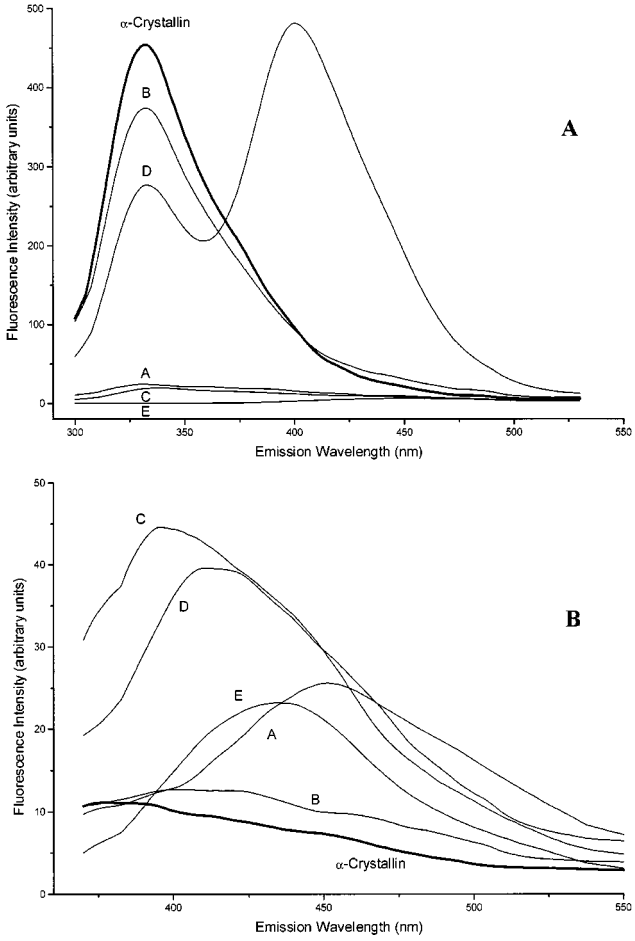

Figure 2 Tryptophan (A) and non-tryptophan (B) emission spectra of modified and unmodified α-crystallin

α-Crystallin was incubated in the absence or presence of 100 mM each of MDA (A), acetaldehyde (B), methylglyoxal (C), aspirin (D) and CHD (E), and the tryptophan (λ_{ex} = 280 nm) and non-tryptophan (λ_{ex} = 340 nm) emission spectra were determined.

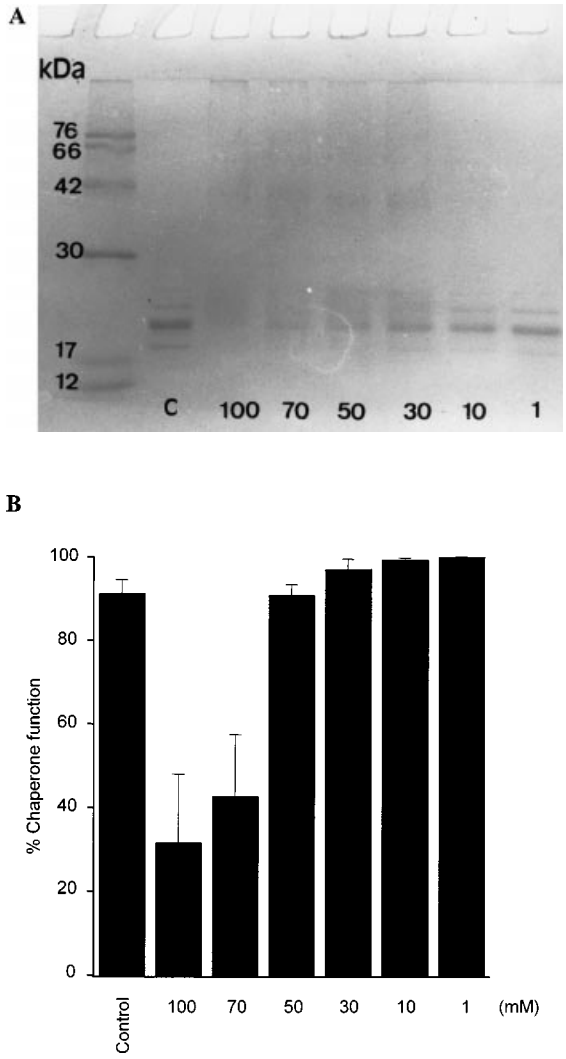

Figure 3 Effect of methylglyoxal on protein modification and chaperone function of α-low crystallin

α-Low crystallin was incubated in the absence or presence of various concentrations of methylgloxal for 24 h prior to either SDS/PAGE analysis (**A**) or determining chaperone function (**B**). Chaperone function was determined by investigating the ability of α-crystallin (100 μg) to protect against heat-induced aggregation of β-low crystallin (250 μg) at 55 °C. Results are means ± S.E.M. and are expressed as protection relative to light scattering of β-low crystallin alone. In (**A**), molecular-mass markers (in kDa) are indicated on the left.

After 72 h of incubating α-crystallin with 100 mM methylglyoxal, there was a complete loss of tryptophan fluorescence, and new non-tryptophan fluorescence was observed with maximum emission at approx. 400 nm, (Figures 2A and 2B). Only slight yellowing of α-crystallin was observed upon incubation with increasing methylglyoxal concentrations and time, and tryptophan fluorescence was lost at low concentrations of methylglyoxal that did not become yellow. Amino acid analysis showed that methylglyoxal modified arginine and lysine residues. At 1 mM (3 days), methylglyoxal blocked 45% of arginine and 15% of lysine residues (Table 1), although chaperone function was retained, whereas, at 100 mM methylglyoxal, only 22% of arginine and lysine residues remained and there was negligible chaperone function (Figure 3).

Incubation of α-crystallin with aspirin

Incubation of aspirin (1–100 mM) with α-crystallin at 37 °C over 72 h did not cause any gross changes to the two α-crystallin subunit bands (Figure 1; lower concentrations are not shown). There was no change in chaperone function of α-crystallin after 72 h of incubation with 100 mM aspirin (results not shown). Amino acid analysis revealed a small decrease in lysine residues (Table 1). The acetyl group would be removed from lysine during the acid hydrolysis required before amino acid analysis. Tryptophan fluorescence decreased at the normal emission wavelength; the second peak close to 400 nm, is due to the aromatic ring of aspirin, which may shield the tryptophan residue of α-crystallin (Figures 2A and 2B).

Incubation of α-crystallin with CHD

As methylglyoxal affected the properties of α-crystallin, a more specific arginine-residue modifier was investigated. Incubation of CHD (1–100 mM) with α-crystallin at 37 °C over 72 h did not cause any gross modification of α-crystallin (Figure 1; results for lower concentrations are not shown), and there was no change in chaperone function of α-crystallin (results not shown). There was

an extensive dose-dependent decrease in arginine residues (70 % after 100 mM CHD for 72 h) on incubation with CHD (Table 1). Incubation with 100 mM CHD abolished tryptophan fluorescence, whereas there was an increase in non-tryptophan fluorescence (Figures 2A and 2B). Again, tryptophan fluorescence was lost at low concentrations of CHD that did not become yellow. Further incubation to 7 days did not cause a decrease in chaperone function, even with only 12 % of arginine residues remaining. After 14 days of incubation, there was no chaperone function. This may be due to CHD modifying both arginine (approx. 10 % remaining) and lysine residues (60 % remaining).

DISCUSSION

Products of lipid oxidation, including MDA, increase with age and cataract in the lens [12]. Interestingly, myopic cataract has a higher concentration of MDA than senile cataract and an increased level of MDA in the vitreous humour and lens [29]. However, the formation of MDA within the lens during cataractogenesis is doubtful, as MDA arises only from the oxidation of polyunsaturated hydrocarbon lipids, which compose less than 3 % of lens membrane lipids [30]. The retina, which is very rich in polyunsaturated fatty acids, particularly at the rod outer segment, could be the main source of lipid oxidation products, especially in retinal degeneration, such as retinitis pigmentosa. MDA produced in the present study could easily diffuse to the lens and may account for the opacities in the posterior or equatorial area of the lens, typical of myopia [31]. Also, injection of peroxidative products, such as docosahexenoic acid, in the vitreous caused posterior subcapsular cataract in the lens of rabbits within 24 h [32]. This may also explain the risk of cataract as a consequence of retinal degeneration or retinal surgery. Cross-linking of α-crystallin by MDA has been reported previously [33]. On storage, MDA cyclizes to form more reactive molecules that can cross-link proteins. Cross-linking would decrease chaperone function. In the present study with freshly prepared MDA, no cross-linking was seen and chaperone function was retained.

The dicarbonyl methylglyoxal readily forms adducts with α-crystallin. Reaction with methylglyoxal resulted in aggregation, non-disulphide covalent cross-linking and a decrease of tryptophan fluorescence and increase of non-tryptophan fluorescence of α-crystallin. These are similar changes to those found during the formation of cataract in human lenses [1], incubated lenses *in vivo* [34] and lens protein incubated *in vitro* [35]. Arginine residues are the major sites of reaction, although modifications of lysine and cysteine residues are also observed (Table 1; [36]), contributing to a major loss of positive charge. α-Crystallin chains contain approx. 8 % (αB-crystallin) and 7.5 % (αA-crystallin) arginine residues. Adducts that lead to immobilization of the C-terminus during cross-linking may also impede the solubilizer role of the C-terminus and lead to insolubilization. The gross modifications observed highlight the effect of a dicarbonyl modifier. The modification of both arginine and lysine residues means that specific amino acids which may be involved in chaperone function cannot be pinpointed, only that a gross change in structure is observed with methylglyoxal.

There could also be a very rapid formation of advanced glycation end-products. *In vitro* experiments have shown that methylglyoxal undergoes a rapid Maillard reaction, initially through a reversible Schiff's base, with proteins to form covalently cross-linked aggregates [36]. The high reactivity of methylglyoxal with proteins suggests that it is one of the major glycating agents present *in vivo*. Effective defence systems exist intracellularly to reduce methylglyoxal toxicity. The GSH-dependent glyoxalase converts methylglyoxal into D-lactate [14]. The activities of the glyoxalase enzymes within the lens decrease with age [37]. Methylglyoxal concentration is elevated in lens, red blood cells and kidneys in diabetes, whereas GSH levels decrease [37].

Acetaldehyde is the primary oxidative metabolite of ethanol, predominantly produced during first pass-through the liver via the enzyme alcohol dehydrogenase. Ethanol-derived acetaldehyde spills over from the liver into the systemic circulation. Numerous studies have demonstrated the covalent binding of acetaldehyde to a variety of proteins under physiological conditions, such as serum albumin [38]. Such a reaction scheme could also explain the cross-linking of proteins that has been reported to occur in the presence of acetaldehyde in human erythrocyte membrane proteins [39]. However, in the present study, no cross-linking was seen and chaperone function was retained. The lens has defence systems against acetaldehyde attack and, in particular, contains aldehyde dehydrogenase [40]. This cytoplasmic enzyme oxidizes aldehydes to less harmful acids. Aldehyde dehydrogenase found in the lens can also degrade MDA as well as acetaldehyde [41]. This may suggest an alternative mechanism of alcohol-induced cataract, such as lipid peroxidation of lens membranes or dehydration [16].

Aspirin was found to have a serendipitous anti-cataract effect during a study on rheumatoid arthritis patients [42]. Epidemiological studies support the protective effects of aspirin, and other non-steroidal anti-inflammatories, such as paracetamol and ibuprofen, in preventing or delaying cataract [43]. Acetylation of lysine residues has been proposed to account for the action of aspirin in inhibiting cataract formation, and the acetyl moiety of aspirin has been shown to bind to the lens proteins and to cause a decrease in the ε-amino groups [44]. This would block the reaction of lysine with other modifiers, such as glycation and carbamylation of lens proteins. However, cyanate also reacts with lysine residues [45] and would also lower the protein charge, yet aspirin and cyanate cause grossly different effects on the lens. Secondly, ibuprofen, which does not contain an acetyl group, can also protect against cataract [46].

The lack of effect of both aspirin and MDA on chaperone function indicates that this activity does not depend entirely on the lysine residues of α-crystallin. The fact that incubation of cyanate with α-crystallin caused non-reducible cross-linking suggests that the mechanism of aspirin may be via the inhibition of disulphide-bond formation [10]. Qin et al. [47] found stable acetylated and carbamylated cysteine residues, though the acetylated form of cysteine is more stable than the carbamylated form, which could prevent the formation of disulphide bonds and cause the protection. Human αA-crystallin has only two cysteine residues, whereas αB-crystallin has none; however, rabbit αA-crystallin, used in the present study, has only one cysteine residue at position 131, indicating that the cross-linking is probably non-disulphide cross-linking.

Only extensive modification of both the arginine and lysine residues by CHD caused a total decrease in chaperone function. The results of the modifications of the amino acids in the present paper (Table 1) suggest that blocking either most arginine residues alone (CHD treatment for 7 days) or most lysine residues alone (MDA) does not lead to a decrease in chaperone function, but when both are blocked (e.g. methylglyoxal and CHD treatment for 14 days), a decrease in chaperone function is observed. Both arginine and lysine residues would contribute to the positive charge of α-crystallin. At 7 days of CHD treatment, 88 % of arginine residues are modified, suggesting the remaining arginine residues may be inaccessible. In theory they could be

essential to chaperone function. Site-directed spin-label studies of αA-crystallin mutants revealed a periodic variation in solvent accessibility along the sequence 109–120, which demonstrates β-strand conformation [48]. In oligomeric proteins, subunit interfaces tend to contain charged groups, especially arginine residues (found within the sequence mentioned above) that are normally involved in salt bridges and/or hydrogen bonds [49]. The sequence Arg^{112}–Arg^{116} is highly conserved and is buried with virtually no accessibility to the aqueous solvent [48]. Equivalent strands from different subunits are in close proximity, which indicates a subunit interface is critical for the assembly of oligomers. Of the two point mutations of αA- (Arg116Cys) and αB-crystallin (Arg120Gly), the αA mutant has been shown to be grossly oligomerized (2000 kDa), with disrupted tertiary structure and 8-anilinonaphthalene-1-sulphonic acid ('ANS') binding [21,22], and the αB mutant was larger (720–1400 kDa) than the wild-type [23]. Both mutants have impaired chaperone function. The two point mutations of αA- (Arg116Cys) and αB-crystallin (Arg120Gly), that lead to congenital cataract and desmin-related myopathy [19,20], may therefore disrupt subunit oligomerization. The obvious assumption that arginine itself is important may not be valid. The structural differences between the wild-type and mutant forms of α-crystallin may arise from such a large amino acid substitution (arginine to cysteine for the αA mutant and arginine to glycine for the αB mutant), which leads to gross structural changes. Our results show that α-crystallin with 88 % of the arginine residues modified by CHD, leaving the equivalent of two remaining arginine residues, retains chaperone function, whereas when just another 2 % is modified, leaving one unmodified arginine residue, all chaperone function was lost. Our modified α-crystallin was highly oligomerized/aggregated, suggesting Arg^{116} was modified, since the αA-crystallin mutant Arg116Cys was grossly oligomerized [21,22]. However, the extensive modification may indicate that something other than arginine is involved in the loss of chaperone function. In our experiments, it may be the overall positive charge.

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