γ III-Crystallin is the primary target of glycation in the bovine lens incubated under physiological conditions

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Several mechanisms have been proposed for the way in which glucose and its metabolites cause cataract, retinopathy and other complications of diabetes, the most convincing being glycation. Glycation, the reaction of sugars with free amino groups of proteins, is one of a variety of non-enzymic post-translational modifications. The aim of the present study was to identify some of the most reactive proteins in the lens when incubated under physiological conditions. Fresh intact bovine lenses were incubated with [¹⁴C]glucose in a conventional tissue-culture medium with added antibiotics. After 3 and 6 days of incubation, the water-soluble proteins were separated by size-exclusion chromatography. Glycated proteins from the water-soluble fractions were separated by using a sugar affinity column (Affi-Gel 601). Then the radioactive fractions were identified on SDS/polyacrylamide gels. In addition, the whole bovine lenses were incubated with 10 mM fructose and glucose for 3 and 6 days. The glycated proteins from the water-soluble fractions in parallel with the radioactive fractions were separated by affinity chromatography, and were identified further by amino-acid sequencing. A progressive uptake of radioactive label showed that the majority of proteins incorporating both glucose and fructose were water-soluble fractions. Chromatography and SDS/polyacrylamide gel results showed that α - and γ -crystallin and some proteins of a mean molecular mass of 36–37 kDa incorporated sugars early during incubation. After 6 days of incubation, more crystallins were glycated compared with 3 days, in particular β -crystallin. Affinity-chromatography results indicated that proteins with subunit

masses of 36 kDa and 20 kDa were possibly radiolabelled at an early stage. The purified glycated proteins following incubation with both glucose and fructose, which corresponded to 20 kDa and 36 kDa bands on SDS/polyacrylamide gels, were sequenced by Edman degradation. N-terminal sequences of both 20 kDa bands were Gly-Lys-Ile-Thr, characteristic of γ -crystallins, but the N-termini of both 36 kDa bands were blocked. Further sequencing after digestion of 36 kDa bands with trypsin and running on HPLC revealed that the glucose sample gave the peptide sequences as Gly-Glu-Tyr-Pro-Asp-Tyr-Gln-Gln and Tyr-Glu-Leu-Pro-Asn-Tyr-Arg, which match with bovine γ IIIb-crystallin. The peptide sequence Tyr-Glu-Leu-Pro-Asn-Tyr-Arg is only present in the published sequence of bovine γ IIIb-crystallin and not in any other type of γ -crystallin. The fructose sample gave the peptide sequences Ile-Thr-Phe-Tyr-Glu-Asp-Arg, Arg-Gly-Asp-Tyr-Pro-Asp-Tyr-Gln-Gln-Trp, Gln-Tyr-Leu-Leu-Arg and Val-Val-Asp-Leu-Tyr, which all matched with bovine γ IIIa-crystallin. The sequence Val-Val-Asp-Leu-Tyr only appears in the sequence of bovine γ IIIa-crystallin. γ III-Crystallin is the most susceptible lens protein to glycation. The primary target of glucose is γ IIIb-crystallin, whereas that of fructose is γ IIIa-crystallin. The early glycation of γ III-crystallin by glucose and fructose could result in structural alterations, leading to aggregation of crystallin and eventually cataract formation.

Key words: cataract, fructose, glucose, glycation, lens, post-translational modification.

INTRODUCTION

Cataract, the major cause of blindness worldwide, is associated with conformational changes and the unfolding of proteins in the lens [1]. Glycation, the non-enzymic reaction of proteins with sugars, is a post-translational protein modification that has been implicated in a variety of age- and diabetes-related complications, including cataract. It is of fundamental importance in aging and in pathological conditions where sugar levels are elevated. Sugars cause the unfolding, aggregation and cross-linking of proteins, resulting in destabilization of the protein structure allowing further modification. Since the proteins of the lens possess very long lives, post-translational modification by sugars can modify almost all proteins given the opportunity and the time.

Lens crystallins, α -, β -, and γ -crystallins, are abundant major proteins in the lens and have a crucial role in maintaining the transparency of the lens and its refractive properties. Their post-translational modification has been proposed as an index of aging

and cataractogenesis [2]. Most studies have focused on glycation of lens crystallins by using high concentrations of sugars. γ -Crystallin was shown to be more susceptible to glycation than α - and β -crystallin by *in vitro* glycation of rat lens soluble fraction with 50 mM glucose or glucose 6-phosphate for > 5 days, and in streptozotocin-diabetic rats during various stages of cataract development [3]. α -Crystallin and β -crystallin may be modified progressively with increasing duration of glycation [3,4]. Using bovine lenses (prenatal, newborn, adult and aged) van Boekel and Hoenders [5] accumulated evidence that all categories of crystallin are glycated, although not to the same extent, and suggested that early glycation products are derived mainly from γ -crystallin. The α A subunits of α -crystallin seem to be glycated to a higher extent compared with the α B subunits.

The glycation sites of the major lens crystallins have been identified through *in vitro* glycation studies, including α A, α B [6], β B₂ [7] and γ B [8]. Lys¹¹ and Lys⁷⁸ of α A-crystallin and Lys⁹⁰ and/or Lys⁹² of α B-crystallin were the fast-reacting glycation

Abbreviations used: MEM, minimal essential medium; TCA, trichloroacetic acid; td, elution time; TFA, trifluoroacetic acid.

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sites [6]. Glucose and fructose have similar glycation specificity on β B₂-crystallin. These were at lysine residue positions 10, 75, 100, 107, 120, 139, 167 and 171 by both glucose and fructose, whereas glycated lysine residues 119 and 47 or 67 were detected only after fructation [7]. The primary site of glycation of bovine γ B-crystallin by fructose was identified as the α -amino group of the N-terminal glycine [8].

Following studies on the major structural proteins of the lens, we have turned our attention to an extensive complement of the normal housekeeping enzymes in lens that may be more susceptible to modification because they have not been selected to be long-lived proteins. Glycation inactivates a number of enzymes *in vitro* [9–13]. Some dehydrogenases in bovine lens homogenates may be targeted preferentially by fructose (C. Napper and J. J. Harding, unpublished work). More recently, Swamy-Mruthinti [14] reported that glycation decreased calmodulin binding to lens transmembrane protein, the major intrinsic protein, which may affect the Ca²⁺-mediated cellular processes leading to lens opacification. The glycation of enzymes and some receptor proteins in the lens may have a much more important role than glycation of structural proteins in the development of cataract.

Glycation of structural proteins or enzymes, as may occur in diabetic or aging lenses, could make those proteins more susceptible to further modifications. To provide an insight into the primary targets of lens proteins in the process of post-translational modification, we attempted to identify the proteins preferentially modified by glycation. We have used fresh bovine lenses for this work, as they are readily available shortly after death. Earlier studies showed that α -crystallin and β -crystallin were glycated at an earlier stage by using homogenates of bovine lens incubated with 10 mM fructose. Affi-Gel results indicated a protein of molecular mass 20 kDa, which could correspond to an α - or γ -crystallin, and a band of 35 kDa, which could be an enzyme, preferentially targeted by glycation (C. Napper and J. J. Harding, unpublished work). The goal of the present study, and a major difference from the earlier studies on glycation, was to identify primary target proteins in lens by using whole intact lens incubated with lower concentrations of sugars, together with radiolabelled sugars as tracers under physiological conditions. After being isolated by size-exclusion chromatography and further affinity column separation, the preferentially modified proteins with higher radioactivity were identified by amino-acid-sequence analysis.

MATERIALS AND METHODS

Materials

Bovine lenses were obtained from a local abattoir. Affi-Gel 601, Econo-Pac Blue prepacked Cartridge and Silver Stain Plus Kit were obtained from Bio-Rad Laboratories (Hemel Hempstead, U.K.). Sephacryl S-300HR, [¹⁴C]glucose and [¹⁴C]fructose were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Liquid Scintillation Cocktail [EcoLite(+)TM] was obtained from ICN Pharmaceuticals (Basingstoke, U.K.). Fructose, glucose and all other chemicals were obtained from Sigma (Poole, Dorset, U.K.) or BDH–Merck (Lutterworth, Leics., U.K.) and were of the highest grade available.

Incubation of lens with ¹⁴C-labelled or unlabelled glucose and fructose

The fresh bovine lenses were dissected out within 4–5 h of slaughter. The incubation solutions were based on a conventional

tissue culture minimal essential medium (MEM) with the following ionic composition (mM): CaCl₂, 1.0; KCl, 5.4; MgSO₄, 1.7; NaCl, 136.4; NaH₂PO₄, 1.2, buffered at pH 7.4 with 10 mM Hepes, with added antibiotics (Penicillin, Streptomycin and Amphotericin B) [15,16]. In preliminary incubations, we tried different concentrations of sugars and incubation times to determine which conditions would provide enough incorporation of radiolabelled sugar for measurement of radioactivity in the lens proteins. We also tried whole bovine lenses incubated with 50 μ Ci of [¹⁴C]fructose and 10 mM unlabelled fructose in a final volume of 10 ml of MEM for 24 h in a 37 °C shaking waterbath, and with 10 mM glucose and 100 μ Ci of [¹⁴C]glucose in a final volume of 10 ml of MEM for 3 days in a 37 °C shaking waterbath, but insufficient radioactivity was incorporated. To increase radiolabelling in the experiments described below, only [¹⁴C]glucose was used, at 100 μ Ci and 250 μ Ci. In the first incubation, a bovine lens was incubated with 100 μ Ci of [¹⁴C]glucose without any unlabelled glucose in a final volume of 9 ml for 3, 6 and 9 days in a 37 °C shaking waterbath.

In the second incubation, in order to get enough label incorporated into lens proteins from radiolabelled sugar, two lenses in each cell were incubated with 250 μ Ci of [¹⁴C]glucose (1.25 ml) in a final volume of 10 ml of MEM with 100 μ l of antibiotics added after 0 and 3 days under the same conditions as above. In additional experiments, bovine lenses were incubated with 10 mM fructose and 10 mM glucose without radiolabelled sugars in a final volume of 10 ml of MEM.

At the end of each incubation, the lenses were removed from the medium, and were then weighed in pre-weighed polythene pots. The lenses were washed with MEM at least five times to remove free radiolabelled sugar.

Isolation of crystallins

Crystallins were isolated by the method of Slingsby and Bateman [17] using Sephacryl S-300HR columns (usually 120 cm $\times$ 2.7 cm, but 100 cm $\times$ 3.7 cm where stated). Decapsulated bovine lenses were homogenized gently in 7 vol. of 50 mM sodium phosphate buffer, pH 6.7, containing 0.2 M KCl in a hand-operated glass homogenizer. After centrifugation at 22 440 *g* for 40 min at 4 °C, the supernatant was loaded on to the column and eluted at 30 ml/h. Fractions of 5 ml were collected. The fractions corresponding to the radioactivity peaks were pooled and were dialysed against three changes of distilled water at 4 °C, then freeze-dried and stored at –20 °C.

Radioactivity measurements

The protein-bound radioactivity was determined in a liquid scintillation spectrometer. Samples (100 μ l of total proteins/soluble proteins) were taken into 1.0 ml of 10 % (v/v) TCA (trichloroacetic acid), and were left overnight at 4 °C to precipitate the protein. The following morning, residues were collected on Whatman GF/C microfibre filter discs under vacuum, and washed with 30 ml of 5 % (v/v) TCA. Blanks consisted of filter papers washed with 30 ml of 5 % (v/v) TCA. Each disc was placed into a scintillation vial and dried at 70 °C for 30 min before adding 4.0 ml of EcoLite(+)TM. The total amount of radioactivity was measured in a scintillation counter.

Three 50 μ l aliquots of the soluble proteins (supernatants) were removed and added to 4.0 ml of scintillation fluid in mini scintillation vials and the number of [¹⁴C]glucose disintegrations per unit time were measured using a scintillation counter in order to confirm the total amount of radiolabelled glucose in the solutions.

Separation of dehydrogenases

The freeze-dried protein fractions with the highest radioactivity after size-exclusion chromatography were separated further on a 5 ml Econo-Pac DEAE Affi-Gel blue prepacked cartridge using an Econo™ Gradient Pump (Bio-Rad). The cartridge was equilibrated with degassed 50 mM potassium phosphate buffer (pH 7.1). A 1 ml sample of each fraction (5–12 mg/ml) was loaded onto the column at a flow rate of 60 ml/h and fractions (1.5 ml) were collected. A volume of 15 ml of degassed application buffer (50 mM potassium phosphate buffer, pH 7.1) was washed through the column, followed by a NaCl gradient of 50 ml (0.1–1.0 M NaCl) with application buffer. Then 1 M NaCl with 10 ml of application buffer was run through, followed by 20 ml of application buffer at 120 ml/min. Absorbances were then measured at 280 nm and the radioactivity of each fraction was determined using the method above. Protein peaks (A_{280}) and the area with a high radioactivity were pooled. After dialysis and freeze-drying, pooled fractions were kept for SDS/PAGE.

Separation of glycated proteins

Glycated and non-glycated proteins were separated by affinity chromatography on a pre-equilibrated Affi-Gel 601 column (27 cm × 0.7 cm) with the degree of incorporation being monitored in a parallel incubation, which contained 250 μ Ci of [14 C]glucose. The freeze-dried material was re-dissolved in 3–4 ml of column buffer (0.125 ammonium acetate, pH 8.5). The bound proteins were eluted first with 50 ml of 0.2 M ethanoic acid at a rate of 25 ml/h and 3 ml fractions were collected, and then bound proteins were eluted further with 50 ml of 0.5 M ethanoic acid. The absorbance of column fractions was determined at 280 nm and 50 μ l was taken from each fraction to measure radioactivity (see above). Fractions contributing to peaks with higher absorbance at 280 nm and radioactivity were freeze-dried and then were examined by SDS/PAGE.

SDS/PAGE

SDS/PAGE was performed according to Laemmli [18] under reducing conditions using a Mini-PROTEAN II Dual Slab Cell Electrophoresis Unit (Bio-Rad). A 12% (w/v) polyacrylamide running gel and a 4% (w/v) polyacrylamide stacking gel were used. Protein (2 mg/ml) was dissolved in the sample buffer containing 5% (w/v) 2-mercaptoethanol and was then incubated in a boiling water bath for 5 min. Both Coomassie Brilliant Blue stain (R-250; Merck) and silver stain were used to detect the polypeptide bands, then gels were scanned and photographed. Protein in the bound fractions was identified further by amino-acid sequencing.

Amino-acid-sequence analysis

The amino-acid-sequence analysis was performed as previously described [19]. Samples of the bound fraction, 30 μ l (2 mg/ml), were loaded on a 4–12% (w/v) NuPAGE gel run with Mes buffer before blotting on to a PVDF filter for N-terminal sequencing. The bands of interest were excised and cut into pieces, then subjected to sequencing on an Applied Biosystems Procise 494A.

In an attempt to identify the N-terminally blocked proteins, they were digested with trypsin and then sequenced on the Applied Biosystems Procise 494A protein sequencer. The upper bands (36 kDa) from bound fractions were excised and digested *in situ* with bovine trypsin [sequencing grade, modified to minimize autolysis (catalogue number 1418025); Roche Diagnostics,

Table 1 Effect of dialysis on radioactivity incorporated into supernatants

'Before' and 'after' refer to dialysis with four changes of purified water (5 litre each time). Values refer to radioactivity in supernatants and washings of water-soluble proteins. The values at 6 days were obtained in a separate experiment. For each counting (c.p.m.), 50 μ l was used.

	Radioactivity (c.p.m.)		
	3 days	6 days	9 days
Before (B)	35 330 050 $\pm$ 925 750	15 762 950 $\pm$ 189 000	26 349 750 $\pm$ 200 200
After (A)	1 195 200 $\pm$ 45 200	620 500 $\pm$ 5000	1 116 000 $\pm$ 32 400
A/B	3.4 %	3.9 %	4.2 %

Lewes, U.K.] for 16 h at 37 °C. The digested fragments were then run on microbore HPLC (Aquapore C18 column, 150 mm × 0.5 mm) to separate the derived peptides using a TFA (trifluoroacetic acid)/acetonitrile gradient and collecting elutant directly on to a PVDF membrane (HPLC was an Applied Biosystems 173A microbore system with integral blotter). Using this system, the chromatogram from the chart recorder was directly compared with the piece of PVDF membrane containing the blotted peptides and peptides of interest were excised, treated with Polybrene® and sequenced on an Applied Biosystems Procise 494A protein sequencer using standard peptide cycles.

RESULTS

Reaction of lenses with radiolabelled sugars

Incubation with 100 μ Ci of [14 C]glucose

The bovine lens was incubated with 100 μ Ci of [14 C]glucose for 3, 6 and 9 days, i.e. longer than in preliminary experiments. The radioactivity of each portion from bovine lens is shown in Table 1. The incorporation of label into protein from both homogenates and supernatants increased over this time, and the majority of the proteins incorporating glucose were water-soluble, confirming what had been found in preliminary experiments with 10 mM sugar (results not shown).

The vast majority of counts in supernatants plus washes of water-insoluble fractions were free radiolabelled glucose (Table 1). The counts after dialysis indicate that there was little benefit in continuing the incubation beyond 3 days.

The supernatants were then separated by size-exclusion chromatography. The elution profile of supernatants from whole lens after 3, 6 and 9 days of incubation lenses on a Sephacryl S-300HR column are shown in Figures 1–3. At 3 days, the major radioactivity was associated with α - and γ S-crystallin fractions (Figures 1). However, the distribution seemed different with labelling of γ BC-crystallin at 6 and 9 days, and more radioactivity went into α _L-crystallin (Figures 2 and 3). It appeared that α - and γ S-crystallin were attacked by glucose at an early stage (3 days of incubation; Figure 1). The loss of this labelling after 6 and 9 days is presumably due to aggregation of the glycated protein (Figures 2 and 3).

After separation by size-exclusion chromatography, the pooled fractions corresponding to the higher radioactivity were dialysed and freeze-dried and then loaded onto an Affi-Gel Blue column to bind dehydrogenases. However, there was still no significant detectable radioactivity in bound fractions after being run on the Blue column, indicating that dehydrogenases were not the primary target(s) of glycation in this experiment (results not shown), although earlier results on lens homogenates showed that dehydrogenases incorporated radiolabel from glucose at an early

Figure 1 Elution profile of whole bovine lens supernatant, incubated for 3 days with 100 µCi of [¹⁴C]glucose, on a Sephacryl S-300HR column (100 cm × 3.7 cm)

Protein (solid line) and radioactivity (broken line) are shown. Crystallins are indicated above the relevant peaks.

Figure 2 Elution profile of whole bovine lens supernatant, incubated for 6 days with 100 µCi of [¹⁴C]glucose, on a Sephacryl S-300HR column (100 cm × 3.7 cm)

Protein (solid line) and radioactivity (broken line) are shown. Crystallins are indicated above the relevant peaks.

stage (C. Napper and J. J. Harding, unpublished work). The much lower amounts of enzymes compared with the major structural proteins (e.g. crystallins) are difficult to detect. SDS/PAGE results showed no remarkable difference between bound and unbound fractions corresponding to the first higher radioactivity peak (α -crystallin), and there were no visible bands in the bound fraction corresponding to the second higher radioactivity (γ -crystallin), according to the elution profile on a Sephacryl S-300HR column (Figures 1–3) by using both Coomassie Blue and silver stain methods (results not shown). Autoradiography after 8 weeks showed negative results.

Incubation with 250 µCi of [¹⁴C]glucose

In order to increase the amount of [¹⁴C]glucose incorporated by lens proteins, the next incubation used a higher amount of radiolabelled glucose (250 µCi). The radioactivity in the supernatant from bovine lens after 3 and 6 days of incubation is shown

Figure 3 Elution profile of whole bovine lens supernatant, incubated for 9 days with 100 µCi of [¹⁴C]glucose, on a Sephacryl S-300HR column (100 cm × 3.7 cm)

Protein (solid line) and radioactivity (broken line) are shown. Crystallins are indicated above the relevant peaks.

Table 2 Radioactivity of different portions of bovine lens incubated with 250 µCi of [¹⁴C]glucose after 3 and 6 days (precipitated counting)

Portion of lens	Radioactivity (c.p.m.)	
	3 days	6 days
Supernatant	381 054 ± 5494	656 994 ± 16 146
Water-insoluble	82 822 ± 16 450	199 547 ± 19 937
Washing of water-insoluble	28 160	65 000

in Table 2. It appeared that the most radioactivity was in the supernatant. The counts in the supernatant from 6 days were greater than those from 3 days, suggesting further a progressive uptake of radioactive label.

Elution profiles of water-soluble proteins and radioactivity after 3 and 6 days of incubation with 250 µCi of [¹⁴C]glucose are shown in Figures 4 and 5. The fractions corresponding to γ - and α -crystallin had incorporated most radiolabelled glucose, but the counts were low. There was higher radioactivity in γ -crystallin at 6 days than at 3 days (Figure 4). The separation of some dehydrogenases was attempted on a Affi-Gel Blue column, but the bound fractions showed no detectable radioactivity. We then tried an Affi-Gel 601 column to separate glycosylated proteins from non-glycosylated proteins.

The two higher radioactivity fractions (γ - and α -crystallin) from Sephacryl chromatography (Figures 4 and 5) were pooled and freeze-dried, and then loaded on to an Affi-Gel 601 column to separate glycosylated proteins from non-glycosylated proteins. The elution profile showed that the vast majority of proteins corresponding to higher radioactivity were unbound (results not shown). The radioactivity of the bound fraction separated from α -crystallin was too low to be detected, but there was a very small amount of radioactivity from γ -crystallin in samples after both 3 and 6 days of incubation (results not shown), which might be from some free [¹⁴C]glucose in the γ -crystallin fractions. SDS/PAGE results showed that bound and unbound fractions presented almost the same bands, except for the bound fraction separated from α -crystallin at 6 days. It revealed a 36 kDa band in the bound fraction (results not shown).

Figure 4 Elution profile of supernatant from whole bovine lens, incubated for 3 days with 250 μCi of [^{14}C]glucose, on a Sephacryl S-300HR column (120 cm $\times$ 2.7 cm)

Protein (solid line) and radioactivity (broken line) are shown. Crystallins are indicated above the relevant peaks.

Figure 5 Elution profile of supernatant from whole bovine lens, incubated for 6 days with 250 μCi of [^{14}C]glucose, on a Sephacryl S-300HR column (120 cm $\times$ 2.7 cm)

Protein (solid line) and radioactivity (broken line) are shown. Crystallins are indicated above the relevant peaks.

In order to increase the chances of getting enough glycosylated proteins labelled by 250 μCi of [^{14}C]glucose, the supernatants from 3 and 6 days of incubation were loaded directly on to an Affi-Gel 601 column. The elution profiles are shown in Figures 6 and 7. Most radioactivity was in the bound fractions, whereas most protein (A_{280}) was in the unbound fractions, as expected. After 6 days, only the second bound fraction had increased. It is possible that the free radiolabelled sugars caused this increased radioactivity in the later stage of elution. The first bound fraction had the same radioactivity as after 3 days, suggesting again that there was little benefit in continuing the incubation beyond 3 days.

SDS/PAGE results showed that proteins had subunits with a 20 kDa band and a 36 kDa band (Figure 8). The 20 kDa band corresponds to α -crystallin and γ -crystallin, whereas the 36 kDa band could be a dehydrogenase subunit or dimer of γ -crystallin, which need to be identified further by amino-acid sequencing. Autoradiography showed negative results again.

Figure 6 Elution profile of supernatant (2 ml) of bovine lens, incubated for 3 days with 250 μCi of [^{14}C]glucose, on an Affi-Gel 601 column

Protein (solid line) and radioactivity (broken line) are shown. A, 0.125 M ammonium acetate; B, 0.2 M ethanoic acid; C, 0.5 M ethanoic acid.

Figure 7 Elution profile of supernatant of bovine lens, incubated for 6 days with 250 μCi of [^{14}C]glucose, on an Affi-Gel 601 column

Protein (solid line) and radioactivity (broken line) are shown. A, 0.125 M ammonium acetate; B, 0.2 M ethanoic acid; C, 0.5 M ethanoic acid.

In order to have enough glycosylated proteins with non-radiolabelled sugars in parallel with radiolabelled ones as described above in lens, bovine lenses were incubated with 10 mM glucose and 10 mM fructose without any radiolabelled sugars for 3 and 6 days. The water-soluble proteins were loaded on an Affi-Gel 601 column under the same conditions as above. The unbound and bound proteins were then separated (Figure 9). Again, the great majority of the protein was unbound and came straight through the column. Bound and unbound fractions were pooled even where the absorbance trace showed no sign of the unbound fraction. SDS/PAGE showed that bound (glycosylated) fractions contained mainly 20 kDa and 36 kDa bands (Figure 10), which were the same as the results of incubation with radiolabelled sugars (Figure 8). SDS/PAGE analysis of the radiolabelled and non-radiolabelled bound fractions indicated that the majority of glycosylated fractions consist of 36 kDa and 20 kDa polypeptides (Figures 8 and 10). There was relatively high radioactivity in the bound fractions (Figures 6 and 7), indicating that the bound fractions are targeted at an early stage. The parallel experiments with non-radiolabelled

Figure 8 SDS/PAGE of water-soluble fraction from bovine lens, incubated with 250 μ Ci of [14 C]glucose, after affinity chromatography on an Affi-Gel column

(A) Coomassie-Blue stain. (B) Silver stain. Lane 1, molecular mass standard markers; lane 2, unbound fraction after 3 days of incubation; lane 3, bound fraction after 3 days of incubation; lane 4, molecular mass standard markers; lane 5, unbound fraction after 6 days of incubation; lane 6, bound fraction after 6 days of incubation.

Figure 9 Elution profile of supernatant of bovine lens, incubated for 3 days with 10 mM glucose, on an Affi-Gel 601 column

Similar profiles were obtained for the supernatants after incubation with glucose for 6 days and for the supernatants after incubation with 10 mM fructose for 3 and 6 days (results not shown). A, 0.125 M ammonium acetate; B, 0.2 M ethanoic acid; C, 0.5 M ethanoic acid.

Figure 10 SDS/PAGE of water-soluble fraction from bovine lens, incubated with 10 mM glucose and 10 mM fructose, after affinity chromatography on an Affi-Gel column

(A) Incubation for 3 days. (B) Incubation for 6 days. Lane 1, molecular mass standard markers; lane 2, unbound fraction with glucose; lane 3, bound fraction with glucose; lane 4, second bound fraction with glucose; lane 5, molecular mass standard markers; lane 6, unbound fraction with fructose; lane 7, bound fraction with fructose; lane 8, second bound fraction with fructose.

sugars further suggest that the 20 kDa and 36 kDa bands are the proteins targeted primarily. The identification of these glycosylated proteins (of approx. molecular mass 20 kDa and 36 kDa) was pursued by amino-acid sequencing.

Identification of glycosylated proteins by sequencing

In an attempt to compare the primary targets of different sugars, the purified proteins glycosylated by both glucose and fructose, which correspond to 20 kDa and 36 kDa bands on SDS/polyacrylamide gels, were sequenced by Edman degradation and two N-termini were identified. Only peptide sequences from the 20 kDa bands were obtained and proved to be γ -crystallin, as both the N-terminal sequences were Gly-Lys-Ile-Thr.

Although the 20 kDa bands were the most abundant components in both bound fractions after separation by affinity chromatography, the identity of the upper bands (36 kDa) would be much more interesting as they represent minor proteins, possibly enzymes, in the lens. However, they appeared to have a blocked N-terminus. Thus upper bands from both glucose- and fructose-treated lenses were digested by trypsin after separation by SDS/PAGE. They were then run further on microbore HPLC to separate the derived peptides [Aquapore C18 column (150 mm $\times$ 0.5 mm) using a TFA/acetonitrile gradient]. The chromatography of fractions glycosylated by glucose is shown in Figure 11.

The glucose sample peaks were taken at 72.3 min and 79.5 min for sequencing. The peak glucose elution time (td) 72.3 min (arrow a in Figure 11) gave the sequences Gly-Glu-Tyr-Pro-Asp-Tyr-Gln-Gln and Tyr-Glu-Leu-Pro-Asn-Tyr-Arg. The peak

Figure 11 Chromatography of digested 36 kDa bands from fractions glycated by glucose, after Affi-Gel 601 column separation, on an Aquapore C18 column

The digested peptides were run on microbore HPLC (Aquapore C18 column, 150 mm $\times$ 0.5 mm) to separate the derived peptides using a TFA/acetonitrile gradient and collecting eluant directly on to a PVDF membrane (HPLC was an Applied Biosystems 173A microbore system with integral blotter). Arrows a and b represent the samples that were sequenced. Peak a gave the sequences Gly-Glu-Tyr-Pro-Asp-Tyr-Gln-Gln and Tyr-Glu-Leu-Pro-Asn-Tyr-Arg. Peak b gave no assignable sequences due to its heterogeneity.

Figure 12 Identification of peptides obtained from glucose-glycated bovine lens protein as being derived from γ IIIb-crystallin

(a) Amino-acid sequence of bovine γ IIIb-crystallin, from SwissProt. (b) Identified peptide sequences from glucose samples of bovine γ IIIb-crystallin. Highlighted residues indicate matching sequences of bovine γ III-crystallin. The asterisk (*) indicates a residue (Glu) that was not matched in the sequence analysis.

glucose td 79.5 min (arrow b in Figure 11) gave no assignable sequences due to its heterogeneity.

Both the sequences for the peak taken at 72.3 min can be identified in the database (SwissProt) as being from γ -crystallin. The first is not an exact match and corresponds to four different bovine γ -crystallins, but the Tyr-Glu-Leu-Pro-Asn-Tyr-Arg is found only in γ IIIb-crystallin (Figure 12), also known as γ D-crystallin [20], thus clearly identifying this protein as a primary target of glycation by glucose in bovine lens.

The sequence Tyr-Glu-Leu-Pro-Asn-Tyr-Arg, identified in the glucose sample, has only been reported in the bovine γ IIIb-crystallin sequence [20].

The fructose sample gave peaks which eluted at 64.7 min (Figure 13, arrow a) and 70.4 min (Figure 13, arrow b). The peak eluted at 64.7 min gave the sequences Gly-Tyr-Leu-Leu-Arg, possibly Gln-Tyr-Leu-Leu-Arg, Val-Val-Asp-Leu-Tyr and Ile-Thr-Phe-Tyr-Glu-Asp-Arg. The peak eluted at 70.4 min gave the sequences Gly-Tyr-Leu-Leu-Arg, possibly Gln-Tyr-Leu-Leu-Arg and Arg-Gly-Glu-Tyr-Pro-Asp-Tyr-Gln-Gln-Trp. Sequence similarities are apparent in both fructose peaks. However, many peptides still formed a plateau that may cause assignment problems. Even in the peak that eluted at 72.3 min, there were other unassignable sequences. The sequences of possibly identified proteins are shown in Figure 14.

The sequence Val-Val-Asp-Leu-Tyr only appears in reported bovine γ IIIa-crystallin [20]. Gln-Tyr-Leu-Leu-Arg is present in all γ -crystallins, unlike Gly-Tyr-Leu-Leu-Arg, indicating that Gln-Tyr-Leu-Leu-Arg is probably the identified peptide sequence.

Figure 13 Chromatography of digested 36 kDa bands from fractions glycated by fructose, after Affi-Gel 601 column separation, on an Aquapore C18 column

The digested peptides were run on microbore HPLC (Aquapore C18 column, 150 mm $\times$ 0.5 mm) to separate the derived peptides using a TFA/acetonitrile gradient and collecting eluant directly on to a PVDF membrane (HPLC was an Applied Biosystems 173A Microbore system with integral blotter). Arrows a and b represent the samples that were sequenced. The peak fructose td 64.7 min (arrow a) gave the sequences Gln-Tyr-Leu-Leu-Arg, Val-Val-Asp-Leu-Tyr and Ile-Thr-Phe-Tyr-Glu-Asp-Arg. The peak fructose td 70.4 min (arrow b) gave the sequences Gln-Tyr-Leu-Leu-Arg and Arg-Gly-Glu-Tyr-Pro-Asp-Tyr-Gln-Gln-Trp.

Figure 14 Identification of peptides obtained from fructose-glycated bovine lens protein as being derived from γ IIIa-crystallin

(a) Amino-acid sequence of bovine γ IIIa-crystallin, from SwissProt. (b) Identified peptide sequences from fructose samples of bovine γ IIIa-crystallin. Highlighted residues indicate matching sequences of bovine γ III-crystallin. The asterisk (*) indicates a residue (Glu) that was not matched in the sequence analysis.

The present data suggested that the primary protein in lens glycated by glucose is probably γ IIIb-crystallin, but that fructose initially glycates γ IIIa-crystallin.

DISCUSSION

The present sequencing of glycated bovine lens proteins revealed that the protein initially glycated by glucose is γ IIIb-crystallin, identified by the peptide sequences Gly-Asp-Tyr-Pro-Asp-Tyr-Gln-Gln and Tyr-Glu-Leu-Pro-Asn-Tyr-Arg. The latter sequence is only present in the published sequence of bovine γ IIIb-crystallin and not in the other types of γ -crystallin, supporting the match with bovine γ IIIb-crystallin. However, proteins from the bovine lenses incubated with fructose gave peptide sequences Ile-Thr-Phe-Tyr-Glu-Asp-Arg, Arg-Gly-Asp-Tyr-Pro-Asp-Tyr-Gln-Gln-Trp, Gln-Tyr-Leu-Leu-Arg and Val-Val-Asp-Leu-Tyr, which were matched with bovine γ IIIa-crystallin, since the sequence Val-Val-Asp-Leu-Tyr is unique to bovine γ IIIa-crystallin [20].

These sequences relate to early sequences reported by Hay et al. [20] and deposited in SwissProt, but these deposited sequences contain a glutamic residue in two peptides where we have identified an aspartic residue. Hay's sequences of γ III- and γ IV-crystallin were assembled from partial recombinant bovine γ -crystallin sequence evidence [20]. Only the first 29 and 45 residues of the native γ IIIa and γ IIIb polypeptides respectively were derived from sequential Edman degradation and were supported

by the co-linearity of their sequences with those predicted from the γ C- and γ D-crystallin genes. The discrepancy between the present and the published sequences was present in residue 61.

Furthermore, the experimental masses obtained for bovine γ IIIa- and γ IVa-crystallins by electrospray MS did not correspond to the theoretical masses calculated from the published sequences [20,21]. The theoretical mass of bovine γ IIIa-crystallin was calculated as 20743 Da and that of γ IIIb-crystallin as 20749 Da from sequences. The MS of γ III-crystallin yielded a major peak at 20748 Da, which is close to the theoretical mass of both γ IIIa- and γ IIIb-crystallin. A second peak corresponded to a mass of 21 009 Da, which is 266 Da greater than γ IIIa-crystallin and 260 Da greater than γ IIIb-crystallin. The mass of isolated γ IIIb-crystallin [21,22] corresponded to the calculated mass of the recombinant γ D-crystallin, supporting the assignment by Hay et al. [20]. The greater mass could not result from any probable post-translational modification, but is more likely to be due to errors in the published sequence. The multiple errors may be throughout the sequence [1,22]. We conclude that an error of residue 61 may be present in the published sequence, that is a glutamic residue should be an aspartic residue in peptides (Gly-Asp-Tyr-Pro-Asp-Tyr-Gln-Gln) of bovine γ IIIa-crystallin and γ IIIb-crystallin. These errors in the database may subsequently lead to confusion, particularly in the identification of γ -crystallin. Encouragingly, the correct sequence of γ IVa-crystallin has now been determined [1].

It is probable that γ IIIa-crystallin is not an abundant protein in the bovine lens and it has been incorrectly assigned [21]. The results of the present study suggest that the primary targets of glycation in the bovine lens are both γ IIIa-crystallin and γ IIIb-crystallin. The identified γ III-crystallins at 36 kDa are possibly a glycation-induced dimer of the major 18–20 kDa γ -crystallins. However, the reason for the difference in targets of glucose and fructose remains unclear.

The γ IIIb-crystallin peptides identified in this work come from the inter-domain region of this two-domain protein, and from a contact region between molecules in the crystal [23]. The γ -crystallins have a group of conserved arginine residues in the inter-domain region, including Arg¹⁴² identified here in peptide Tyr-Glu-Leu-Pro-Asn-Tyr-Arg¹⁴² (Figure 12). In the crystal, the sequence Pro-Asp-Tyr-Gln-Gln, identified in the present study, interacts with the surface of a second symmetry-related molecule [23].

The water-soluble proteins incorporating both glucose and fructose at an early stage under the conditions described are mainly γ -crystallins, which is consistent with studies *in vitro*, indicating that γ -crystallin is an extremely vulnerable target for glycation, even in the presence of sugars at a lower concentration [3]. This may appear surprising as α - and β -crystallin have more lysine residues than γ -crystallin, and are found in the lens cortex, whereas γ -crystallin is largely confined to the central nucleus; however, α - and β -crystallin have blocked α -amino groups, and in γ -crystallin this is the site for preferential glycation [8]. γ -Crystallins, one of the major crystallin groups in the mammalian lens, are a subgroup of $\beta\gamma$ -crystallins, although homology between β - and γ -crystallin is weak [1]. Three-dimensional structures have been reported for many γ -crystallins that are characterized by two domains with paired Greek key motifs. γ -Crystallins, monomers of 20 kDa (except for γ s-crystallin), are synthesized in the early stages of development and are abundant in the lens nucleus, where the very long-lived structural proteins are found. Post-translational modifications, including glycation, are generally slow reactions, which have a more damaging effect on γ -crystallins. Regarding the lysine content of the different crystallins, γ -crystallin showed relatively

high levels of early glycation products in the aging of bovine lens proteins (prenatal, calf and up to 20 years old) [5]. However, there is no significant conformational change of γ -crystallin resulting from N ϵ -(carboxymethyl)lysine formation, an advanced glycation end product in the human lens, but its conformational stability decreased [24]. The lens nucleus is rich in γ -crystallin and its stable conformation can withstand most insults and the γ -crystallin remains soluble. Glycation of γ -crystallins could result in conformational changes, which in turn, could lead to aggregates or cross-linking, and eventually cataract formation. γ IIIb-Crystallin, known as γ D-crystallin, was found in mutated forms in two human cataracts [25,26]. In one, it appears that the mutant has the normal fold, but readily crystallizes, and it is the crystals that scatter the light in the cataract [26]. As these proteins are major components of human lens [27], it would be of interest to know whether they are preferentially glycosylated in human diabetic cataract. Unfortunately, clear fresh human lenses are not available for the type of experiments reported here, and analysis of human cataracts would show many glycosylated proteins, not just the primary targets.

It is possible that an extensive complement of the normal housekeeping enzymes, receptor proteins etc. in the lens are preferentially modified by glycation, based on our previous studies; however, it might not be possible to detect these under our conditions. The further investigations using different modification agents might help to elucidate this possible phenomenon.

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REFERENCES

- Harding, J. J. (1997) Lens. In *Biochemistry of the Eye* (Harding, J. J., ed.), pp. 94–135, Chapman and Hall Medical, London.
- Thampi, P., Hassan, A., Smith, J. B. and Abraham, E. C. (2002) Enhanced C-terminal truncation of α A- and α B-crystallins in diabetic lenses. *Invest. Ophthalmol. Visual Sci.* **43**, 3265–3272.
- Swamy, M. S. and Abraham, E. C. (1991) Differential glycation of rat α -, β - and γ -crystallins. *Exp. Eye Res.* **52**, 439–444.
- Cherian, M. and Abraham, E. C. (1993) *In vitro* glycation and acetylation (by aspirin) of rat crystallins. *Life Sci.* **52**, 1699–1707.
- van Boekel, M. A. M. and Hoenders, H. J. (1992) *In vivo* glycation of bovine lens crystallins. *Biochim. Biophys. Acta* **1159**, 99–102.
- Abraham, E. C., Cherian, M. and Smith, J. B. (1994) Site selectivity in the glycation of α A- and α B-crystallins by glucose. *Biochem. Biophys. Res. Commun.* **201**, 1451–1456.
- Zhao, H. R., Smith, J. B., Jiang, X. Y. and Abraham, E. C. (1996) Sites of glycation of β B₂-crystallin by glucose and fructose. *Biochem. Biophys. Res. Commun.* **229**, 128–133.
- Pennington, J. and Harding, J. J. (1994) Identification of the site of glycation of γ -II-crystallin by [¹⁴C]fructose. *Biochim. Biophys. Acta* **1226**, 163–167.
- Blakytyn, R. and Harding, J. J. (1992) Glycation (non-enzymic glycosylation) inactivates glutathione reductase. *Biochem. J.* **288**, 303–307.
- Ganea, E. and Harding, J. J. (1995) Molecular chaperones protect against glycation-induced inactivation of glucose-6-phosphate dehydrogenase. *Eur. J. Biochem.* **231**, 181–185.
- Heath, M. M., Rixon, K. C. and Harding, J. J. (1996) Glycation-induced inactivation of malate dehydrogenase: protection by aspirin and a lens molecular chaperone, α -crystallin. *Biochim. Biophys. Acta* **1315**, 176–184.
- Hoshi, A., Takahashi, M., Fujii, J., Myint, T., Kaneto, H., Suzuki, K., Yamasaki, Y., Kamada, T. and Taniguchi, N. (1996) Glycation and inactivation of sorbitol dehydrogenase in normal and diabetic rats. *Biochem. J.* **318**, 119–123.
- Yan, H. and Harding, J. J. (1997) Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. *Biochem. J.* **328**, 599–605.
- Swamy-Mruthinti, S. (2001) Glycation decreases calmodulin binding to lens transmembrane protein, MIP. *Biochim. Biophys. Acta* **1536**, 64–72.
- Owers, J. and Duncan, G. (1979) The viability of the bovine lens in organ culture. *Exp. Eye Res.* **28**, 739–742.
- Crompton, M., Rixon, K. C. and Harding, J. J. (1985) Aspirin prevents carbamylation of soluble lens proteins and prevents cyanate-induced phase separation opacities *in vitro*: a possible mechanism by which aspirin could prevent cataract. *Exp. Eye Res.* **40**, 297–311.

- 17 Slingsby, C. and Bateman, O. A. (1990) Rapid separation of bovine β -crystallin subunits β -B₁, β -B₂, β -B₃, β -A₃ and β -A₄. *Exp. Eye Res.* **51**, 21–26
- 18 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 19 Crawford, C., Brown, N. R. and Willis, A. C. (1990) Investigation of the structural basis of the interaction of calpain II with phospholipid and with carbohydrate. *Biochem. J.* **265**, 575–579
- 20 Hay, R., Andley, U. P. and Petrash, J. M. (1994) Expression of recombinant bovine γ B-, γ C- and γ D-crystallins and correlation with native proteins. *Exp. Eye Res.* **58**, 573–583
- 21 Kilby, G. W., Truscott, R. J. W., Aquilina, J. A., Sheil, M. M., Riley, M. L. and Harding, J. J. (1995) Electrospray ionisation mass spectrometry of lens crystallins: verification and detection of errors in protein sequences of bovine γ -crystallins. *Eur. Mass Spectrom.* **1**, 203–208
- 22 Riley, M. L., Harding, J. J., Kilby, G. W., Truscott, R. J. W., Aquilina, A. and Sheil, M. M. (1996) Molecular masses of γ -crystallins. *Ophthalmic Res.* **28** (suppl. 2), 131–135
- 23 Chirgadze, Y. N., Driessen, H. P. C., Wright, G., Slingsby, C., Hay, R. E. and Lindley, P. F. (1996) Structure of the bovine eye lens γ D (γ -IIIb)-crystallins at 1.95 Å. *Acta Crystallogr.* **D52**, 712–721
- 24 Liang, J. J. and Fu, L. (2001) Conformational study of N ϵ -(carboxymethyl)lysine adducts of recombinant γ C-crystallin. *J. Protein Chem.* **20**, 641–645
- 25 Stephan, D. A., Gillanders, E., Vanderveen, D., Freas-Lutz, D., Wistow, G., Baxevanis, A. D., Robbins, C. M., Vanauken, A., Quesenberry, M. I., Baily-Wilson, J. et al. (1999) Progressive juvenile-onset punctate cataracts caused by a mutation of the γ D-crystallin gene. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1008–1012
- 26 Kmoch, S., Brynda, J., Asfaw, B., Bezouska, K., Novak, P., Rezacova, P., Ondrova, L., Filipec, M., Sedlacek, J. and Elleder, M. (2000) Link between a novel human γ D-crystallin allele and a unique cataract phenotype explained by protein crystallography. *Hum. Mol. Genet.* **9**, 1779–1786
- 27 Lampi, K. J., Ma, Z., Shih, M., Shearer, T. R., Smith, J. B., Smith, D. L. and David, L. L. (1997) Sequence analysis of β A3, β B3, and β A4 crystallins completes the identification of the major proteins in young human lens. *J. Biol. Chem.* **272**, 2268–2275

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