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The reaction of glutathione with the eye-lens protein γ-crystallin

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Lens cells contain high concentrations of thiol-rich proteins, γ-crystallins and reduced glutathione. Solutions of bovine γ-crystallin react avidly with either reduced or oxidized glutathione to form protein–glutathione mixed disulphides. A method of purification of a γ-II crystallin–glutathione adduct containing two mixed disulphide groups is described.

Although ubiquitous, no universal function can be confidently ascribed to glutathione. The eye lens maintains higher concentrations of reduced glutathione within its cells than do other tissues (Waley, 1969). The ratio of protein thiol to reduced glutathione in most species of lens is about 5:1 (Kuck *et al.*, 1982). The requirement for glutathione in maintaining a transparent lens is indicated by the observation that concentrations decline in nearly all forms of cataract (Kinoshita, 1964), with the possibility of some glutathione becoming protein-bound (Harding, 1970; Anderson & Spector, 1978). Furthermore, human cataract formation is often associated with oxidation of lens proteins (Augusteyn, 1981; Harding, 1981; Spector, 1984). Kinoshita (1964) has suggested that the high concentration of glutathione in lenses may serve to protect thiol groups from oxidation to disulphides, even though, as he points out, glutathione was found to be more readily oxidized than bovine lens proteins (Kinoshita & Merola, 1959).

The rate of synthesis of glutathione is high in many tissues, and in eye lenses it has been estimated that half the glutathione is replaced every 28h (Reddy *et al.*, 1966). The role of glutathione as a shuttle for reducing equivalents in coupled redox reactions with glutathione reductase and glutathione peroxidase would not deplete the high concentrations. It follows that glutathione takes part in reactions that involve either degradation or its loss from lens cells (Rathbun *et al.*, 1983). The ability of electrophilic foreign compounds to conjugate with reduced glutathione either spontaneously or catalysed by glutathione *S*-transferases will contribute to these reactions by catabolizing the tripeptide to mercapturic acids, which are excreted (Mannervik *et al.*, 1983; Srivastava *et al.*, 1984). In addition, an inter-organ transport system has been discovered whereby synthesis of glutathione in different tissues of the body is followed by its release into plasma, circulation and uptake elsewhere as required (Rankin *et al.*, 1983). However, there is also recycling within a cell through the activity of the enzymes of the γ-glutamyl cycle (Meister, 1983; Meister & Anderson, 1983). One of these enzymes is an extracellular membrane-bound enzyme γ-glutamyl transpeptidase, which participates in both catabolic and transport processes by reaction with glutathione (Meister, 1983), as do other transport-associated proteins (Beutler, 1983; Akerboom *et al.*, 1984). However, in the lens and erythrocytes there is controversy over whether the physiological substrate is reduced or oxidized glutathione (Meister, 1983; Beutler, 1983).

The ways in which glutathione can react with proteins need to be established for a proper understanding of glutathione metabolism. Homogenates of bovine lens proteins have been shown to undergo a disulphide-exchange reaction with oxidized glutathione to form mixed disulphides (Srivastava & Beutler, 1973). γ-Crystallins are a family of low-M_r intracellular proteins that are synthesized early in development, occupying the core of the eye lens, where they remain with little turnover throughout life (Harding & Dilley, 1976). The concentration of reduced glutathione in the bovine lens is 40–50 μmol/g. We have investigated the reaction of reduced and oxidized glutathione with the γ-crystallins from bovine lens, with particular emphasis on γ-II crystallin, the predominant member of the family. This protein molecule contains seven thiol groups in well-defined environments, as determined by high-resolution X-ray-diffraction analyses (Blundell *et al.*, 1981; Wistow *et al.*, 1983; Summers *et al.*, 1984).

Materials and methods

Materials

Reduced glutathione and oxidized glutathione were obtained from BDH Chemicals, Poole, Dorset, U.K. Oxidized glutathione (grade II) and *p*-hydroxymercuribenzoate were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Sephadex G-75 and sulphopropyl- (SP-)Sephadex C-50 were obtained from Pharmacia Fine Chemicals, London W.5, U.K. DEAE-cellulose (DE-52) was obtained from Whatman, Maidstone, Kent, U.K. All other chemicals were obtained from BDH Chemicals, AnalaR grade wherever possible.

Isolation of γ-crystallins

Calf lenses, weighing approx. 1.5g, were disintegrated by stirring in 50mM-sodium phosphate buffer, pH6.7, containing 0.02% NaN_3. The soluble proteins were separated by centrifugation at 17000*g* for 1h at 5°C. Fractionation of monomeric γ-crystallins from monomeric βs-crystallin was achieved by gel filtration on a Sephadex G-75 column (1000mm × 70mm diam.) with the lens extraction buffer as eluent by essentially the method of Björk (1961). Fractions containing γ-crystallin were combined and concentrated to a volume of 20ml by ultrafiltration in an Amicon cell equipped with a YM10 membrane. The mixture of γ-crystallins was separated into γ-I, γ-II, γ-III and γ-IV crystallins by ion-exchange chromatography on SP-Sephadex in sodium acetate buffer, pH5.0, with an NaCl gradient as described by Björk (1964). γ-II crystallin was either prepared in this way or alternatively all buffers used in the isolation were 0.005% (v/v) with respect to 2-mercaptoethanol. γ-III crystallin was further chromatographed into the components IIIa and IIIb by ion-exchange chromatography on DEAE-cellulose as described previously (Slingsby & Miller, 1983).

Reactions of γ-crystallins with glutathione

Solutions of γ-II, γ-IIIb and γ-IV crystallins were dialysed exhaustively against distilled water followed by equilibration by dialysis against 50mM-Tris/acetate buffer, pH8.5. The protein solutions were adjusted to a concentration of 1mg/ml by measurement of the u.v. absorption at 280nm, by using an average absorption coefficient $A^{1\%}_{1cm}$ of 21 for γ-crystallin (Björk, 1964). A solution of reduced glutathione (4mg/ml) or oxidized glutathione (8mg/ml) was freshly prepared in 50mM-Tris/acetate buffer, pH8.5, and a 25μl portion (325nmol) of each was added to separate 250μl (12.5nmol) portions of purified γ-crystallin solutions. The reaction mixture was placed in a stoppered 4ml test tube and left at 20°C for 1h, with occasional shaking. No precautions were taken to remove bivalent cations from the solutions. The solutions were then frozen at −15°C for at least 18h followed by thawing immediately before analysis by isoelectric focusing. The effect of varying the concentration of reduced glutathione relative to protein was assayed with γ-II crystallin. A series of solutions were prepared containing 2mg amounts of reduced glutathione in 10ml, 2ml, 1ml and 0.2ml; 20μl portions of each were added to separate 250μl portions of γ-II crystallin (1mg/ml). Furthermore, 40μl, 100μl and 200μl samples of reduced glutathione (10mg/ml) were added to 250μl portions of γ-II crystallin (1mg/ml). The reaction mixtures were subsequently treated as described above. The effect of the buffer on the reaction was monitored by equilibrating γ-II crystallin solutions by dialysis against 50mM-Tris/acetate buffer, pH7.5, or 50mM-sodium phosphate buffer, pH8.0. A 250μl portion of the protein (1mg/1ml) was then treated with 25μl of reduced glutathione (4mg/ml) as described above.

Reduction of γ-crystallin–glutathione adduct with dithiothreitol

Samples (250μl) of γ-II, γ-IIIb and γ-IV crystallins (1mg/ml) previously equilibrated with 50mM-Tris/acetate buffer, pH8.5, were each treated with 20μl of either reduced glutathione (4mg/ml) or oxidized glutathione (8mg/ml) for 1h at 20°C, after which time 20μl of dithiothreitol (20mg/1ml) was added and each mixture was left for 10min at 20°C before being frozen.

Large-scale preparation and isolation of γ-II crystallin–glutathione adduct

A 30mg sample of γ-II crystallin that had been isolated in the presence of 0.005% (v/v) 2-mercaptoethanol was used as the starting product. The protein in a volume of 24ml was dialysed exhaustively against distilled water at 4°C followed by dialysis against 50mM-Tris/acetate buffer, pH8.5. To this solution was added 0.6ml of reduced glutathione (4mg/ml), and the mixture was left at 20°C for 5h and then frozen for 18h at −15°C. Anion-exchanger DE-52 DEAE-cellulose was equilibrated in 25mM-Tris/acetate buffer, pH8.0, and poured to form a 200mm × 15mm diam. column at 8°C. With the equilibration buffer as eluent the position of elution of untreated γ-II crystallin, equilibrated in the same buffer, was determined. Meanwhile the γII crystallin/reduced glutathione mixture was thawed and equilibrated by dialysis against 25mM-Tris/acetate buffer, pH8.0, at 8°C and introduced to the column. Elution was carried out with 160ml of buffer at a flow rate of 24ml/h, and fractions were collected every 4ml. The first major protein peak emerged after the position of the untreated γ-II crystallin. Stepwise elution was then employed with first

160ml of 50mM-Tris/acetate buffer, pH7.5, followed by 160ml of 50mM-sodium phosphate buffer, pH4.5. Fractions comprising the main protein peaks were pooled and analysed by isoelectric focusing.

Determination of thiol content

The number of thiol groups in the γ-II crystallin molecule was determined by titration, by using the spectrophotometric method of Boyer (1954) as described by Torchinsky (1981) with application to γ-II crystallin as described by Blundell *et al.* (1983). The titration was performed on a solution of γ-II crystallin that had been isolated in the absence of any added reducing agent. The rapidly reacting thiol groups were determined by recording the increase in absorbance at 250nm within 1min of addition of *p*-hydroxymercuribenzoate. Thereafter the increase in absorption after successive additions of titrating agent required 30–60min intervals, making the end point difficult to determine. Total thiol content was thus determined in the presence of 0.5% sodium dodecyl sulphate, in which case the increase in absorption at 250nm could be reliably measured 5min after the addition of titrating agent, although 15–30min intervals were required as the end point was approached.

Analysis of γ-crystallin–glutathione adducts by isoelectric focusing

Isoelectric focusing was performed on LKB Ampholine PAG plates, pH3.5–9.5, that had been subjected initially to focusing at 25mA for 30min. Samples (10μl) were pipetted directly on to the gel surface near the anode side. Focusing was then resumed at an initial value of 50mA. The voltage was allowed to rise but restricted to a maximum of 1.1kV. Pharmacia markers were run.

Results and discussion

Bovine γ-crystallins are a family of closely related, monomeric, slightly basic proteins. Separation of the major component γ-II crystallin by chromatography in acidic buffers containing 2-mercaptoethanol results in a protein fraction with isoelectric point near 7.8 together with trace amounts of components of pI near 7.3 (Fig. 1, lane g). In order to investigate the reactions of protein with glutathione, preparations of γ-II crystallin were also prepared in buffers from which all reducing agents were excluded during the isolation. This procedure also yields predominantly a protein of isoelectric point near 7.8, although there is an increase in the trace components of pI near 7.3 (Fig. 1, lane c).

Reaction of glutathione with a protein to form a mixed disulphide will result in the acquisition by the protein of an extra negative charge. Bovine γ-II crystallin was treated with a moderate excess of either reduced or oxidized glutathione at pH8.5 in 50mM-Tris/acetate buffer. The progress of reaction was assessed by using isoelectric focusing by the appearance of proteins of more negative charge concomitant with the depletion of the native protein band of pI7.8. γ-II crystallin isolated in the absence of reducing agents (Fig. 1, lane c) reacts with reduced glutathione to form a mixture of products comprising protein molecules that have one, two or three bound glutathione molecules as deduced from their decreasing isoelectric points of pI7.3, 6.6 and 6.0 (Fig. 1, lane b). Evidence that the reaction was covalent was based on performing the isoelectric-focusing experiment in the presence of 6M-urea, whereby the negative charges remained on the reaction products. Furthermore, when the reaction products are treated with excess dithiothreitol, the negative charges are completely removed and the isoelectric point characteristic of native protein is recovered (Fig. 4, lanes g and h), indicating that the glutathione was covalently bound through a mixed disulphide bridge. Similar products are observed when γ-II crystallin is reacted with oxidized glutathione (Fig. 1, lane a) which can also be reduced back to native protein with dithiothreitol.

Whereas these results are consistent with the hypothesis that bovine γ-II crystallin possesses accessible cysteine residues that are available for reaction with oxidized glutathione by a disulphide-exchange mechanism (Torchinsky, 1981), it was surprising that the protein also appears to react with reduced glutathione to form a derivative containing up to three mixed disulphide groups. Although spontaneous oxidation of glutathione in air is sluggish, it is possible that γ-II crystallin reacts sufficiently avidly with oxidized glutathione by disulphide exchange that air oxidation of glutathione is effectively promoted by rapid reaction of the disulphide with protein thiol groups. The evidence points to the mixed disulphide being a very favourable product.

Further experiments were performed in order to investigate the reaction of reduced glutathione with γ-II crystallin. The ratio of reduced glutathione to protein was varied and the formation of products was followed by isoelectric focusing. When 1 equivalent of reduced glutathione was treated with 1 equivalent of γ-II crystallin, then the major product was a protein molecule with one glutathione molecule bound (Fig. 2, lane h). However, some protein molecules have an additional second site labelled, thus accounting for some unmodified protein as a result of unavailability of reagent. When 5 equivalents of glutathione are used no protein escapes reaction and the predominant protein species contains two mixed

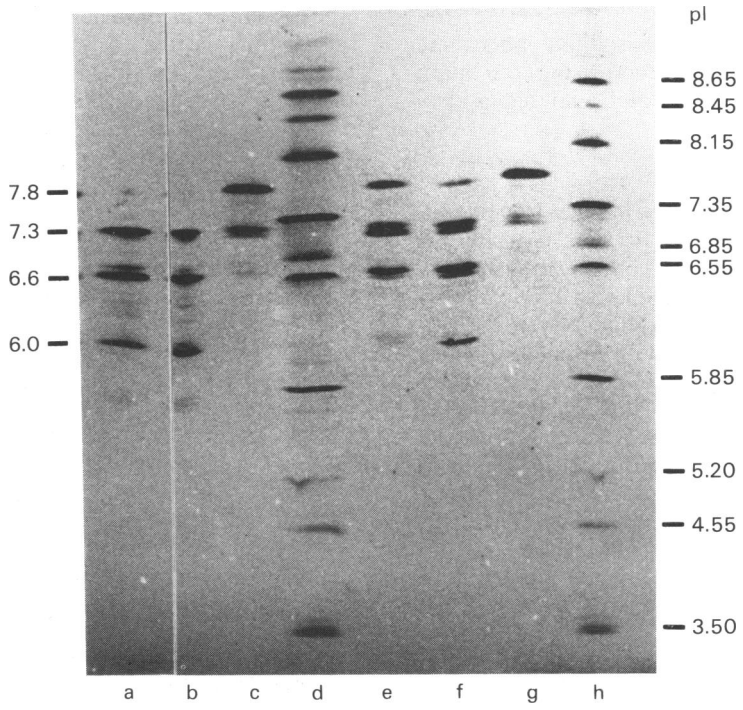

Fig. 1. *Isoelectric focusing of different preparations of γ-II crystallin with either reduced or oxidized glutathione* The anode is at the bottom of the gel. Lane a, products of reaction of γ-II crystallin (12.5 nmol), isolated in the absence of reducing agents, with oxidized glutathione (325 nmol); lane b, products of reaction of γ-II crystallin (12.5 nmol), isolated in the absence of reducing agents, with reduced glutathione (325 nmol); lane c, untreated γ-II crystallin isolated in the absence of reducing agents; lane d, marker mixture (20 μl) (Pharmacia), pI values given on the right; lane e, products of reaction of γ-II crystallin (12.5 nmol), isolated in the presence of 2-mercaptoethanol, with oxidized glutathione (325 nmol); lane f, products of reaction of γ-II crystallin (12.5 nmol), isolated in the presence of 2-mercaptoethanol, with reduced glutathione (325 nmol); lane g, γ-II crystallin isolated in the presence of 2-mercaptoethanol; lane h, marker mixture (10 μl).

disulphide groups (pI 6.6), with some molecules have one or three (Fig. 2, lane g). The reaction proceeds similarly with 10 (Fig. 2, lane f) or 50 equivalents (Fig. 2, lane e) of reduced glutathione, although when a very large excess is used, as for example 500 equivalents (Fig. 2, lane b), then the original protein band at pI 7.8 is largely recovered. These results indicate that γ-II crystallin possesses at least three cysteine residues that avidly and stoichiometrically react with reduced glutathione. A very large excess of glutathione results in less extensively modified protein products, presumably because any mixed disulphide formed could now undergo further rearrangement with the excess reduced glutathione, as these conditions are now favourable for disulphide exchange.

The number of mixed disulphides formed per protein molecule appeared to be largely independent of the time allowed for the reaction, which was varied between 15 min and 6 h at 20°C. However, the relative amounts of the protein adduct with three mixed disulphide groups, pI 6.0, was always higher if the reaction mixture had been frozen at −15°C before analysis by isoelectric focusing. Consequently the protocol followed for comparing different sets of conditions always included one freezing step. It was also observed that, if solutions of γ-crystallin that contained 0.005% (v/v) to 2-mercaptoethanol were frozen, then some irreversible precipitation occurred on thawing, which did not occur with γ-crystallin solutions frozen in the absence of reducing agent. Taken together, these results indicate that the reactivity of certain γ-crystallin cysteine residues towards thiol groups is increased during freezing and thawing.

The extent of the reaction with glutathione was diminished by lowering the pH of the 50 mM-Tris/acetate buffer from 8.5 to 7.5, as shown by the observation that only trace amounts of protein adduct containing three glutathione molecules were formed (results not shown). Nevertheless, at a pH approaching physiological (7.5) the reaction proceeded such that no unmodified protein (pI 7.8) remained and the products were comprised of protein bands at pI 7.3 and 6.6 representing protein

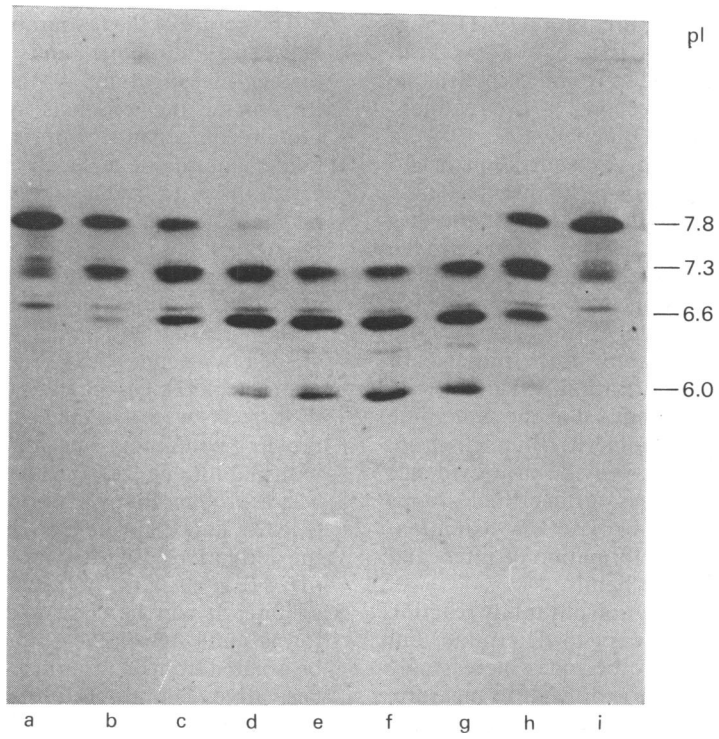

Fig. 2. *Effect of varying the relative concentration of reduced glutathione on the reaction with γ-II crystallin*
The anode is at the bottom of the gel. All lanes show the reaction products after the reaction of 1 equivalent of γ-II crystallin, isolated in the absence of reducing agents, with the following equivalents of reduced glutathione at pH 8.5: lane b, 500; lane c, 250; lane d, 100; lane e, 50; lane f, 10 lane g, 5; lane h, 1; lanes a and i are the control γ-II crystallin solutions with no added glutathione.

molecules with one and two mixed disulphide groups. However, if the reaction was carried out in 50mM-sodium phosphate buffer at pH 8.0, then analysis of the reaction products indicated that only one mixed disulphide group was formed and a large proportion of the protein was unmodified. These results indicate that, at a pH that promotes the S^- anion, and in the presence of a large cation that may stabilize it, the γ-II crystallin favours more extensive reaction with glutathione.

The reaction of glutathione with preparations of γ-II crystallin was dependent to some extent on previous exposure to certain reducing agents. A sample of γ-II crystallin that had been isolated in buffers containing 0.005% 2-mercaptoethanol yet dialysed exhaustively into mercaptan-free buffer displayed a predominant component on isoelectric focusing with the same pI of 7.8 as protein that had never been exposed to the reducing agent (Fig. 1, lanes c and g). Reaction of this preparation of γ-II crystallin with reduced glutathione yielded protein adducts with one, two and three additional negative charges in a way comparable with the protein that had not been exposed to reducing agent (Fig. 1, lanes b and f). However, there was a marked depletion of reaction products when the reaction was performed with oxidized glutathione (Fig. 1, lane e). These results indicate that exposure of γ-II crystallin to 2-mercaptoethanol during the isolation procedure resulted in the blocking of some protein cysteine residues that were consequently unavailable for reaction with oxidized glutathione via a disulphide-exchange mechanism. The results are consistent with the previous hypothesis that γ-II crystallin possesses cysteine residues that are reactive towards low-M_r thiols such that a mixed disulphide is formed. Hence the mixed-disulphide derivative of γ-II crystallin with 2-mercaptoethanol was susceptible to a disulphide exchange with additional reduced glutathione (Fig. 1, lane f) yet less reactive towards oxidized glutathione (Fig. 1, lane e).

Although in the presence of some reducing agents γ-II crystallin appears to form mixed disulphides, an intriguing question remains as to what is the oxidation state of the untreated protein. For this purpose a sample of γ-II crystallin was isolated in the absence of any added reducing agent, and the number of rapidly reacting thiol groups per molecule was determined by titration with *p*-hydroxymercuribenzoate, a reagent that cannot be involved in a disulphide-exchange

mechanism (Boyer, 1954; Torchinsky, 1981). Preparations of γ-II crystallin that had never been exposed to reducing agent were indistinguishable from samples that had received a pretreatment with excess dithiothreitol (Blundell *et al.*, 1983) in that both samples titrated the equivalent of 3.5 rapidly reacting thiol groups per molecule. These results support the idea that γ-II crystallin has surface thiol groups that were mainly reduced in the intact lens and furthermore can maintain their reduced state in the absence of added reducing agents during isolation. Only after a period of 3 weeks at ambient temperature in the presence of air did the thiol-group titration value for γ-II crystallin fall by 1 equivalent yet at the same time the protein remained monomeric (Blundell *et al.*, 1983). These observations provide firm evidence that γ-II crystallin possesses surface thiol groups that are resistant to immediate air oxidation, which would result in the formation of inter- and intra-molecular disulphide bonds.

The end point of the titration of rapidly reacting thiol groups is relatively easy to determine, but thereafter the reaction rate becomes increasingly sluggish and the end point is difficult to measure. Consequently the titration of γ-II crystallin was carried out in the presence of 0.5% sodium dodecyl sulphate. On the basis of two titrations an average value of 6.0 (±0.4) thiol groups were titrated per protein molecule. This result is inconsistent with there being 1 equivalent of an intramolecular disulphide bridge per protein molecule. The data are consistent with the possibility that all cysteine residues are reduced under the conditions of isolation, with partial blocking of the equivalent of one thiol group through steric hindrance or alternatively through some limited oxidation.

However, the data presented here demonstrate how γ-II crystallin comprises cysteine residues that in the presence of the low-M_r thiols glutathione and 2-mercaptoethanol undergo a reaction leading to the formation of a mixed disulphide. Reduced glutathione (redox potential −0.25 V) reacts with unprotected γ-II crystallin to form a mixed disulphide that can be reduced back to native protein with dithiothreitol (redox potential −0.33 V). Reaction of γ-crystallin with low-M_r thiols to form mixed disulphides is also consistent with our previous observation that when the protein is isolated in the presence of 2-mercaptoethanol there is blocking of a surface thiol group (Blundell *et al.*, 1983). Titration of γ-II crystallin that had been isolated in the presence of 2-mercaptoethanol with *p*-hydroxymercuribenzoate indicated the loss of 1 equivalent of thiol group compared with protein isolated in the absence of reducing agent.

The nature of the favourable product of reaction between glutathione and γ-crystallin requires further investigation. An attempt was made to fractionate the products of the reaction into protein components comprising equal numbers of bound glutathione molecules. After reaction of γ-II crystallin with reduced glutathione the excess reagent was removed by dialysis. A column of DEAE-cellulose ion-exchange resin was prepared on which the protein derivatives were chromatographed (Fig. 3*a*). Stepwise elution with buffers of both increasing ionic strength and decreasing pH yielded two major peaks (Fig. 3*a*, 1 and 3) and one minor peak (2). On the basis of isoelectric focusing these peaks were assigned as follows: 1 and 2 were proteins containing one mixed disulphide group with glutathione (Fig. 3*b*, lanes b and c) and peak 3 was a protein comprising two mixed disulphides (Fig. 3*b*, lane a). Protein material corresponding to the derivative with three mixed disulphide groups, pI 6.0 (Fig. 3*b*, lane e) was not recovered from this column. It can be concluded that γ-II crystallin forms stable derivatives with glutathione that can be isolated from one another, thus enabling protein derivatives containing either one or two mixed disulphide groups to be obtained pure.

X-ray-diffraction data have been collected from freshly prepared crystals of γ-II crystallin grown in the presence of 3 mM-dithiothreitol, and a high-resolution (0.16 nm) electron-density map has been calculated (Summers *et al.*, 1984). Interpretation of this map leads to the conclusion that γ-II crystallin contains seven thiol groups per molecule. Bovine γ-II crystallin is thus richly endowed with thiol groups at positions 15, 18, 22, 32, 41, 78 and 109 in the sequence: two of these cysteine residues, at positions 32 and 78, can be described as inaccessible to bulky reagents, as they have zero accessibility to water molecules, whereas cysteine-18 is close to both cysteine-78 and cysteine-22, with cysteine-15 nearby (Wistow *et al.*, 1983; Blundell *et al.*, 1983). As the lens contains high concentrations of reduced glutathione it is concluded that, if certain γ-II-crystallin cysteine residues become accessible to glutathione in the presence of molecular oxygen, oxidation would proceed.

Complete cDNA sequences of γ-crystallins from bovine (Bhat & Spector, 1984), human (Schoenmakers *et al.*, 1984), rat (Moormann *et al.*, 1982; Schoenmakers *et al.*, 1984), mouse (Lok *et al.*, 1984) and frog (Tomarev *et al.*, 1984) are now known. Comparison of these sequences show that cysteine residues 18, 32, 41, 78 and 109 are conserved in nearly all sequences, whereas cysteine residues 15 and 22 are frequently replaced by other residues. Partial chemical sequences are available for two other members of the γ-crystallin family from bovine lens, γ-III and γ-IV, which indicate

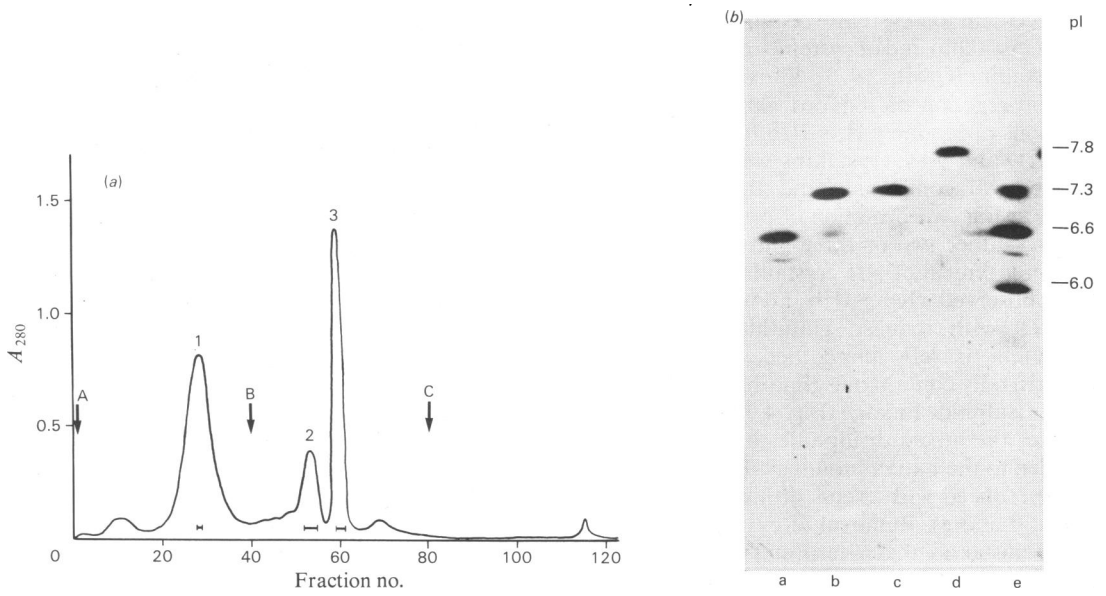

Fig. 3. *Separation of the products of reaction of γ-II crystallin with reduced glutathione*
(*a*) Ion-exchange chromatography of γ-II crystallin–glutathione adducts on a DEAE-cellulose column equilibrated with 25mM-Tris/acetate buffer, pH8.0. The arrows mark the elution of the column with the following buffers: A, 160ml of 25mM-Tris/acetate buffer, pH8.0; B, 160ml of 50mM-Tris/acetate buffer, pH7.5; C, 160ml of 50mM-sodium phosphate buffer, pH4.5. Fractions were collected every 4ml. The horizontal bars indicate the pooled fractions corresponding to peaks 1, 2 and 3. (*b*) Identification of the protein peaks obtained from (*a*) by isoelectric focusing. The anode is at the bottom. The deduced isoelectric points are shown on the right. Lane a, peak 3 from (*a*); lane b, peak 2 from (*a*); lane c, peak 1 from (*a*); lane d, untreated γ-II crystallin after passage through the DEAE-cellulose column; lane e, unfractionated γ-II crystallin–glutathione adducts.

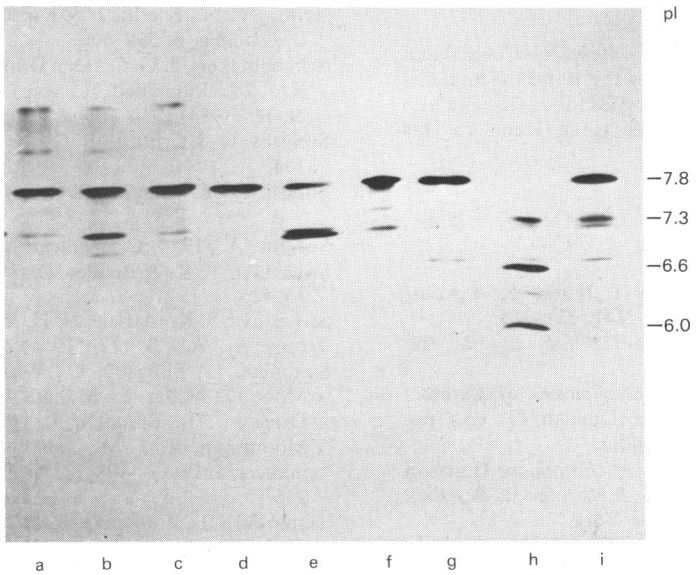

Fig. 4. *Reaction of reduced glutathione with different members of the bovine γ-crystallin family*
The anode is at the bottom. The isoelectric points are shown on the right. Lanes c, f and i show the preparations of γ-IV, γ-IIIb and γ-II crystallins after isolation by ion-exchange chromatography in the absence of added reducing agents. Lanes b, e and h show respectively the isoelectric points of the products after the reaction of 12.5nmol of γ-IV, γ-IIIb and γ-II crystallins each with 325nmol of reduced glutathione at pH8.5. Lanes a, d and g respectively show the effect of excess dithiothreitol on each of the mixtures of γ-IV, γ-IIIb and γ-II crystallins after reaction with reduced glutathione.

the replacement of cysteine residues 15 and 22 from γ-IV crystallin and of cysteine residue 22 from γ-III crystallin (Croft, 1973; Slingsby & Croft, 1978). The reaction of reduced glutathione with preparations of bovine γ-II, γ-IIIb (a subcomponent of γ-III) and γ-IV crystallins were performed under identical conditions, and the results are indicated in Fig. 4. Formation of protein adducts, comprising either two or three glutathione molecules bound, typical of γ-II crystallin (Fig. 4, lane h) are not observed when γ-IIIb and γ-IV crystallins are treated with reduced glutathione. Instead, some protein is left unmodified, whereas the product can only contain one glutathione molecule in mixed-disulphide linkage (Fig. 4, lanes b and e). In all cases the mixed disulphides are completely regenerated to the native proteins γ-IV, γ-IIIb and γ-II when reduced with excess dithiothreitol (Fig. 4, lanes a, d and g). Removal of cysteine residues from the cluster in the *N*-terminal domain of γ-crystallin is associated with a decrease in the sites available for reaction with glutathione.

It is suggested that γ-crystallins, in particular γ-II crystallin, can readily react with reduced glutathione, resulting in its oxidation. This work has shown how complexes of glutathione with lens components can be purified. These reactions require attention in view of the observed oxidation of glutathione associated with its active transport out of the lens (Beutler, 1983).

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