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Conformational Changes in Human Lens Proteins in Cataract

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The reactivity of protein thiol groups in human lens and the susceptibility of the proteins to tryptic digestion were investigated. Both were found to be greater in some cataractous lenses, indicating that lens proteins have unfolded during cataractogenesis. Almost all the tyrosine in the proteins of the normal human lens reacts with tetranitromethane and is therefore probably on the outside of the major lens proteins.

The urea-insoluble protein isolated from normal human and most cataractous human lenses is largely an artifact, produced during extraction by aerobic oxidation of protein thiols to disulphide cross-links (Harding, 1972). The isolation of different proportions of this protein from different types of cataractous human lens, normal lens, rat lens and ox lens (Dische, 1965; Manski *et al.*, 1968; Pirie, 1968; Harding, 1969, 1972) indicates that the proteins in each case have different susceptibilities to oxidation in air. For example, about half of the cataractous human lenses collected at operation yield more urea-insoluble protein than does normal human lens (Pirie, 1968). It was therefore decided to examine the reactivities of protein thiol groups in normal and cataractous human lens. The results of this study, described below, suggest that the proteins unfold during cataractogenesis. The degree of unfolding was examined by estimating the susceptibility to tryptic digestion of the proteins (Rupley, 1967) and the reactivity of the tyrosine residues towards tetranitromethane (Sokolovsky *et al.*, 1966). Reactivity of functional groups has often been used as a probe of protein conformation (see Cohen, 1970; Radda, 1971).

Materials and Methods

Lenses

Human cataractous lenses were those removed in the Oxford Eye Hospital. They were graded as described by Pirie (1968) and van Heyningen (1972). Lenses graded as group II were used in this study; these lenses have a visible nucleus with a pale cortex. Group II lenses form a large proportion of cataracts removed in Oxford.

For the thiol-reactivity experiments, group II lenses were further subdivided into those with few cortical opacities and those with many cortical opacities.

Lenses referred to as 'normal' were clear lenses obtained at *post mortem*.

Rate of carboxymethylation of lens thiol groups

Each decapsulated lens was homogenized and then carboxymethylated *in vacuo* in a Thunberg tube by addition of 2ml of 1% iodoacetic acid (recrystallized) (BDH Chemicals Ltd., Poole, Dorset, U.K.) in 0.1M-$NaHCO_3$, adjusted to pH8.8.

After a known time (0–240min) the tube was opened and 2ml of 10% (w/v) trichloroacetic acid was added to both stop the reaction and precipitate the protein. After standing at 4°C for 18h the protein was collected by centrifugation and dissolved in 6M-guanidinium chloride. Portions of this solution were assayed for unchanged thiol groups in 7M-urea, by using the 5,5′-dithiobis-(2-nitrobenzoic acid) reagent of Ellman (1959), as described by Harding (1970). To check that precipitation of lens proteins with trichloroacetic acid caused no change in content of protein thiol groups, total thiol groups were determined directly in a portion of a dialysed lens extract, and also on the protein precipitated from it by trichloroacetic acid and re-dissolved in guanidinium chloride. After precipitation 96% of the original protein thiol groups were recovered.

Labelling the fast-reacting thiol groups of lens

Each decapsulated lens was partially carboxymethylated as described above, except that the iodoacetic acid included 10μCi of iodo[2-^{14}C]acetate (The Radiochemical Centre, Amersham, Bucks., U.K.). After a time (15–45min) the Thunberg tube was opened and the contents were acidified by addition of 0.24ml of conc. HCl to stop the reaction, before dialysis against water (four changes of 200ml) for 24h. The first two changes of water were kept at pH4.0 to stop further reaction. The dialysed proteins were then fractionated into α-, β- and γ-crystallins and the urea-soluble and urea-insoluble fractions of the water-insoluble proteins of the lens, by using the isoelectric-precipitation and gel-filtration methods described by Harding (1969). In one experiment the

dialysed protein was freeze-dried and a portion taken for amino acid analysis.

An end-window Geiger–Müller tube was used to measure radioactivity. Radioactivity counting at 'infinite thinness' was carried out by adding either 0.05ml or 0.02ml of solution to a 4cm² planchet, superimposing a circle of paper tissue and drying under an i.r. lamp. Dried soluble proteins were dissolved in conc. formic acid. Insoluble proteins were hydrolysed in 6M-HCl *in vacuo* for 18h at 110°C. The dried hydrolysate was dissolved in water and 0.02ml samples were taken for counting.

Tryptic digestions

Each lens was decapsulated before being homogenized in 12ml of water. The pH was adjusted to 8.1, then a further 0.7ml of 2mM-NaOH was added before addition of 0.3ml of a solution (10mg/ml in 1mM-HCl) of trypsin [from bovine pancreas, twice-crystallized, salt-free; Boehringer (London) Ltd., London W.5, U.K.]. The pH was then maintained at 8.1 by measured additions of 2mM-NaOH throughout digestion at 20°C. This method for monitoring peptide cleavage was introduced by Waley & Watson (1953).

Progress curves were obtained by plotting volume of 2mM-NaOH added against time.

Nitration of tyrosine in lens proteins

Each lens was decapsulated before being ground in 10ml of 0.05M-tris–HCl, pH 8.0, in an all-glass manual homogenizer. To the suspension was added 0.5ml of 0.84M-tetranitromethane (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) in 95% (v/v) ethanol (Sokolovsky *et al.*, 1966). After mixing, a 0.2ml sample was removed and added to 50μl of 0.4M-cysteine, to stop the reaction, before dialysis against water for 3h (three changes of 5 litres). Further 0.2ml samples were removed at time-intervals while the reaction proceeded at 20°C, and were treated similarly, all being dialysed together.

The dialysed samples were transferred to hydrolysis tubes; 0.75ml of 8M-HCl was added to each. The tubes were sealed under vacuum before incubation at 110°C for 18h. The hydrolysates were dried over a steam bath and re-dissolved in 1.0ml of 0.05M-tris–HCl, pH 8.0, plus 60μl of 0.1M-NaOH.

The absorbance of these solutions at 428nm (the $\lambda_{max.}$ of nitrotyrosine) was measured by using a Unicam SP.500 spectrophotometer.

Amino acid analysis

Amino acid analyses were performed with a Locarte amino acid analyser, by the method of Spackman *et al.* (1958).

Results

Reactivity of lens thiol groups

The results of experiments on the rate of carboxymethylation of lens thiol groups showed that the protein thiol groups of the cataractous lenses reacted more rapidly with iodoacetate than those of normal lenses from persons of the same age range (Fig. 1). The cataractous lenses, all of group II, fell into two sub-groups depending on whether the cortex had few or many cortical opacities; the latter had less total protein thiol groups. In both sub-groups 40–50% of the original thiol groups were carboxymethylated within 20min, whereas in normal lenses only about 10% had reacted in that time and, even after 2h, less than 25% of the protein thiol groups of normal lens had reacted with iodoacetate. The cataractous lenses have a greater proportion of protein thiol groups that will react with iodoacetate under non-denaturing conditions, at pH 8.8 in aqueous buffer, and also these thiol groups react more rapidly than those of normal human lens (Fig. 1).

Labelling the reactive thiol groups with iodo[2-^{14}C]acetate showed that all major lens protein fractions in both normal and cataractous (group II) lenses (the three water-soluble fractions and the water-insoluble protein) had some reactive thiol groups. On average the thiol groups of α-crystallin were more highly labelled than those of other fractions, but, because α-crystallin has relatively few thiol groups, most of the reactive thiol groups were present in β- and γ-crystallin. Amino acid analysis of partially carboxymethylated protein, compared with the original protein, showed that neither histidine nor methionine had reacted under these conditions.

Tryptic digestions

The amount of alkali required to maintain constant pH during tryptic digestion is proportional to the number of peptide bonds split (Waley & Watson, 1953).

The initial slopes of the progress curves were 0.141±0.027ml of 2mM-NaOH/min per g wet wt. of lens for normal human lenses and 0.229±0.082ml of 2mM-NaOH/min per g wet wt. of lens for group II cataractous lens (Table 1). The difference between the means is significant (Student's t test, $P<0.01$). Thus the proteins of group II cataractous lenses are digested more rapidly than those of normal lenses. No difference was found between the group II sub-groups.

Nitration of tyrosine

The progress curves for nitration of tyrosine in human lens proteins were similar irrespective of whether the lens was cataractous or not. The initial

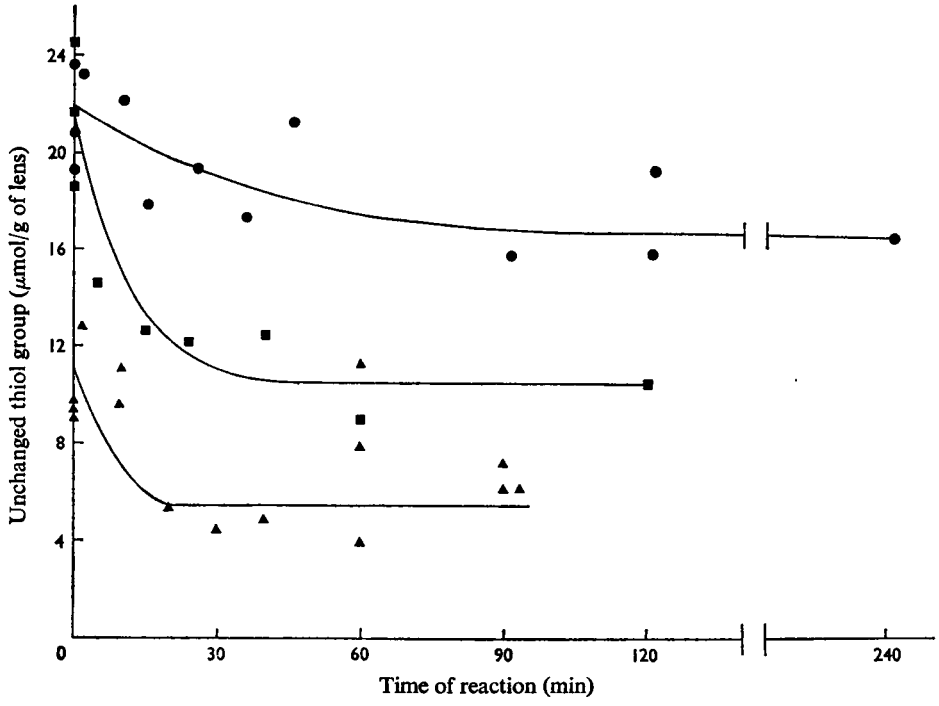

Fig. 1. *Rate of carboxymethylation of lens protein thiol groups*

Lenses were treated with iodoacetate. Unchanged thiol groups were determined at zero time and at intervals thereafter. ●, Normal lens; ■, cataract, group II with few cortical opacities; ▲, cataract, group II with many cortical opacities.

Table 1. *Tryptic susceptibility of and rate of nitration of human lens proteins*

Results are expressed as means±S.D. for the numbers of determinations given in parentheses. The difference between the mean initial slopes for tryptic digestion is significant (Student's t test, $P<0.01$).

	Initial slopes of progress curves	
Lens	Tryptic digestion (ml of 2mM-NaOH/min per g of lens)	Nitration (μmol of nitrotyrosine/min per g of lens)
Normal	0.141±0.027 (10)	1.50±0.18 (4)
Group II cataract	0.229±0.082 (9)	1.74±0.27 (4)

rates were not significantly different: 1.50±0.18μmol of nitrotyrosine/min per g for normal lens and 1.74±0.27μmol of nitrotyrosine/min per g for group II lens (Table 1). The final extent of nitration (after 90 or 120min) was 60–70μmol of nitrotyrosine/g of lens for normal and cataractous lenses. The total tyrosine content of the lens was shown to be about 69μmol/g wet wt.

Amino acid analysis of a hydrolysate of the nitrated proteins from a normal human lens showed that little tyrosine remained; after phenylalanine was a peak not found in un-nitrated proteins. This peak was in the position of authentic nitrotyrosine (Koch–Light Laboratories). The relative areas of the tyrosine and nitrotyrosine peaks showed that 97% of the tyrosine of normal human lens proteins had reacted with tetranitromethane.

Discussion

The results shown in Fig. 1 demonstrate that the cataractous lenses have a greater proportion of their thiol groups that react with iodoacetate under non-denaturing conditions than clear post-mortem lenses.

Most protein thiol groups in the normal lens are unreactive and must be 'buried' within the protein structure. Thiol groups appear to be uncovered in cataractogenesis, and the revealed thiol groups in cataractous lens also appear to react more rapidly.

The urea-insoluble protein isolated from human lens is formed by aerobic oxidation of protein thiol groups during isolation (Harding, 1972). The larger amount of urea-insoluble protein isolated from the cataractous lens presumably results from the greater number of reactive thiol groups in the proteins of such lenses. This change of reactivity indicates that during cataract development a lens protein(s) changes from its native conformation. The protein chains have, at least partly, unfolded, exposing previously 'buried' thiol groups to reagents in the aqueous environment.

The suggested conformational change was confirmed by the increased susceptibility of proteins in group II cataractous lenses to tryptic digestion. As far as I am aware the only other reports of a change of conformation of protein in a diseased state relate to the unfolding of haemoglobin to form Heinz bodies in certain congenital haemolytic anaemias (Jacob *et al.*, 1968).

In many proteins some tyrosine residues do not react with tetranitromethane or other reagents (Sokolovsky *et al.*, 1966; Sokolovsky & Vallee, 1967; Johansen *et al.*, 1967; Nilsson & Lindskog, 1967; Chang *et al.*, 1971; Christen *et al.*, 1971; Dorner, 1971; Helman & Givol, 1971; see also Di Prisco *et al.*, 1970) and such tyrosine residues are generally said to be buried within the protein. It was surprising therefore to find that at least 97% of the tyrosine in the normal lens was available to tetranitromethane, indicating that almost all the tyrosine residues of all major lens proteins are probably on the outside of these proteins. Such an arrangement would be most unusual, although it would provide the lens proteins with the possibility of hydrophobic interactions, which may be necessary to arrange them in an ordered fashion required for clarity. However, the assumption that reactivity of tyrosine residues with tetranitromethane necessarily means that they are on the outside of the protein molecule has been questioned in two recent papers. The three-dimensional structure of cytochrome *c* showed that the two reactive tyrosine residues are buried within the molecule (Dickerson *et al.*, 1971). As tetranitromethane is somewhat hydrophobic it has been suggested that a tyrosine residue in a hydrophobic environment on the inside of a flexible protein may be preferentially modified compared with one on the surface (Myers & Glazer, 1971).

Labelling the reactive thiol groups showed that all the crystallins have their conformations altered, and this may well be the result of some general factor. If this is the case it would be surprising if all other proteins, including enzymes, of the lens went unscathed.

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