Biochem. J. (1987) 246, 761–769 (Printed in Great Britain)

Conformational changes induced in lens α- and γ-crystallins by modification with glucose 6-phosphate

Implications for cataract

Howard T. BESWICK* and John J. HARDING†
Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, U.K.

There is good evidence that the non-enzymic chemical modification of proteins plays a role in the aetiology of cataract and diabetic sequelae. This paper presents new evidence that glycosylation of two major lens structural crystallins, α- and γ-crystallins, by glucose 6-phosphate (G6P) induces conformational changes in the proteins. In addition the surface charge on the molecules is altered. These changes would affect protein–protein and protein–water interactions within the lens and could lead to disruption of the short-range order of the lens proteins which is essential for lens transparency. Conformational changes to lens proteins are known to occur in human cataractous lenses but their cause *in vivo* is not established. Cumulative chemical modification of proteins, over a period of decades, is a strong candidate as a causal agent.

INTRODUCTION

There now exists an overwhelming body of evidence indicating that non-enzymic chemical modification of proteins both *in vitro* and *in vivo* plays a role in a variety of diseases including diabetes, cataract and certain steroid- and alcohol-induced conditions. For recent reviews see Harding & Crabbe (1984), Harding (1985) and Cerami & Crabbe (1986).

The proteins most susceptible to this type of damage *in vivo* are those, such as the eye lens crystallins, which show little or no turnover during the lifetime of the individual, although, where the local environment of a residue within a protein is such that its reactivity is greatly increased, proteins with half-lives as short as 1–2 days have been shown to be modified with a resultant alteration of function (Steinbrecher & Witztum, 1984).

The lens is a specialized structure in which the lens crystallins are present at high concentrations. The short-range interactions between crystallins are essential for lens transparency (Delaye & Tardieu, 1983). Any chemical modification which causes conformational changes, especially if it alters the surface charge of the protein, would affect protein–protein and protein–water interactions within the lens possibly perturbing short-range order and compromising the transparency of the lens. The conditions in which lens proteins can be chemically modified *in vivo* are many and varied; they include modification by administered drugs, e.g. steroids (Bucala *et al.*, 1985), and also by reactive metabolites elevated as a result of disease.

Glucose, elevated in diabetes, and cyanate, elevated during renal failure or severe diarrhoea, have both been shown to bind to lens crystallin amino groups, resulting in loss of positive charge from the protein surface and causing conformational changes which expose previously buried thiol groups (Beswick & Harding, 1984; Liang & Chylack, 1984). These exposed thiol groups can participate in inter-protein disulphide bonding *in vitro* (Beswick & Harding, 1984): *in vivo* this could lead to the formation of high-M_r light-scattering aggregates within the lens which would impair its transparency. Diabetes, renal failure and severe diarrhoea are all associated with an increased risk of cataract formation (van Heyningen & Harding, 1986). Conformational changes to lens crystallins have been detected in human cataract (Harding, 1972) and the extent of non-enyzmic glycosylation is elevated in diabetic lens proteins (Kasai *et al.*, 1983). Glucose 6-phosphate (G6P) is a chemically reactive glycolytic intermediate which is membrane-impermeant and known to bind to lens proteins *in vitro* (Stevens *et al.*, 1978). The G6P concentration is elevated in the diabetic lens (Gonzalez *et al.*, 1980). The following studies show the effects of G6P binding on the conformations of α- and γ-crystallins.

α-Crystallin is a polydisperse protein of mean M_r 800000; it is composed of 20000-M_r subunits of two main types A and B, in the approximate ratio of 2:1, that have extensive sequence homology. Both bovine A and B subunits are phosphorylated *in vivo* (Sredy & Spector, 1984). If analysed by isoelectric focusing the phosphorylated α-A1 and -B1 can be resolved from the unphosphorylated α-A2 and -B2. Hence in young animals four major bands are observed, but as the animal ages the α-subunits are successively degraded from the *C*-terminus giving rise to a variety of extra bands on isoelectric focusing (Bloemendal, 1977).

γ-Crystallins are a major class of lens water-soluble crystallins which, unlike α-crystallins, are found predominantly in the nucleus of the lens. The γ-crystallins are of particular interest since the conformational changes detected in human cataractous-lens crystallins were found in lenses with nuclear cataracts (Harding, 1972). The γ-crystallins are monomeric in solution with an approximate M_r of 20000 but with different isoelectric

Abbreviations used: G6P, glucose 6-phosphate; DETAPAC, diethylenetriaminepenta-acetic acid.

* Present address: National Eye Institute, Building 6, Room 208, National Institutes of Health, Bethesda, MD 20205, U.S.A.
† To whom correspndence should be addressed.

points in the range 7–8 (Björk, 1961). Isoelectric focusing resolves γ-crystallins into between five and 13 protein components (Bours *et al.*, 1982). The γ-crystallins are the only major crystallin class to possess a free *N*-terminus (Mok & Waley, 1968). Once amino acid sequences of the different γ-crystallins became available it was shown that there was considerable homology between the different proteins, and the bovine γ-crystallins were products of different ancestrally related genes (Slingsby & Croft, 1978).

The three-dimensional structure of one of the γ-crystallins (γ-II) has been published (Blundell *et al.*, 1981). The authors commented that γ-crystallin showed the highest internal symmetry of all the proteins studied by X-ray diffraction. The molecule consists of two homologous domains each of which consists of two similar 'greek key' motifs. The polypeptide backbones of the motifs in each domain contribute to the formation of two antiparallel β-pleated sheets. The structure is consistent with the secondary structure predicted from c.d. data (Horwitz *et al.*, 1977). The γ-II molecule has a total of seven free thiol groups, some of which could participate in intramolecular disulphide bond formation under oxidizing conditions with little effect on protein conformation (Blundell *et al.*, 1981). Another striking feature of the molecule is the network of positive and negative charges on the protein surface which probably contributes to the stabilization of the protein tertiary structure (Wistow *et al.*, 1983). The amino acid and gene sequence homology of some γ- and β-crystallins from lenses of various species indicates that they can be considered as a single β/γ family of proteins (Tomarev *et al.*, 1982; Inana *et al.*, 1983; Berbers *et al.*, 1984; Harding & Crabbe, 1984; Schoenmakers *et al.*, 1984).

MATERIALS AND METHODS

Lenses

Bovine lenses were obtained from Clays (Buckingham, Bucks., U.K.) on the day the animals were slaughtered and stored at −20 °C until used.

Chemicals

5-Hydroxymethylfurfural, thiobarbituric acid, G6P and urea were obtained from Sigma Chemical Co. Ampholines were purchased from Pharmacia, U.K. All chemicals used to make polyacrylamide gels were purchased from BDH Chemicals and were of electrophoretic grade.

Preparation of bovine α- and γ-crystallins

Bovine lenses (net weights 2–2.2 g) were decapsulated and hand-homogenized in 0.1 M-phosphate buffer, pH 7.4, containing final concentrations of 0.5% NaN_3 and 5 mM-DETAPAC, a metal-ion chelator. The homogenate was centrifuged at 12000 *g* for 30 min at 4 °C. The resulting supernatant contained the total water-soluble lens crystallins. α- and γ-Crystallins were isolated by gel chromatography on a TSK-HW-55 (BDH) column (110 cm × 2.5 cm). The elution buffer was identical with the homogenization buffer, a flow rate of 15 ml/h being used. Fractions from several runs were pooled to provide enough α- and γ-crystallins for the experiments. The chromatographic profiles and SDS/polyacrylamide gels indicated that both α- and γ-crystallin preparations were uncontaminated by other crystallins, especially β-crystallin.

Incubation of α- and γ-crystallin with G6P

The pooled crystallins were concentrated by ultrafiltration under positive N_2 pressure and divided into four equal fractions. Incubations containing final concentrations of 0, 20 mM-, 50 mM- and 100 mM-G6P in column buffer (pH 7.4) were in sealed vessels at 37 °C in a shaking water bath. At 0, 3, 6 and 12 days after starting the incubations portions were removed for thiol determination and tryptophan fluorescence analysis. The effects of the presence and absence of O_2 were not studied directly, but DETAPAC would decrease metal-ion-catalysed oxidation.

Quantification of extent of glycosylation

At the end of the incubation period (12 days) the remaining sample was exhaustively dialysed over a period of 24 h against three changes of 5 litres of distilled water. The protein was then freeze-dried and duplicate 2 mg samples were assayed using the thiobarbituric acid colorimetric method (Subramaniam *et al.*, 1980).

C.d.

C.d. spectra were recorded digitally from 340 to 255 nm (near-u.v.) and from 250 to 220 nm (far-u.v.) using a Jasco J-41C spectropolarimeter with a J-DPY data processor as detailed in Beswick & Harding (1984). The spectra presented were recorded in 10 mM-phosphate buffer, pH 7.4, and represent the averages of at least four scans. The scans are presented as plots of c.d. absorption coefficient (Δa) in $ml \cdot mg^{-1} \cdot cm^{-1}$ against wavelength in nm. All plots are corrected to a final protein concentration of 1 mg/ml. (Spectra are accurate to less than 5%.)

Tryptophan fluorescence

Tryptophan fluorescence was measured using a Perkin–Elmer 3000 spectrofluorimeter with a xenon lamp interfaced to a recorder via a servo mechanism. All samples were in 10 mM-phosphate buffer, pH 7.4, and were adjusted to equal absorbance at 280 nm before measuring their fluorescence. Emission spectra from 260 to 450 nm were recorded using an excitation wavelength of 280 nm. The area under the single emission peak was measured for each sample and the quantum yields were calculated (Liang & Chakrabarti, 1982).

Quantification of protein thiol groups

Protein thiol groups were measured in the presence of freshly made 8 M-urea using Ellman's reagent (Sedlak & Lindsay, 1968).

SDS/polyacrylamide gel electrophoresis

These were slab gels with a 5% stacking gel and a 15% running gel. Running and staining conditions were as in Beswick & Harding (1984). Each sample was run twice, once after reduction with dithiothreitol. These samples were heated at 90 °C for 5 min in sample buffer containing 100 mM-dithiothreitol and then loaded directly on to the gel. Comparison of the two tracks allows the detection of any inter-protein disulphide-bond formation. These reducing conditions were sufficient to cleave the disulphide bonds of carbamoylated α-crystallin (Beswick & Harding, 1984).

Table 1. Quantification of G6P bound to α-crystallin and total thiol groups detectable under denaturing conditions (8 M-urea)

The thiobarbituric acid method and Ellman's reagent were used respectively. Duplicate assays were performed on dialysed freeze-dried protein; all duplicates were within 7% of each other.

Assay sample	Incubation period ...	G6P bound (mol/mol of 20000-M_r subunits) 12 days	Thiol content (mol/mol of 20000-M_r subunits) 0 days	12 days
α-incubated control		0	0.71	0.69
α+20 mM-G6P		0.46	0.71	0.72
α+50 mM-G6P		1.12	0.71	0.71
α+100 mM-G6P		1.60	0.71	0.65

Isoelectric focusing

Isoelectric focusing was performed in 7% acrylamide gels in the presence of 8 M-urea using a pH gradient of 4–8. Gels were cast on Gelbond PAG (Pharmacia). The inter-electrode distance was approx. 10 cm. The gels were prefocused for 500 V·h at a power rating of 6W before loading the protein samples. Once loaded the samples were focused for 1000 V·h at a power rating of 10 W. All other procedures used are outlined in the Pharmacia handbook 'Isoelectric Focusing: Principles and Methods'.

RESULTS

From the chromatogram (not shown) it was evident that the proteins used in these experiments were predominantly α- and γ-crystallins respectively, essentially free of other crystallin classes and higher-M_r material. This is also apparent by examination of the incubated control tracks in the SDS/polyacrylamide-gel analysis (Fig. 2). On SDS/polyacrylamide gels the α-crystallin A and B subunits resolve as two major bands, the slower component being the B subunits and the faster component the A subunits, despite the fact that they are known to have extensive homology in their amino acid sequences and almost identical M_r values (Bloemendal, 1977). The anomalous behaviour on SDS/polyacrylamide-gel electrophoresis is probably due to the SDS failing to unfold the B subunits completely. In SDS/polyacrylamide gels containing 6 M-urea, the A and B chains coincide (van der Ouderaa *et al.* 1974).

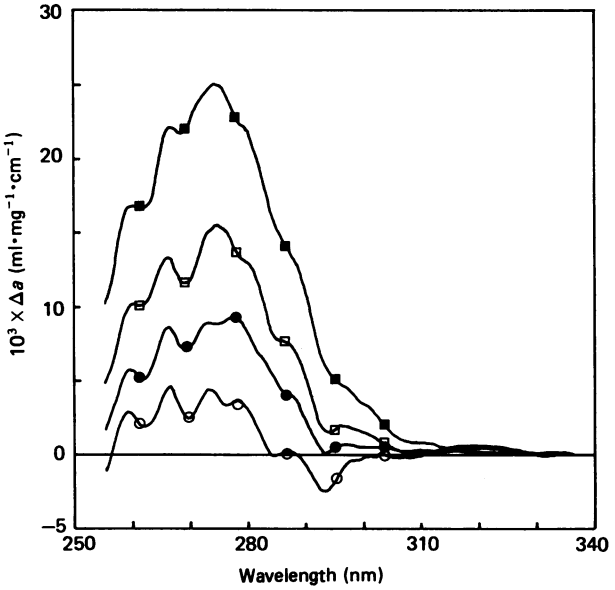

Fig. 1. Near-u.v. c.d. spectra of the α-crystallin samples after 12 days incubation

All analyses are in 10 mM-phosphate buffer, pH 7.4, and at equal protein concentration. The spectra are marked: ○, α-crystallin-incubated control; ●, α-crystallin incubated with 20 mM-G6P; □, α-crystallin incubated with 50 mM-G6P, ■, α-crystallin incubated with 100 mM-G6P.

α-Crystallin

After 12 days incubation, the extent of glycosylation in each of the α-crystallin samples were estimated by using the thiobarbituric acid method. The results are shown in Table 1. The extent of modification increases in approximately direct proportion to the G6P concentration used in the incubation. There is ample evidence that the main sites of non-enzymic glycosylation of proteins are the amino groups (Harding, 1985). The α-amino group of α-crystallin is acetylated (Bloemendal, 1977) and so the reaction here is presumably with the ε-amino groups of lysine. The α-subunits have an average of eight lysine residues each and the maximum extent of modification is 20% of the total lysine. The α-crystallin became yellow during the incubation with G6P but at a much lower rate than the γ-crystallin (see below).

Further evidence for chemical modification of α-crystallin is provided by isoelectric focusing (results not shown). Identities were assigned to the major bands by comparison with published staining patterns for bovine α-crystallin (van Kleef *et al.*, 1976). Various α-B bands disappear from the G6P-incubated samples and a smear of more acidic proteins appear at the anode. After incubation in 100 mM-G6P for 12 days, most of the main α-B bands have disappeared. This behaviour is consistent with G6P binding to lysine amino groups. This reaction not only removes one positive charge but also adds a further two negative charges due to the phosphate group on G6P. The result is that the modified proteins become considerably more anodic.

Fig. 1 shows the near-u.v. c.d. spectra for the protein samples after 12 days incubation. There is a clearly visible relationship between the magnitude of the

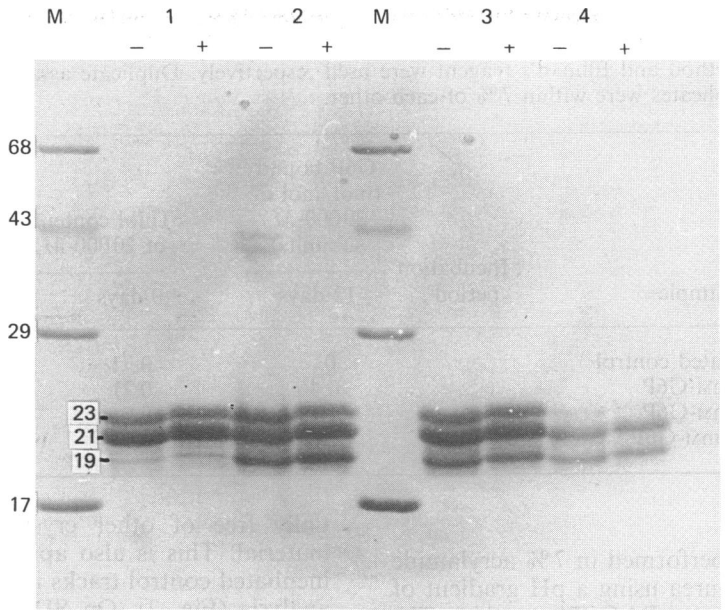

Fig. 2. SDS/15% polyacrylamide-gel electrophoresis of the α-crystallin samples after 12 days incubation

The samples were dissolved in sample buffer in which dithiothreitol was either present (+) or absent (−) and then run on the gel. M denotes the calibration marker tracks. The M_r values ($\times 10^{-3}$) are labelled for the major bands. Samples are: tracks 1, α-crystallin-incubated control; tracks 2, α-crystallin incubated with 20 mM-G6P; tracks 3, α-crystallin incubated with 50 mM-G6P; tracks 4, α-crystallin incubated with 100 mM-G6P.

dichroism and the extent of modification by G6P (Table 1). The bands from 290 to 305 nm are usually attributable to tryptophan transitions. The band at 293 nm is probably a tryptophan ^{1}-L_a band. The intensity of this band increases with increased glycosylation. If the bound G6P is close to a tryptophan residue it is possible that one of the sugar hydroxy groups could hydrogen-bond to the tryptophan ring nitrogen atom. Such interactions have been documented for aqueous mixtures of sugar polyols and lysozyme tryptophan residues (Ikeda & Hamaguchi, 1970). The result was always an increase in the intensity of the ^{1}-L_a band similar to that seen in Fig. 1. The striking increase in the intensity of the bands in the phenylalanyl region (270–255 nm) and in the tyrosyl region (275–282) must reflect the altered environment of these residues within the protein. There is also some loss of fine structure in the tyrosyl region as the extent of glycosylation increases. These changes in the c.d. spectra indicate a progressive change in the conformation of α-crystallin induced by reaction with G6P. The c.d. spectra show tails above 310 nm. This can sometimes be indicative of disulphide bond formation, the tail being the extremity of a broader band with its maximum above 240 nm masked by other chromophores. However, this was not supported by the thiol assays performed with Ellman's reagent. The assays were in 8 M-urea and for all incubations there appeared to be little or no loss of thiols after 12 days incubation (Table 1). The control value of 0.69 thiol group/subunit is close to the expected value for α-crystallin, which has approximately two A subunits each containing one thiol group to every B subunit, which lacks thiol groups (Harding & Dilley, 1976). It appears therefore that 8 M-urea makes the thiol groups completely accessible. In addition, at the higher G6P concentrations, there was no evidence of any disulphide-bonded protein in samples with no dithiothreitol present on the SDS/polyacrylamide gel (Fig. 2). There is a little disulphide-bonded protein in the 20 mM-G6P-incubated protein track; however, the bulk of this material does not appear to have been formed at the expense of the main A and B subunit bands (Fig. 2). We therefore conclude that there is little or no contribution of disulphides to the observed c.d. spectra. The far-u.v. c.d. spectra appeared identical with one another (not shown), indicating that any conformational changes occurring are affecting the tertiary/quarternary structure of α-crystallin, not the secondary structure of the subunits.

These changes in the c.d. of the protein samples after incubation with G6P were accompanied by altered tryptophan fluorescence, indicating a decreased quantum yield for tryptophan in the modified proteins, suggesting an altered environment for the tryptophan (Fig. 3).

The SDS/polyacrylamide-gel-electrophoretic analysis in Fig. 2 shows a decrease in the α-B band as the extent of glycosylation increases. This corresponds closely to the behaviour seen during isoelectric focusing, where most of the major α-B bands move as a consequence of their altered isoelectric points as a result of chemical modification. The loss of the α-B band is linked to the appearance of another band of lower apparent M_r. This band of lower apparent M_r is not likely to be have been the result of random proteolysis since (*a*) the extent of the new band formation appears to be directly related to the extent of modification and is not present in the incubated control, (*b*) proteolysis would probably produce a smear on the gel whereas the new band is distinct, and (*c*) the major lens proteinase is Ca^{2+}-dependent (Harding & Crabbe, 1984) and would therefore be inhibited by the DETAPAC present during all incubations. It is possible that the G6P-modified protein behaves anomalously on SDS/polyacrylamide-gel electrophoresis.

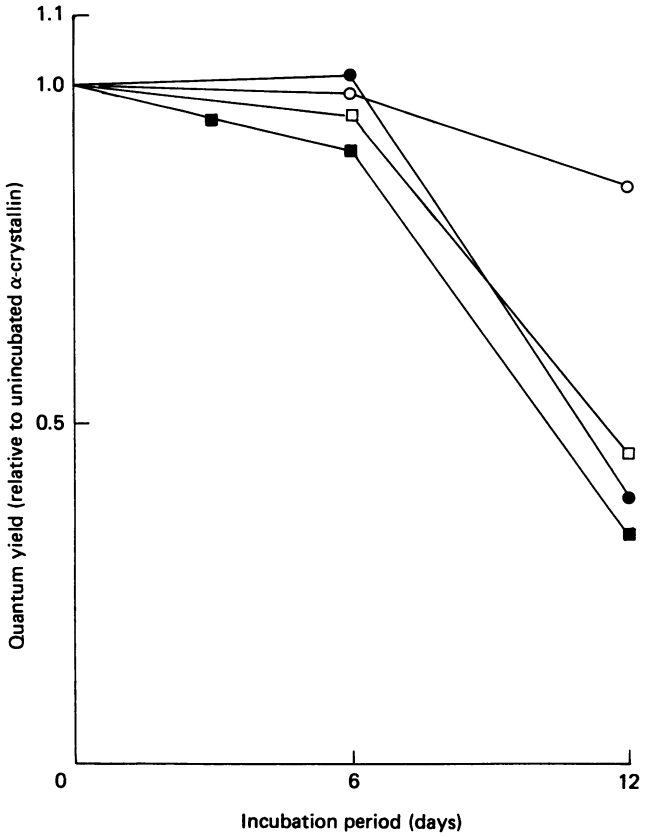

Fig. 3. Changes in quantum yield of α-crystallin during incubation

Results are expressed relative to the quantum yield of freshly prepared α-crystallin. All samples were in 10 mM-phosphate buffer, pH 7.4. The samples are: ○, α-crystallin-incubated control; ●, α-crystallin incubated with 20 mM-G6P; □, α-crystallin incubated with 50 mM-G6P; and ■, α-crystallin incubated with 100 mM-G6P.

γ-Crystallin

Quantification of the extent of glycosylation of γ-crystallin after 12 days incubation using the thiobarbituric acid assay reveals a progressive increase in the extent of modification that is directly related to the G6P concentration. The values, in order of the increasing concentration of G6P (0, 20, 50 and 100 mM) used in the incubations, are 0.0, 0.34, 0.82 and 1.12 mol of G6P bound/mol of γ-crystallin. It is evident that G6P modifies γ-crystallin to a lesser extent than it does α-crystallin under similar conditions (Table 1), as reported previously (Stevens *et al.*, 1978). At the end of the experiment the incubations containing G6P were clear and distinctly yellow; no colour was visible in the incubated control. Initial investigations of the yellow chromophore revealed it to have an absorption peak with a broad maximum at 442 nm; the amount of the chromophore formed was proportional to the G6P concentration and the bulk of it was not removed by exhaustive dialysis (Fig. 4). With an excitation wavelength of 442 nm, the chromophore showed no fluorescence emission over the region 450–800 nm. The magnitude of the absorption at 442 nm is less than 0.08 even in the incubations containing 100 mM-G6P. It is likely therefore that the concentration of chromophore is low. The chromophore appears to be bound covalently to the γ-crystallin. The yellow chromophore does not appear if 100 mM-G6P is incubated in the absence of protein but under conditions otherwise identical with those used in these experiments. The exact chemical identity of the chromophore is unknown; however, during the preparation of glucosyl-glycine adducts for a previous set of experiments, it was noticed that the purified adduct was yellow (Beswick & Harding, 1985). It is therefore possible that these chromophores are similar and are both late products formed by rearrangement of the initial Schiff bases and Amadori products formed during non-enzymic glycosylation.

Isoelectric-focusing gels (results not shown) also provide evidence that G6P has modified either the *N*-terminal or the ε-amino groups of γ-crystallin. Chemical modification of amino groups by G6P results in a net gain of three negative charges by the protein, and it is apparent from the gel that, as the extent of modification increases, protein bands with isoelectric points between 7 and 8 disappear. This correlates with the appearance of new anodic bands with isoelectric points between 6 and

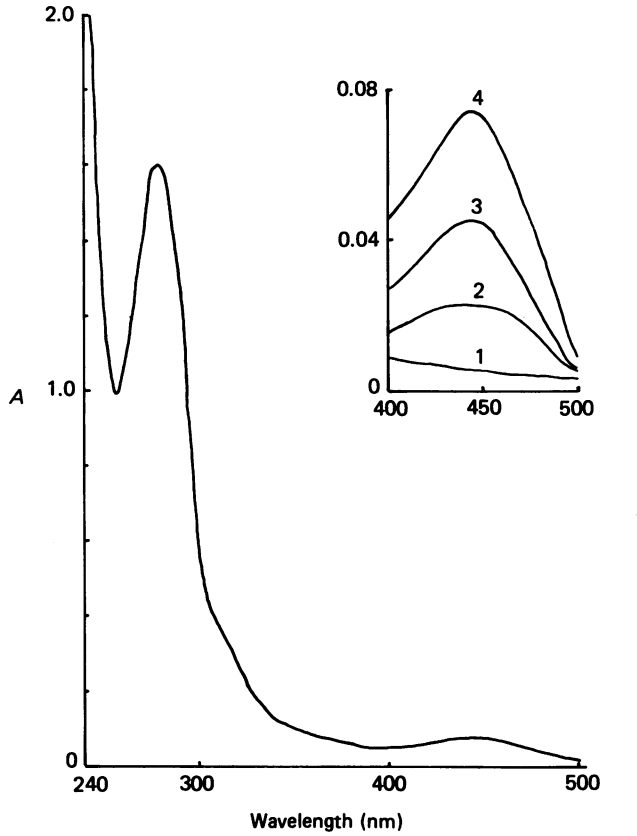

Fig. 4. Complete absorption spectrum (240–500 nm) for the 100 mM-G6P 12-day incubated sample, after exhaustive dialysis

All samples were in 10 mM-phosphate buffer, pH 7.4. The yellow chromophore has a broad maximum at approx. 442 nm. The inset shows scans in the region of the yellow chromophore absorption for all samples on a more sensitive absorption range after 12 days incubation at equal protein concentrations (0.7 mg/ml). The spectra labelled are: 1, γ-crystallin-incubated control; 2, γ-crystallin incubated with 20 mM-G6P; 3, γ-crystallin incubated with 50 mM-G6P; 4 γ-crystallin incubated with 100 mM-G6P.

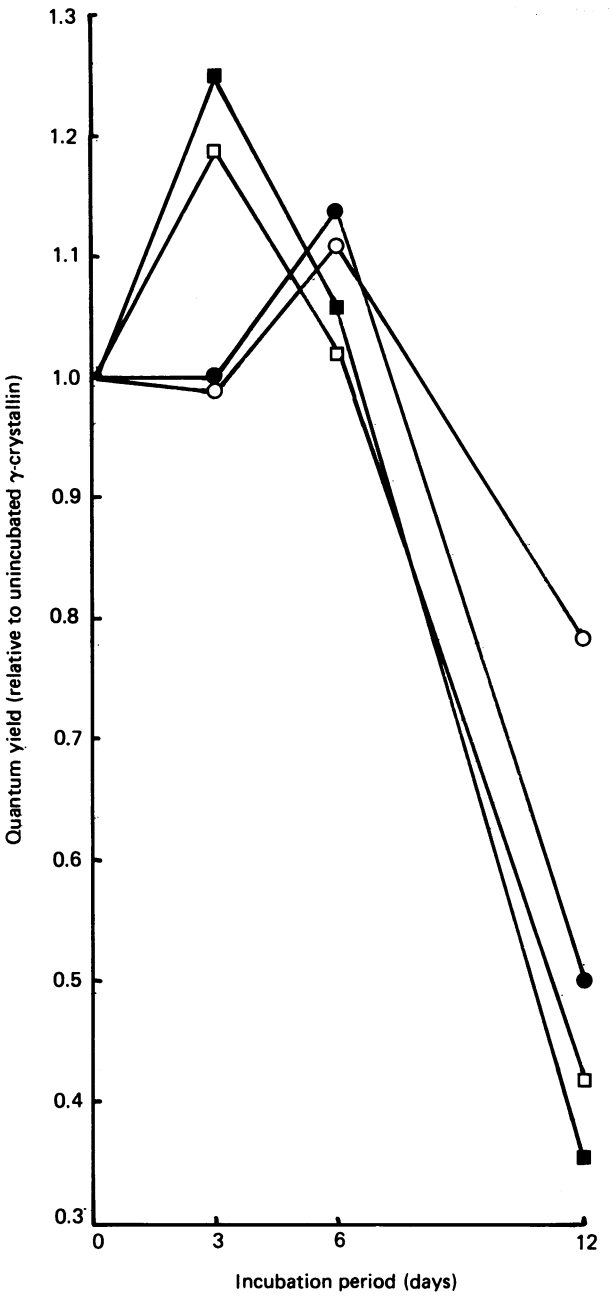

Fig. 5. Quantum yields of γ-crystallin at various times after the start of incubation

These are expressed relative to the quantum yield of freshly prepared unincubated γ-crystallin. All samples were in 10 mM-phosphate buffer, pH 7.4. The samples are: ○, γ-crystallin-incubated control; ●, γ-crystallin incubated with 20 mM-G6P; □, γ-crystallin incubated with 50 mM-G6P; ■, γ-crystallin incubated with 100 mM-G6P.

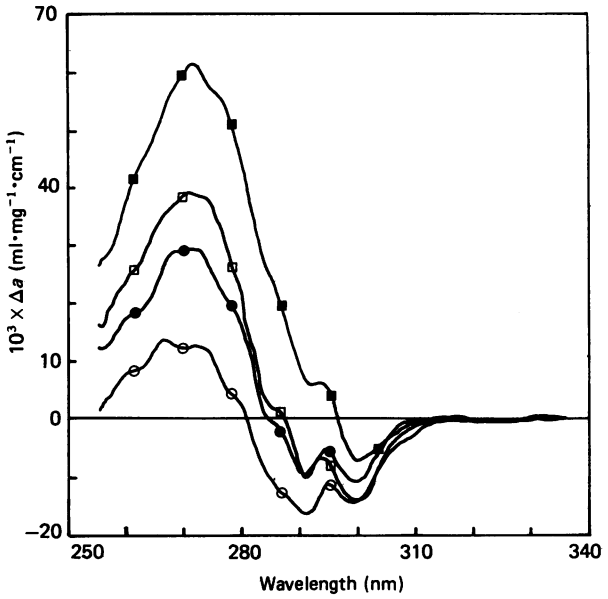

Fig. 6. Near-u.v. c.d. spectra of γ-crystallin samples after 12 days incubation

All samples have been exhaustively dialysed and are in 10 mM-phosphate buffer, pH 7.4. The plots are adjusted to represent equal protein concentrations. The samples are: ○, γ-crystallin incubated control; ●, γ-crystallin incubated with 20 mM-G6P; □, γ-crystallin incubated with 50 mM-G6P; ■, γ-crystallin incubated with 100 mM-G6P.

4.5. The incubated control γ-crystallin contains a considerable amount of material with isoelectric points below 7, which is not usually reported for γ-crystallin samples. The exact origins of these bands are unknown; however, they are derived from γ-crystallin rather than contaminating crystallins of a different class. The evidence supporting this is: (*a*) the good resolution of the γ-crystallins during the initial TSK-HW-55 gel chromatography separation; (*b*) the SDS/polyacrylamide gels show the γ-crystallin-incubated control as a single clear major band of M_r 21000 (results not shown).

Changes in the tryptophan fluorescence of the incubated γ-crystallin samples were monitored (Fig. 5). All samples show an initial increase in quantum yield relative to an unincubated control sample of γ-crystallin; this is followed by a sharp decrease. The decrease occurs more rapidly in the 50 mM and 100 mM-G6P-incubated samples compared with the other samples. The incubated control sample does show a decrease in quantum yield but it is much less than that for the G6P-incubated samples (Fig. 5). The decrease could be the result of the G6P binding to a location close to a tryptophan residue. Such a site exists in the fourth motif of γ-crystallin where Lys-154 appears to be close to Trp-148 (Blundell *et al.*, 1981). Decreased quantum yields have usually been associated with the chromophore being in a more polar environment in the protein, i.e. more surface-exposed.

The near-u.v. c.d. spectra of the 12-day-G6P-incubated samples show striking changes relative to the incubated control (Fig. 6). The general form of the changes appears similar to those observed during the G6P modification of α-crystallin in that the dichroism of the protein is increased over the entire spectrum and is accompanied by some loss of fine structure. The changes cover the phenylalanyl, tyrosyl and tryptophanyl regions of the near-u.v. c.d. spectrum. Although the extent of glycosylation of γ-crystallin is less than that of α-crystallin, the changes in dichroism appear to be relatively greater.

Taking the tryptophan fluorescence and c.d. data together, there is little doubt that non-enzymic chemical modification of γ-crystallin by G6P results in conformational changes to γ-crystallin. The far-u.v. c.d. spectra

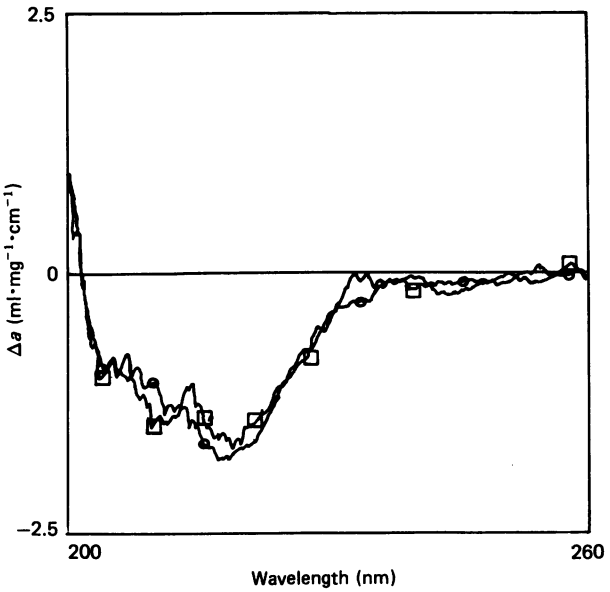

Fig. 7. Far-u.v. c.d. spectra of γ-crystallin after 12 days incubation

Symbols are as in Fig. 6.

of the samples after 12 days of incubation were characteristic of β-sheet and showed no significant changes due to chemical modification, indicating no gross alterations of secondary structure (Fig. 7).

The assays of the free γ-crystallin thiol groups carried out in 8 M-urea reveal a loss in the free thiol groups. The extent of this loss is directly related to the extent of glycosylation and observed conformational changes (Table 2). In the freshly prepared unincubated γ-crystallin used in these experiments, only 2.8 mol of thiol group/mol of γ-crystallin were detected. This may at first seem a little strange since γ-crystallin has seven free thiol residues (Wistow *et al.*, 1983). However it is known that the gross conformation of the γ-crystallins is unaltered after a 1 h incubation in 8 M-urea (Horwitz *et al.*, 1977). It is therefore likely that Ellman's reagent does not have access to all the protein thiols of γ-crystallin under the assay conditions used. Nevertheless there is clearly a loss of protein thiol groups. However, it was evident from SDS/polyacrylamide-gel studies that this is not associated with an increase in inter-protein disulphide bonding (results not shown). The three-dimensional structure of γ-crystallin indicates that certain cysteines, particularly Cys-23 and Cys-74, or Cys-23 and Cys-18, are close enough to one another to form intra-protein disulphide bonds (Blundell *et al.*, 1981). Normally, in bovine or human γ-crystallins, these thiol groups remain reduced (Yu, 1977) but the induced conformational changes could slightly alter the position of the cysteine residues to make disulphide-bridge formation easier.

DISCUSSION

These experiments indicate that G6P will readily bind to lens proteins resulting in an alteration of the isoelectric point and alteration of the tertiary/quaternary structure of the protein as detected by differences in the near-u.v. c.d. spectra and tryptophan fluorescence of the modified samples compared with the incubated controls. These G6P-induced changes in conformation do not result in any detectable increase in the extent of inter-protein disulphide bonding (Fig. 2). The latter result differs from the results obtained by Stevens *et al.* (1978) with solutions of total crystallins where extensive inter-protein disulphide bonding occurred producing large aggregates which scattered light. They state, however, that the extent of aggregate formation in their experiments is not simply related to the degree of glycosylation; other factors, such as the presence of O_2, are involved. The protein–protein and protein–water interaction around a G6P-modified protein would almost certainly be altered and this could in turn affect the short-range order necessary for lens transparency. The conformational changes reported are noticeably different in nature to those reported for carbamoylation of bovine lens α-crystallin (Beswick & Harding, 1984). This is probably due to the fact that, whereas G6P and cyanate both react primarily with lysine amino groups under our experimental conditions, they may react with different lysines within the protein. During cataractogenesis, it is possible to envisage two or more modifying agents acting in an additive or even synergistic manner. It is also possible that some chemical modifications could cause conformational changes which protect them against further damage by other chemical agents. Day *et al.* (1979) noticed that acetylation of serum albumin by aspirin inhibited glycosylation of the protein *in vitro*. The basis of this inhibition is not simply competition for identical binding sites since the major human serum albumin modification site for aspirin is Lys-199 whereas the major glycosylation site is Lys-525 (Walker, 1976; Garlick & Mazer, 1983). It is therefore probable that acetylation induces a conformational change which masks the glucose-binding site. Chemical damage to the

Table 2. Changes in thiol content of γ-crystallin during incubation with G6P

All assays were performed in duplicate, the duplicates being within 10% of each other. The assays were performed in 8 M-urea at pH 8.

Sample	Incubation period ...	Thiol content (mol/mol of γ-crystallin) 0 days	6 days	12 days
γ-incubated control		2.83	2.76	2.75
γ+20 mM-G6P		2.80	2.60	2.40
γ+50 mM-G6P		2.80	2.30	2.40
γ+100 mM-G6P		2.80	2.00	1.50

lens proteins of the type discussed would be slow to accumulate and this correlates with the slow progress of cataract, which mainly affects people of 60 years or above.

Modification of γ-crystallin by G6P results in the alteration of protein tertiary structures facilitating the loss of thiol groups. The yellow chromophore detected in these experiments is different to that isolated from old human lenses (Monnier & Cerami, 1983). It may represent an early browning product or simply be characteristic of the G6P–γ-crystallin adduct. As indicated by its behaviour in 8 M-urea and the characteristics of its three-dimensional structure, γ-crystallin is structurally very stable. In earlier unpublished studies performed by the authors, it was found that modification by cyanate had no detectable effect on the conformation of γ-crystallin despite higher levels of modification being achieved. This may reflect the fact that modification by cyanate results in the net gain of one negative charge by γ-crystallin (loss of one positive charge) whereas modification by G6P results in a net gain of three. This would result in a much greater disruption of the surface network of charges on γ-crystallin which could destabilize the native conformation. Such an alteration in surface charge would almost certainly affect interactions between γ-crystallin molecules in the nucleus of the lens and nearby proteins and surrounding water. The attached sugar phosphate would also affect the hydration of the γ-crystallin. These two events could result in regions of differing density and therefore differing refractive index within the lens, which in turn would result in light-scattering and a decreased efficiency of light transmission through the lens. In addition, carbohydrate polyols have a much greater potential for forming extra hydrogen bonds to neighbouring atoms than does cyanate, which might also contribute to the observed differences in the effect of modification by the two ligands. The observed changes in conformation induced by non-enzymic chemical modification of γ-crystallin by G6P occur at ratios of less than 1 mol of G6P bound/mol of γ-crystallin and so it is likely that modifications of a single amino group results in the changes reported. As well as the effects of chemical modifications on the structural crystallins of the lens, it is possible that *in vivo* other proteins such as enzymes could be affected. Evidence already exists that modification of lens α-crystallin drastically affects its conformation and chemical properties, and this may partly explain the high risk of cataract associated with severe diarrhoea or renal failure (Harding & Rixon, 1980; Beswick & Harding, 1984; van Heyningen & Harding, 1986).

Diabetes is an established cataract risk factor. Work has been published suggesting that glycosylation of lens protein may play a role in cataractogenesis and other diabetic complications (Brownlee *et al.*, 1984). This paper provides new evidence in support of these ideas. Cataract in humans over the age of 60 probably has multifactorial origins, and the non-enzymic chemical modification of lens proteins almost certainly plays a role in cataractogenesis.

We are grateful to the Royal National Institute for the Blind for their support, and to Dr. S. R. Martin for producing the c.d. spectra.

REFERENCES

Berbers, G. A. H., Hoekman, W. A., Bloemendal, H., de Jong, W. W., Kleinschmidt, T. & Braunitzer, G. (1984) Eur. J. Biochem. **139**, 467–479
Beswick, H. T. & Harding, J. J. (1984) Biochem. J. **223**, 221–227
Beswick, H. T. & Harding, J. J. (1985) Biochem. J. **226**, 385–389
Björk, I. (1961) Exp. Eye Res. **1**, 145–154
Bloemendal, H. (1977) Science **197**, 127–138
Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnell, B. & Wistow, G. (1981) Nature (London) **289**, 771–777
Bours, J., Vornhagen, R., Herlt, M. & Rink, H. (1982) Curr. Eye Res. (1982) **1**, 651–658
Brownlee, M., Vlassara, H. & Cerami, A. (1984) Ann. Intern. Med. **101**, 527–537
Bucala, R., Gallati, M., Manabe, S., Cotlier, E. & Cerami, A. (1985) Exp. Eye Res. **40**, 853–863
Cerami, A. & Crabbe, M. J. C. (1986) Trends Pharmacol. Sci. **7**, 271–274
Day, J. F., Thorpe, S. R. & Baynes, J. W. (1979) J. Biol. Chem. **254**, 595–597
Delaye, M. & Tardieu, A. (1983) Nature (London) **302**, 415–417
Garlick, R. L. & Mazer, J. S. (1983) J. Biol. Chem. **258**, 6142–6146
Gonzalez, A. M., Sochor, M. & McLean, P. (1980) Biochem. Biophys. Res. Commun. **95**, 1173–1179
Harding, J. J. (1972) Biochem. J. **129**, 97–100
Harding, J. J. (1985) Adv. Protein Chem. **37**, 247–334
Harding, J. J. & Crabbe, M. J. C. (1984) in The Eye, vol. 1B (Davson, H., ed.), pp. 207–492, Academic Press, New York
Harding, J. J. & Dilley, K. J. (1976) Exp. Eye Res. **22**, 1–73
Harding, J. J. & Rixon, K. C. (1980) Exp. Eye Res. **31**, 567–571
Horwitz, J., Kabasawa, I. & Kinoshita, J. H. (1977) Exp. Eye Res. **25**, 199–208
Ikeda, K. & Hamaguchi, K. (1970) J. Biochem. (Tokyo) **68**, 785–794
Inana, G., Piatigorsky, J., Norman, B., Slingsby, C. & Blundell, T. (1983) Nature (London) **302**, 310–315
Kasai, K., Nakamura, T., Kase, N., Hiraoka, T., Suzuki, R., Kogure, F. & Shimoda, S.-I. (1983) Diabetologia **25**, 36–38
Liang, J. N. & Chakrabarti, B. (1982) Biochemistry **21**, 1847–1852
Liang, J. N. & Chylack, L. T. (1984) Biochem. Biophys. Res. Commun. **123**, 899–906
Mok, C. C. & Waley, S. G. (1968) Exp. Eye Res. **7**, 148–153
Monnier, V. M. & Cerami, A. (1983) Biochim. Biophys. Acta **760**, 97–103
Schoenmakers, J. G. G., den Dunnen, J. T., Moormann, R. J. M., Jongbloed, R., van Leen, R. W. & Lubsen, N. H. (1984) Ciba Found. Symp. **106**, 208–218
Sedlak, J. & Lindsay, R. H. (1968) Anal. Biochem. **25**, 192–197
Slingsby, C. & Croft, L. R. (1978) Exp. Eye Res. **26**, 291–304
Sredy, J. & Spector, A. (1984) Exp. Eye Res. **39**, 653–664
Steinbrecher, U. P. & Witztum, J. L. (1984) Diabetes **33**, 130–134
Stevens, V. J., Rouzer, C. A., Monnier, V. M. & Cerami, A. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2918–2922
Subramaniam, C. V., Radhakrishnamurthy, B. & Berenson, G. S. (1980) Clin. Chem. **26**, 1683–1687
Tomarev, S. I., Krayer, A. S., Skryabin, K. G., Bayer, A. A. & Gause, G. C. (1982) FEBS Lett. **146**, 315–318

van der Ouderaa, F. J. G., de Jong, W. W. & Bloemendal, H. (1974) Mol. Biol. Rep. **1**, 365–367

van Heyningen, R. & Harding, J. J. (1986) Lancet **1**, 1111–1113

van Kleef, S. M., Willems-Thijssen, W. & Hoenders, H. J. (1976) Eur. J. Biochem. **66**, 477–483

Walker, J. E. (1976) FEBS Lett. **66**, 173–175

Wistow, G., Turnell, B., Summers, L., Slingsby, C., Moss, D., Miller, L., Lindley, P. & Blundell, T. (1983) J. Mol. Biol. **170**, 175–202

Yu, N. T. (1977) Crit. Rev. Biochem. **4**, 229–280

Received 28 January 1987/31 March 1987; accepted 10 June 1987