Site of carbamoylation of bovine γ -II-crystallin by potassium [^{14}C]cyanate

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One possible route to cataract formation may be via the carbamoylation of lens proteins due to increased concentrations of cyanate in the body resulting from uraemia associated with renal failure and with severe diarrhoea. Carbamoylation of γ -II-crystallin, which is found in the lens core, could alter the surface charge network of the molecules, resulting in aggregation, increased light-scattering and hence cataract. We have attempted to locate the site(s) of carbamoylation in γ -II-crystallin. γ -II-Crystallin was isolated by gel chromatography and ion-exchange chromatography. γ -II-Crystallin was then carbamoylated by incubation with potassium [^{14}C]cyanate, followed by citraconylation and digestion with trypsin to give peptides that were separated by high-resolution ion-exchange chromatography. The amino acid compositions of the radioactive peptides were compared with the expected peptide composition for γ -II-crystallin. The radioactive peptide compositions, which agreed with the theoretical peptides, all matched with the *N*-terminal region of γ -II-crystallin and had in common the presence of the *N*-terminal glycine residue. It appears that the α -amino group of the *N*-terminal glycine was the main site of carbamoylation. This site forms part of the charge network on the surface of γ -II-crystallin.

INTRODUCTION

The vertebrate lens consists of an inner nuclear core and an outer cortex enclosed within a collagenous elastic capsule. A single layer of epithelial cells divide, elongate, differentiate and overlay older cells that are retained throughout life (Harding & Crabbe, 1984). Lens proteins remain within the lens, and so an octagenarian will possess some lens proteins 80 years old. These proteins will be susceptible to post-synthetic non-enzymic modification due to changes in their environment. In the mammal the major soluble lens proteins are the crystallins (α , β and γ) located within the fibre cells (Wistow & Piatigorsky, 1988).

Cataract is a lens opacity that impairs vision. In nuclear cataract there are conformational changes in the crystallins (Harding, 1972) and an increase in cross-linking (especially disulphide-bond formation) between crystallins leading to the formation of high-molecular-mass aggregates (Harding, 1973).

Various causative factors have been implicated in different types of cataractogenesis. One such factor (particularly for cases in tropical countries) may be an increase in the extent of carbamoylation of lens proteins (Harding & Rixon, 1980). Severe diarrhoea leads to high concentrations of urea, which comes into equilibrium with cyanate. Cyanate can modify amino and thiol groups of proteins (Carreras *et al.*, 1976).

Incubation of rabbit lenses with 1 mM-cyanate produced a ring of opacity in 24 h (Kinoshita & Merola, 1973). Oral NaOCN caused cataract in man (Nicholson *et al.*, 1976). In addition, patients with renal failure and therefore a high blood urea concentration have a high risk of cataract (van Heyningen & Harding, 1986).

γ -Crystallin, one of the major structural proteins in the mammalian lens, seemed most susceptible to carba-

moylation by cyanate (Harding & Rixon, 1980). Slingsby & Croft (1973) showed that γ -crystallins are synthesized at an early stage in the development of the lens and are consequently located in the lens core, where many senile and brunescant cataracts form.

γ -Crystallins consist of several homologous proteins including γ -I- to γ -IV-crystallins isolated by Björk (1961, 1964). The complete amino acid sequence of γ -II-crystallin (calf) is known (Croft, 1972; Bhat & Spector, 1984).

The crystal structure has been determined to high resolution [0.26 nm (2.6 Å) (Blundell *et al.*, 1981), 0.19 nm (1.9 Å) (Wistow *et al.*, 1983) and 0.16 nm (1.6 Å) (Summers *et al.*, 1984)], showing that γ -II-crystallin has the highest internal symmetry of any protein so far studied by X-ray crystallography. The protein consists of two similar domains, the *N*- and *C*-terminal domains, connected by a short peptide. Each domain consists of two Greek-key-motif structures. Each motif consists of about 40 amino acid residues and is essentially a β -sheet of four antiparallel strands.

The surface of γ -II-crystallin is largely polar and hydrophilic, with the *C*-terminal domain having a greater preponderance of acidic and basic groups than the *N*-terminal domain. Much of the ionic and polar binding potential of the protein surface is accounted for in intramolecular interactions, with over 50 % of the ionic side chains forming ion-pairs (Wistow *et al.*, 1983). These surface charge interactions may be a major feature responsible for the stability of the molecule. By binding to a specific residue (or residues) cyanate may affect the overall charge distribution of the molecule, causing altered interactions with adjacent molecules and aggregate formation.

The present work was an attempt to identify the site(s) of carbamoylation of γ -II-crystallin. We chose to study γ -II-crystallin because its crystal structure is known,

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because it is especially susceptible to carbamylation, and because it is associated with high-molecular-mass aggregates in cataract.

Carbamylation of γ -II-crystallin may occur at several sites. Thiol groups are a possible site, but reactions of cyanate with thiol groups are rapidly reversible (Stark, 1964). Other possibilities are the ϵ -amino groups of the two lysine residues (Lys-2 and Lys-163) and the *N*-terminal glycine.

MATERIALS AND METHODS

Lenses

Bovine lenses were from Clays of Long Crendon (Thame, Oxon., U.K.) and were stored at -20°C until used.

Materials

KO^{14}CN was obtained from Amersham International, Amersham, Bucks., U.K. Other chemicals were from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma Chemical Co. (Poole, Dorset, U.K.) and were of the best quality available. Bovine trypsin (type III; $2\times$ crystallized) was from Sigma Chemical Co. Sephadex G-50 and SP-Sephadex were from Pharmacia, Milton Keynes, Bucks., U.K. The Dowex AG-50W X4 (-400 mesh) was from Bio-Rad Laboratories, Watford, Herts., U.K.

Isolation of γ -crystallins

Lens nuclei (average mass 0.35 g) were obtained by gently scraping away the softer outer cortex from 12 bovine lenses (mean mass 2 g). They were then homogenized gently in 20 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 50 mM-NaCl, 1 mM-EDTA and 0.05% NaN_3 at room temperature in a hand-operated glass homogenizer. The homogenate was centrifuged at 10000 *g* for 40 min at 4°C before being loaded on to a Sephadex G-50 (fine beaded) column (100 cm $\times$ 5.7 cm). Proteins were eluted with degassed homogenization buffer at a flow rate of 33 ml/h and monitored by reading the absorbance of each fraction at 280 nm. The fractions (11 ml) contributing to each protein peak were pooled and dialysed against four changes of distilled water at 4°C , including one overnight dialysis, freeze-dried and stored at -20°C .

Isolation of γ -II-crystallin

The γ -crystallins were separated by cation-exchange chromatography on an SP-Sephadex C-50 column (84 cm $\times$ 1.8 cm), by a modification of the procedure of Björk (1964).

The freeze-dried γ -crystallin fraction, corresponding to the third peak of the Sephadex G-50 elution profile, was dissolved in 5 ml of 0.2 M-sodium acetate buffer, pH 5, and was loaded on to the column. A linear gradient of degassed sodium acetate buffer ranging from 800 ml of 0.2 M-sodium acetate buffer, pH 5.0, to 800 ml of 0.5 M-sodium acetate buffer, pH 5.0, was created, and the fractions (70 $\times$ 10 ml) were collected at a flow rate of 15 ml/h. The absorbance of each fraction was read at 280 nm to locate the eluted proteins. Fractions corresponding to protein peaks were pooled, dialysed against four changes of distilled water at 4°C and freeze-dried.

Gel electrophoresis

The protein peaks eluted from the Sephadex G50 column and the ion-exchange column were examined by

polyacrylamide-gel electrophoresis in an LKB 2050 Midget electrophoresis unit. The mini-gels consisted of a 15% acrylamide running gel and a 5% stacking gel.

Carbamylation of γ -II-crystallin by KO^{14}CN

The γ -II-crystallin (2.4 mg/ml) was incubated with 25 mM-KOCN and 50 μCi of KO^{14}CN (52 Ci/mol) in 0.1 M-sodium pyrophosphate buffer, pH 8.0, containing 5 mM-diethylenetriaminepenta-acetic acid at 37°C . At zero time four 10 μl portions were removed for determination of total radioactivity. At 0, 3, 24 and 48 h four 25 μl portions were removed and added to 1 ml of 10% (w/v) trichloroacetic acid and left overnight at 4°C to precipitate protein. Residues were collected on Whatman GF/C microfibre filter discs and washed in 5% trichloroacetic acid. Each disc was dried at 70°C for 30 min before being added to 3.5 ml of Scintran cocktail T (BDH Chemicals). Radioactivity was measured by scintillation counting. After 48 h the reaction was stopped by dialysis of the mixture against four changes of distilled water at 4°C followed by freeze-drying.

Citraconylation and trypsin digestion

The freeze-dried protein (15 mg/ml) was redissolved in 0.5 M-phosphate buffer, pH 8.2, and the amino groups were blocked by citraconylation (Dixon & Perham, 1968; Panjwani & Harding, 1978).

Citraconic anhydride (30-fold molar excess over amino groups) was added in four portions at 20 min intervals with stirring, with maintenance of the pH at 8.2–8.3 with 1.6 M-KOH. After addition of the final portion of citraconic anhydride the mixture was stirred for 1 h at 19°C before dialysis. The protein was denatured by heating in a boiling-water bath for 10 min and then freeze-dried. The freeze-dried protein was redissolved (5.5 mg/ml) in 200 mM-ammonium bicarbonate buffer, pH 8.1, containing 1 mM- CaCl_2 (Panjwani & Harding, 1978). Trypsin solution (110 μl of 0.5% trypsin in 0.1 M- NH_4HCO_3) was added to the mixture, before incubation for 3 h at 37°C in a shaking water bath. A further 51 μl of fresh trypsin solution was added, to give an overall protein/trypsin ratio of 50:1, and after a further 4 h incubation the mixture was cooled, acidified to pH 2.6, freeze-dried and redissolved in 3.6 ml of pyridine/acetate buffer (0.02 M in pyridine), pH 3.0. This mixture was stirred for 3 h at room temperature to remove the citraconyl groups before being freeze-dried.

Ion-exchange chromatography of tryptic peptides

The freeze-dried digest was dissolved in 3 ml of distilled water adjusted to pH 2.5 with 3 M-HCl and loaded on to a pre-equilibrated Dowex AG-50W column (89 cm $\times$ 1 cm). Initial elution was with 70 ml of pyridine/acetate buffer (0.02 M in pyridine), pH 3.0, followed by elution with a linear gradient of 500 ml of the previous buffer and 500 ml of pyridine/acetate buffer (1.1 M in pyridine), pH 4.2. This was followed by a second gradient of 250 ml of the pH 4.2 buffer and 250 ml of pyridine/acetate (2.0 M in pyridine) buffer, pH 5.0. Fractions (3.6 ml) were collected at 19°C at a flow rate of 9 ml/h.

From each fraction 0.3 ml samples were added to a scintillation vial containing 3.5 ml of Scintran cocktail T for measurement of radioactivity. Other samples (0.6 ml) were removed and dried down in an oven for the ninhydrin assay to identify peptide-containing fractions. Each dried-down sample was redissolved in 0.6 ml of

Fig. 1. Isolation of γ -crystallin by gel chromatography on Sephadex G-50

For conditions see the text.

distilled water/aq. 1% (v/v) pyridine/aq. 2% (w/v) ninhydrin (1:1:1, by vol.). The stoppered tubes were placed in a boiling-water bath for 20 min, then cooled down, and 0.5 ml of aq. 10% (v/v) pyridine was added. The absorbance was measured at 570 nm. Fractions contributing to radioactive peaks and also ninhydrin-positive peaks were individually freeze-dried. Seventeen peptides plus arginine would be expected from examination of the amino acid sequence.

Amino acid analysis

To each freeze-dried peptide sample 175 μl of 6 M-HCl was added. The mixture was then transferred to

Fig. 2. Ion-exchange chromatography of γ -crystallins on the SP-Sephadex C-50 column for isolation of γ -II-crystallin

For conditions see the text. Shaded areas represent pooled fractions.

hydrolysis tubes, which were sealed under vacuum and placed in an oven (108 $^{\circ}\text{C}$) for 18 h for hydrolysis to occur. Afterwards, the tubes were opened and the solution was rotary-evaporated. The hydrolysate was re-dissolved in pH 2.2 amino acid analysis loading buffer (175 μl) and centrifuged in a Microfuge before being loaded on an LKB 4400 amino acid analyser. Duplicate analyses were performed.

RESULTS

Isolation of γ -II-crystallin

The Sephadex G-50 elution profile for the lens soluble proteins gave three main peaks (Fig. 1). SDS/polyacrylamide-gel electrophoresis showed the first two peaks (A and B) to be mixtures of α - and β -crystallins, whereas the third peak (C) is γ -crystallin free of contamination (gel not shown).

The SP-Sephadex cation-exchanger allows the separation of the different γ -crystallins (γ -I– γ -IV). A typical elution profile is shown in Fig. 2 and resembles that obtained by Björk (1964). All of the fractions pooled showed a single band on SDS/polyacrylamide-gel electrophoresis corresponding to a molecular mass of about 20000 Da (gel not shown). This shows that β s-crystallin is not present, and therefore the four different fractions from the four peaks (D, E, F and G in Fig. 2) were deduced to be γ -I– γ -IV-crystallin respectively by comparison with chromatograms obtained by Björk (1964). The pooled fractions corresponding to peak E, taken to be γ -II-crystallin, were freeze-dried.

Incubation of γ -II-crystallin with KO^{14}CN

The incorporation of the labelled cyanate into γ -II-crystallin was monitored throughout the incubation (Fig. 3), and the reaction was stopped before 1 mol of cyanate bound/mol of protein so that the most reactive site could be identified.

Tryptic peptides of γ -II-crystallin

The tryptic peptides were eluted from Dowex AG-50W by using two linear gradients of pyridine/acetate

Fig. 3. Incorporation of $[^{14}\text{C}]$ cyanate into bovine γ -II-crystallin

For conditions see the text. The error bars are standard deviations for the quadruplicate determinations contributing to each point.

Fig. 4. Fractionation of tryptic peptides on Dowex AG-50W (first attempt)

For conditions see the text.

buffer. In the first attempt to isolate the tryptic peptides, different linear gradients were used, which consisted of 500 ml of 0.2 M-pyridine/acetate buffer, pH 3.1, to 500 ml of 1.1 M-pyridine/acetate buffer, pH 4.2, followed by a second linear gradient of 250 ml of the previous pH 4.2 buffer to 250 ml of 2 M-pyridine/acetate buffer, pH 5.0. The main radioactive peak A was eluted early on and corresponded to a large ninhydrin-positive peak (Fig. 4). The second largest radioactive peak B in Fig. 4 did not coincide with any ninhydrin-positive peak. All of the fractions contributing to the radioactive and ninhydrin-positive peaks were pooled and taken for amino acid analysis. Only peak B was found to correspond to any theoretical peptide, and this appeared to

correspond to the *N*-terminal two residues, Gly-Lys, with lesser amounts of a contaminant (Table 1). As the main peaks were eluted early on, the elution conditions were changed for a second attempted isolation starting with isocratic elution at lower ionic strength, then a shallower gradient to increase the retention times and lead to a better separation (see the Materials and methods section). This time six radioactive peaks (P1 to P6) were eluted (Fig. 5). The main peak, P3, contributed 34% of the total radioactivity eluted. It did not correspond to any of the major ninhydrin-positive peaks. This could be explained if the labelled peptide present had been carbamoylated at the *N*-terminus, which would therefore not react with ninhydrin. The major ninhydrin-positive peptide fractions (a, b and c) were not labelled.

Of the radioactive peaks P1–P6 (Fig. 5) only the P3 and P6 compositions matched with any possible theoretical peptides. None of the ninhydrin-positive peaks (a, b and c) matched with any peptide sequence. The hydrolysate of fraction P3 contained equal amounts of glycine and lysine and only traces of other amino acids. This indicates that the peptide is Gly-Lys, which matches with the first two amino acid residues at the *N*-terminus of γ -II-crystallin. This is the same peptide as in peak B of Fig. 4. Trypsin must have cleaved at Lys-2, indicating that citraconylation, which was performed to prevent trypsin cleaving at lysine, had not been completely successful. Possibly the citraconyl groups were hydrolysed off either during dialysis, when the pH may have fallen, or during the subsequent boiling. The glycine/lysine ratio in the experimental peptide is 1:1 (Table 1). If the amino group of lysine had been carbamoylated, then some of the lysine would have been converted into homocitrulline, and so the area of the lysine peak would decrease and a peak, representing homocitrulline, would appear just before valine (Harding & Rixon, 1980). This

Table 1. Amino acid composition of isolated peptides (fraction B from Fig. 4 and fraction P3 from Fig. 5) that correspond to the *N*-terminal peptide Gly-Lys

Compositions were calculated relative to lysine set to 1 mol/mol. Amino acids with less than 0.2 mol of residue/mol are omitted.

Amino acid	Amino acid composition (mol of residue/mol)		
	Fraction B (Fig. 4)	Fraction P3 (Fig. 5)	Theoretical peptide 1–2
Asp	0.33	0	0
Ser	0.24	0	0
Glu	0.26	0	0
Gly	0.94	0.93	1
Val	0.25	0	0
Leu	0.28	0	0
Lys	1.0	1.0	1

Fig. 5. Fractionation of tryptic peptides on Dowex AG-50W column (second attempt)

For conditions see the text.

has not occurred, and so the cyanate must have labelled the *N*-terminal glycine. Carbamoylated glycine, having a blocked amino group, would not react with ninhydrin, and therefore would not be detected on amino acid analysis. The acid-hydrolysis step would release free glycine (Stark, 1967).

Table 2. Amino acid composition of fraction P6 (Fig. 5) compared with those of two lysine-containing theoretical peptides calculated from the amino acid sequence

Compositions were calculated relative to lysine set to 1 mol/mol. Amino acids with less than 0.2 mol of residue/mol were omitted or set to zero. Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (mol of residue/mol)		
	Fraction P6 (Fig. 5)	Theoretical peptide 1-9	Theoretical peptide 154-168
Asp	0.9	1	2
Thr	0.3	1	0
Ser	0.2	0	1
Glu	1.0	1	0
Gly	1.0	1	2
Ala	0	0	2
Val	0	0	1
Ile	0.5	1	0
Leu	0.6	0	2
Tyr	0.9	1	1
Phe	0.6	1	0
Lys	1.0	1	1
Arg	1.1	1	1
Met	0	0	1
Trp	N.D.	0	1

The amino acid composition of fraction P6, which had 12% of the total eluted radioactivity (Fig. 5), is similar to, but not identical with, the expected composition of residues 1-9 (Table 2). Certainly the composition shows that fraction P6 does not come from the only other expected lysine-containing peptide (residues 154-168; Table 2). We conclude tentatively that fraction P6 is the expected *N*-terminal peptide from residues 1-9 with carbamoylation at the *N*-terminus because there was no homocitrulline present and because it does not correspond to a major ninhydrin-positive peak (Fig. 5). If both peptides P3 and P6 represent γ -II-crystallin that had reacted with cyanate at the *N*-terminus then 46% of the eluted radioactivity has been traced to that site.

DISCUSSION

The radioactive peptides identified in this study matched the *N*-terminal peptide residues 1-2 or residues 1-9. No homocitrulline was found in any of the peptides tested, indicating that the lysine residues had not been carbamoylated. The main site of carbamoylation is the free α -amino group of the *N*-terminal glycine residue. *N*-Terminal carbamoylation of haemoglobin S has been reported (Lee & Manning, 1973).

This result indicates why γ -crystallins are more susceptible to carbamoylation than are α - and β -crystallins. The blocked *N*-termini of α - and β -crystallins protect against carbamoylation, whereas the free *N*-terminal glycine residue of γ -crystallin is modified. Other long-lived proteins, such as collagen and the basic protein of myelin, have blocked *N*-termini, and this could be an effective means of protection against electrophilic attack (Harding, 1985).

γ -II-Crystallin has an extensive network of ion-pairs over its surface (Wistow *et al.*, 1983). This network may

Fig. 6. Positions on the positively and negatively charged residues of bovine γ -II-crystallin

The diagram is taken from Summers *et al.* (1984).

contribute to the stability of the protein, which does not unfold even in high urea concentrations (Horwitz *et al.*, 1977). The charges are evenly balanced around the *N*-terminus (Fig. 6), so that the binding of cyanate to the *N*-terminus would alter this balance and could alter the interactions of γ -II-crystallin with other lens crystallins and surrounding water molecules, even in the absence of a conformational change, although a change in conformation would have a much greater effect. These altered interactions are manifested in the phase-separation effect, whereby opacification occurs in the lens nucleus as a result of separation of cell cytoplasm into protein-rich and protein-poor phases. Cyanate increases the phase-separation temperature leading to opacification (Crompton *et al.*, 1985). Aspirin prevents attack by cyanate and the rise in phase-separation temperature, possibly by acetylating amino groups.

Glucose 6-phosphate produces a conformational change in γ -crystallin, leading to exposure of thiol groups and intermolecular disulphide-bond formation to give high-molecular-mass aggregates (Beswick & Harding, 1987). No such change was found on carbamoylation of γ -crystallin (Beswick & Harding, 1984). Glucose 6-phosphate is thought to bind to the same site as cyanate on γ -II-crystallin (R. Blakytyn & J. J. Harding, unpublished work). Both eliminate the positive charge of the α -amino group, but glucose 6-phosphate introduces 2 negative charges into the protein. The cyanate molecule occupies less space and has less effect on charge, and this may be insufficient to cause the unfolding obtained by the addition of glucose 6-phosphate.

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