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Conformational changes induced in bovine lens α-crystallin by carbamylation

Relevance to cataract

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Carbamylation of lens proteins may contribute to cataractogenesis in certain medical conditions where blood urea is elevated for prolonged periods. This paper reports on the effects of carbamylation on the physicochemical properties of one of the major lens structural proteins, α-crystallin. In particular it is shown that carbamylation alters the tertiary and secondary structure of the protein, leading to an increased reactivity of protein thiols, resulting in interchain disulphide bonding.

Carbamylation of proteins by isocyanic acid at physiological pH values involves the irreversible covalent modification of amino groups (Stark, 1965) and the rapid, but reversible, reaction of thiol groups (Stark, 1964). Carbamylation of essential lysine residues inactivates some enzymes (Carreras *et al.*, 1976) and can inhibit the normal functioning of other proteins (Mellado *et al.*, 1982; Hasilik *et al.*, 1983).

Urea and cyanate have been used to treat sickle-cell anaemia. The beneficial effects were found to be due to carbamylation of the *N*-termini of haemoglobin S (Lee & Manning, 1973). During clinical trials of oral sodium cyanate, some patients developed cataract (Nicholson *et al.*, 1976) and cyanate can also induce posterior subcapsular cataracts in beagles (Kern *et al.*, 1977). In clinical conditions where uraemia occurs, such as renal failure and severe diarrhoea, the blood cyanate will be elevated. Cataract has often been observed in uraemics (Hollwich *et al.*, 1975), and increased levels of carbamylated proteins have been observed in renal-failure patients (Harding, 1984).

Harding (1980) suggested that repeated bouts of diarrhoea could contribute to the high prevalence of cataract in tropical countries, and recent epidemiological studies have confirmed this (Minassian *et al.*, 1984).

Carbamylation *in vitro* of total lens homogenates occurs readily, and there is evidence of carbamylation in some Pakistani lenses (Harding & Rixon, 1980). Carbamylation of protein amino groups would alter the protein surface charge, and this could result in conformational changes and possibly protein unfolding of the kind already observed in human cataract (Harding, 1972).

α-Crystallin is a major lens structural protein isolated as a high-M_r polydisperse polymer composed of two main types of subunits, the A_2 (acidic) subunits and the B_2 (basic) subunits (van der Ouderaa *et al.*, 1973). These subunits have extensive sequence homology and M_r values of approx. 20000. The A_2-chain of bovine α-crystallin has seven lysine residues and one thiol group per subunit, whereas the B_2-chain has ten lysine residues and no thiols per subunit. The approximate ratio of the two subunits (A/B) in the polymer is 2:1 (Harding & Crabbe, 1984).

The present study investigates the effects of carbamylation on the physicochemical properties of α-crystallin. In particular, conformational changes are reported that affect secondary and tertiary structure and result in increased thiol reactivity, with the resultant formation of interchain disulphide bonds. The ways in which the observed changes could contribute to the cataractogenic process are discussed. Since the α-crystallins of mammals are closely related (de Jong *et al.*, 1973), these events may serve as a model for human cataract.

Abbreviations used: SDS, sodium dodecyl sulphate; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

Materials and methods

Lenses

Bovine lenses were obtained from J. Meade and Co., Meat Wholesaler (Slough, Berks., U.K.) on the day the animals were slaughtered, and stored at −20°C.

Chemicals

Potassium cyanate was obtained from BDH Chemicals, Poole, Dorset, U.K., and recrystallized from ethanol. Potassium [^{14}C]cyanate (52mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Scintillation cocktail T and all other chemicals were obtained from BDH or Sigma, and were of the highest purity available.

Preparation of bovine α-crystallin

Each lens was decapsulated, then hand-homogenized in 10ml of distilled water. The homogenates from eight lenses were pooled and centrifuged at 10000*g* for 40min at 4°C. The supernatant containing the water-soluble crystallins was decanted and the pH was adjusted to 4.5 by the dropwise addition of 0.4M-HCl, with constant stirring. After 3h at 4°C the crude α-crystallin was isolated by centrifugation at 8000*g* for 40min at 4°C and purified by gel chromatography on Sephadex G-200 (van Dam & ten Cate, 1966). The purity of the native α-crystallin preparation was confirmed on SDS/polyacrylamide gels before use.

Incubation of α-crystallin with potassium cyanate

All protein incubations were performed at 37°C in 0.1M-sodium pyrophosphate/0.05% (w/v) NaN_3, pH8, in a shaking water bath.

Progress of cyanate binding. Covalent binding of cyanate to α-crystallin was monitored by assaying the radioactivity incorporated into protein from potassium [^{14}C]cyanate. At various times quadruplicate aliquots, each containing 200μg of protein, were removed from the incubation and ejected into 1ml of 10% (w/v) trichloroacetic acid. The protein in the sample was allowed to precipitate overnight at 4°C and recovered by filtration through a 2.5cm-diameter glass-fibre filter circle (Whatman, Maidstone, Kent, U.K.) in a Millipore microanalysis apparatus attached to a vacuum pump. The circle was washed four times in 5% (w/v) trichloroacetic acid and put inside a scintillation-vial insert before drying in an oven at 70°C for 30min. Scintillation cocktail T (3.5ml) was added to the cooled dry vials and the radioactivity incorporated into the trichloroacetic acid-precipitated protein assayed by liquid-scintillation counting.

Carbamylation of α-crystallin for physicochemical studies. Typically 200mg of α-crystallin (20mg/ml) was carbamylated by using unlabelled potassium cyanate (1, 10, 50 or 100mM). A separate sample of α-crystallin was incubated in the same buffer with 100mM-KCl, but otherwise under identical conditions, as an incubated control. At the end of the incubation period (5–7 days) the samples were exhaustively dialysed against distilled water and freeze-dried before use or storage at −20°C.

Circular dichroism

C.d. spectra were recorded digitally from 340 to 255nm (near-u.v.) and from 250 to 220nm (far-u.v.) with a Jasco J41-C spectropolarimeter with a J-DPY data processor having a sensitivity of 0.5 millidegrees/cm and with an instrumental time constant of 4s. The spectra presented here were recorded at 37°C in 10mM-phosphate buffer, pH7.4, and represent the averages for at least four scans. The scans are presented as plots of c.d. absorption coefficient ($\Delta\varepsilon$; units $ml \cdot mg^{-1} \cdot cm^{-1}$) against wavelength in nm. All plots are corrected to a final protein concentration of 1mg/ml. The far-u.v. data was subjected to a secondary-structure analysis by the method of Provencher & Glöckner (1981).

Reduced specific viscosity

Viscosity was measured with a low-shear 30 model rheometer (Contraves, Luton, Beds., U.K.), the output from which was processed by a Rhoescan 20 control unit and passed to an *xy* recorder. The viscosities of α-crystallin solutions, all 40mg/ml, were measured at 37°C ± 0.1°C in 0.1M-pyrophosphate/0.05% NaN_3, pH8. The viscosities of a 100mM-KCl control and solutions of α-crystallin containing final cyanate concentrations of 1, 10, 50 and 100mM were monitored with increasing incubation time at 37°C. The reduced specific viscosities were calculated from the rheographs obtained and have units of litres/g. The viscosity of α-crystallin in 6M-urea was also measured.

Assay of protein thiol groups

Free thiols were determined using DTNB and a slightly modified Sedlak & Lindsay (1968) method in that 0.5M-Tris buffer was used and this contained a final concentration of 10mM-diethylenetriaminepenta-acetic acid, a metal-ion chelator, to inhibit autoxidation of thiol groups. All assays were performed in freshly prepared 6M-urea.

SDS/polyacrylamide-gel electrophoresis

Acrylamide (15%, w/v) slab gels with 5% (w/v)-acrylamide stacking gel were run at constant voltage (100V) for 4–5h, a Tris/glycine buffer system (Laemmli, 1970) being used. The protein bands were revealed by Coomassie Blue staining. The final composition of normal sample buffer used was 2% (w/v) SDS/25mM-Tris/20% (w/v) glycerol/0.005% Bromophenol Blue.

Detection of interchain-disulphide-bonded aggregates on SDS/polyacrylamide gels. Each α-crystallin sample (200μg of protein) was analysed twice on the same gel, once dissolved in normal sample buffer and once dissolved in sample buffer

supplemented with DTT (100mM) to reduce disulphide bonds.

Results

Rate of modification of α-crystallin by isocyanic acid

The rate of modification was monitored by measuring the incorporation of label from potassium [^{14}C]cyanate into trichloroacetic acid-precipitable protein. Initial rates of binding (up to 10mol of cyanate/day per mol of α-subunits) were approximately directly proportional to the cyanate concentration (Fig. 1). After 6 days, 100mM-cyanate had carbamylated 60% of the total lysine and the reaction had ceased, whereas carbamylation with lower cyanate concentrations continued. The contribution of thiols to the total cyanate binding was shown to be small by studies of the rate of carbamylation of α-crystallin that had its thiol groups blocked by iodoacetamide (results not shown).

Carbamylation-induced conformational changes in α-crystallin

(*a*) *Native and incubated control α-crystallin.* The c.d. spectra of native and incubated control α-crystallin are very similar to each other (Figs. 2 and

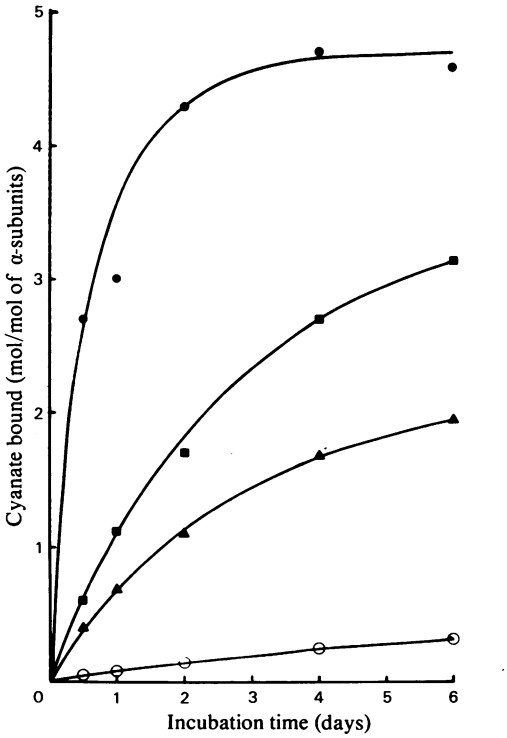

Fig. 1. *Progressive carbamylation of native bovine α-crystallin using various concentrations of potassium cyanate*

α-Crystallin (10mg/ml in 0.1M-pyrophosphate buffer, pH8), was carbamylated in the presence of 2μCi/ml of potassium [^{14}C]cyanate with unlabelled potassium cyanate added to give final cyanate concentrations of 1mM (○), 10mM (▲), 50mM (■) and 100mM (●). The progress of cyanate binding with time was monitored by removal of quadruplicate aliquots at various times and assaying the radioactivity incorporated into protein, from which the total cyanate binding can be calculated. The S.D. values for the quadruplicate aliquots were within 10% of the means. All incubations were at 37°C.

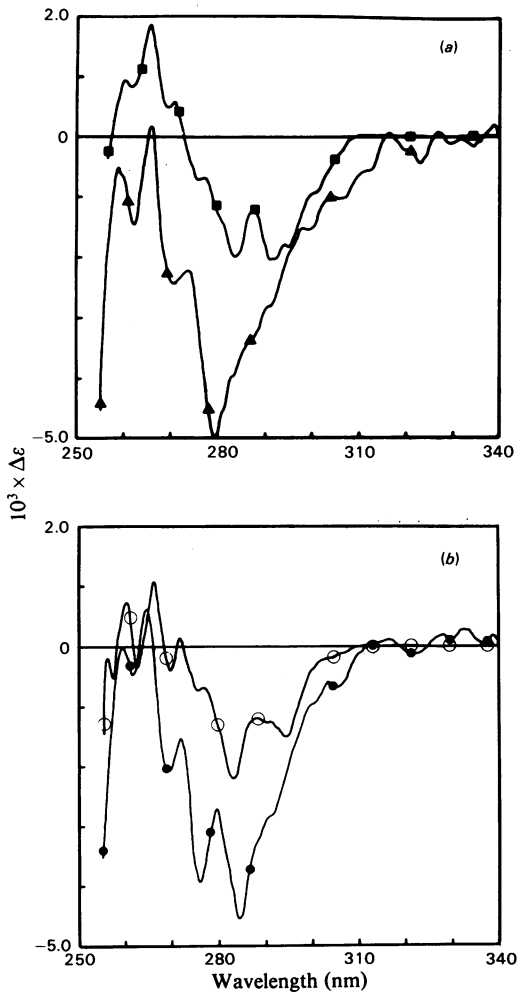

Fig. 2. *Near-u.v. c.d. spectra of carbamylated and control samples of α-crystallin at 37°C, pH7.4, and equal protein concentrations*

(*a*): ■, 100mM-KCl-incubated control α-crystallin; ▲, 100mM-cyanate-carbamylated α-crystallin. (*b*): ○, Native α-crystallin; ●, 50mM-cyanate-carbamylated α-crystallin, Δε units are ml·mg^{-1}·cm^{-1}.

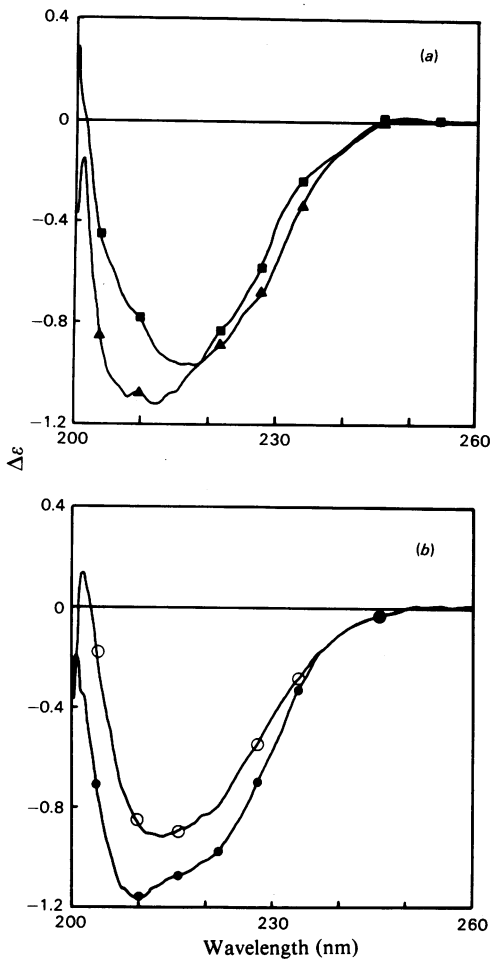

Fig. 3. *Far-u.v. c.d. spectra of carbamylated and control samples of α-crystallin at 37°C, pH 7.4, and equal protein concentrations*

(*a*): ■, 100mM-KCl-incubated control α-crystallin; ▲, 100mM-cyanate-carbamylated α-crystallin. (*b*): ○, Native α-crystallin; ●, 50mM-cyanate-carbamylated α-crystallin. Δε units are as in Fig. 2.

3) and to previously published spectra (Li & Spector, 1967; Horwitz, 1976). Considering the near-u.v. spectra (Fig. 2), the three maxima of 261, 267 and 272nm are due to phenylalanyl transitions. The maximum at 277nm may be a tyrosyl ^{1}Lb transition. The minimum at 283nm arises from tryptophan and tyrosine residues, whereas that at 292–294nm is mainly due to tryptophyl transitions (Strickland, 1974).

In the far-u.v. (Fig. 3) the c.d. spectra show minima at 214nm indicative of the β-sheet secondary structure that is characteristic of all bovine crystallins (Siezen & Argos, 1983).

(*b*) *Changes in the c.d. spectra induced by carbamylation.* The c.d. spectra of α-crystallin modified with final cyanate concentrations of 100mM or 50mM differ significantly from the controls (Figs. 2 and 3). The increase in negative amplitude of the bands due to the phenylalanyl vibronic structure is striking, as are the similar changes in the tryptophyl and tyrosyl regions of the spectra. The c.d. spectrum of the α-crystallin modified using 100mM-cyanate has lost some, but not all, of its fine structure in the tryptophyl/tyrosyl region, indicating partial disruption of tertiary structure. In both carbamylated samples a blue shift of 5–6nm occurred in the tryptophan/tyrosine region, the phenylalanyl region being affected to a lesser extent. This may reflect differential interaction of solvent molecules with the chromophores in the native and carbamylated protein (Strickland, 1974). It is not surprising that solvent interaction should alter, since carbamylation results in the removal of positive charge from the protein surface due to modification of lysine amino groups. Furthermore, the tryptophan residues of α-crystallin are in relatively hydrophilic environments compared with those in other bovine crystallins (Liang & Chakrabarti, 1982) and so changes in the protein–solvent interaction would be expected to affect the c.d. spectrum in this region.

The far-u.v. c.d. spectra of the carbamylated samples also show a blue shift, the new minima appearing at approx. 210nm. The results of secondary-structure predictions based on the far-u.v. c.d. data for control and carbamylated samples are shown in Table 1. The curve-fitting programme used gives very good accuracy for α-helix and β-sheet prediction, whereas the β-turn and random-coil estimates are less reliable. The data indicate a small increase in the α-helical content of the carbamylated samples relative to the controls, primarily at the expense of β-sheet. Carbamylation results in the conversion of a charged residue (lysine) into a relatively hydrophobic amide (homocitrulline). Lysine and other charged residues are α-helix-destabilizing, whereas hydrophobic residues are commonly found in α-helices (Schiffer & Edmundson, 1967), and so one might expect the conversion of lysine into homocitrulline to favour α-helix formation.

The c.d. results demonstrate that the conformation of α-crystallin changes on carbamylation. These changes appear to affect primarily tryptophan and tyrosine chromophores, resulting in changes in tertiary and secondary structure without disrupting either completely. Further support for a carbamylation-induced conformational change was provided by observed changes in tryptophan fluorescence and absorbance spectra (results not shown).

Table 1. *Secondary-structure composition of α-crystallin samples predicted from u.v. c.d. data by the method of Provencher & Glöckner (1981)*

C.d. data were collected at pH7.4 and 37°C.

Sample	Percentage of total structure			
	α-Helix	β-Sheet	β-Turn	Random coil
Native α-crystallin	6.8	42.5	23.4	27.7
Incubated control α-crystallin	5.1	47.8	18.8	28.3
Carbamylated α-crystallin				
50mM-Cyanate	9.1	40.8	23.9	26.2
100mM-Cyanate	12.0	31.8	22.9	33.3

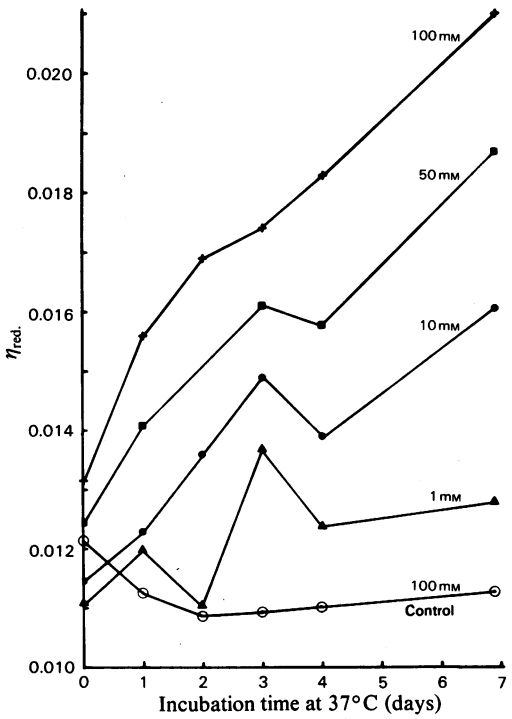

Fig. 4. *Changes in reduced specific viscosity ($\eta_{red.}$) of α-crystallin induced by carbamylation*

All measurements were at pH8 and 37°C, with an α-crystallin concentration of 40mg/ml and final cyanate concentrations of 1mM (▲), 10mM (●), 50mM (■) and 100mM (+). The open circles (○) indicate changes in the incubated control α-crystallin (100mM-KCl).

Changes in reduced specific viscosity induced by carbamylation

All the protein solutions behaved in a Newtonian manner throughout the incubation period. The reduced specific viscosity was unchanged by incubation in 100mM-KCl, but increased in a time- and cyanate-concentration-dependent manner during incubations with potassium cyanate at pH8 and 37°C (Fig. 4). When proteins denature, the viscosity generally increases (Joly, 1965). Such increases can result from protein aggregation, a change in shape or hydration of the protein, an unfolding of the protein chains, or any combination of these. The reduced specific viscosity of α-crystallin in 6M-urea at 37°C is 0.042 litre/g, twice that of α-crystallin carbamylated by 100mM-cyanate for 7 days. Assuming that 6M-urea produces complete denaturation, the observed changes are consistent with a partial unfolding, accompanied by changes in shape and hydration of the protein, which was already indicated by the fluorescence and c.d. results.

Table 2. *Assay of protein thiol groups of α-crystallin using DTNB in 6M-urea, pH8*

Results are means for triplicate experiments; all values were within 15% of each other. The predicted thiol content is based on an A/B subunit ratio of 2:1 in α-crystallin.

	Thiol (mol/mol of α-subunits)
Predicted value	0.67
Native α-crystallin	0.58
Incubated control α-crystallin	0.38
α-Crystallin, carbamylated, 100mM-cyanate	0.08

Carbamylation-induced changes in thiol-group reactivity of α-crystallin

Assay of free thiol groups in carbamylated and incubated control samples of α-crystallin in 6M-urea. The results of these assays, performed after 5-day incubations, indicate that, whereas some of the thiols are oxidized during the preparation and during the control incubation, a much greater loss of free thiol groups is seen in the carbamylated α-crystallin, indicating an apparent increase in the susceptibility of thiol groups in carbamylated α-crystallin to oxidation relative to the controls (Table 2).

SDS/polyacrylamide-gel analysis. In order to determine whether the observed decrease in free thiols was due to interchain-disulphide-bond

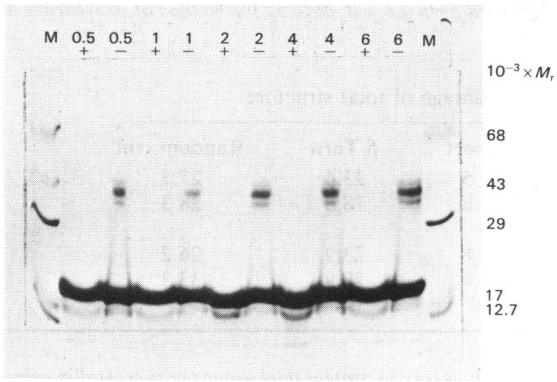

Fig. 5. *Appearance of disulphide-bonded material in control (100mM-KCl) incubations of α-crystallin with increasing incubation time*

The time at which the sample was removed from the incubation mixture (in days) is shown at the head of each gel track. '+' Indicates that DTT was present in the sample buffer; − denotes its absence. Tracks containing M_r calibration markers are shown (M), and their M_r values are indicated at the side of the gel.

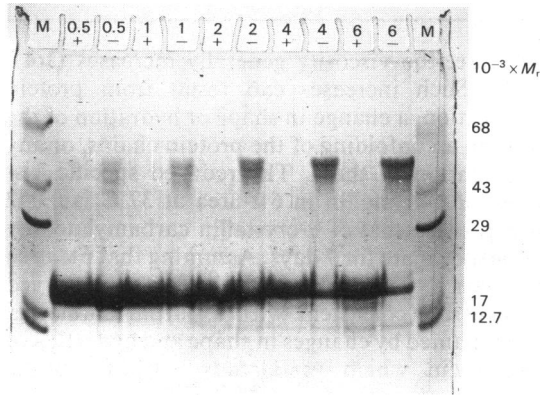

Fig. 6. *Appearance of disulphide-bonded material in 100mM-cyanate incubations of α-crystallin with increasing incubation time*

For details, see Fig. 5.

formation, samples were removed from incubated controls or carbamylation incubation mixes at various times and analysed on SDS/polyacrylamide gels in the presence or absence of dithiothreitol, a disulphide-reducing agent. During the control incubation, disulphide-bonded material is formed that has an apparent M_r of 38000 (Fig. 5). This material does not increase substantially with incubation time; furthermore, it appears to be formed mainly from a minor component (apparent M_r 14000) rather than from the main α-crystallin band. The cyanate-incubated samples show a progressive increase in disulphide-bonded material (M_r 53000), which is formed at the expense of the main α-crystallin band (Fig. 6) and presumably consists of α-crystallin A-subunit dimers. These aggregates should have an M_r of approx. 40000; however, the disulphide bridge between protein chains would prevent complete binding of SDS to the protein dimer, resulting in anomalously high apparent M_r values. Thus it appears that the protein thiols that disappear on carbamylation have been oxidized to protein–protein disulphides.

Conclusions

We have shown that carbamylation of the amino groups of α-crystallin changes its conformation and increases the reactivity of its thiol groups, which then become oxidized to form interchain disulphide bonds. All these changes occur during human cataractogenesis (Harding & Crabbe, 1984). Modification of lysine would eliminate positive charges, changing the protein–protein and protein–water interactions and disrupting any ion-pair networks on the protein surface. These networks are thought to stabilize proteins, including γ-II crystallin of bovine lens (Wistow *et al.*, 1983), and their loss could cause changes in conformation. Carbamylation is thought to predispose towards cataract in populations with elevated blood urea (Harding & Rixon, 1980), but it is also a model for other non-enzymic post-translational changes to proteins in cataract (Harding & Crabbe, 1984). These modifications include glycosylation, deamidation, addition of glutathione, racemization of aspartic acid residues, corticosteroid binding and oxidation of methionine. Other proteins can be modified, and these non-enzymic changes may be factors in various diseases (Harding, 1984). The α-amino groups of proteins are particularly susceptible to many of these chemical changes, and it is possible that the blocked α-amino groups of many long-lived proteins afford protection against chemical attack.

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