Reaction of Tyrosine Oxidation Products with Proteins of the Lens

By ANTOINETTE PIRIE

Nuffield Laboratory of Ophthalmology, University of Oxford

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Oxidation of tyrosine in the presence of bovine lens proteins leads to the formation of brown or black melanoproteins. Both tyrosinase and the oxidizing system of ferrous sulphate–ascorbic acid–EDTA are effective. The fluorescence of the lens proteins is both altered and enhanced by the tyrosine-oxidizing systems. Their fluorescence spectra resemble those of urea-insoluble proteins of human cataractous lens and of 1,2-naphthaquinone–proteins of naphthalene cataract. The lens proteins lose their thiol groups and, in acid hydrolysates of treated β - and γ -crystallins, a substance has been detected chromatographically that behaves similarly to a compound formed when 3,4-dihydroxyphenylalanine (dopa) is oxidized by tyrosinase in the presence of cysteine. Analysis and behaviour of this substance from hydrolysates of lens proteins suggest that it is a compound of cysteine and dopa.

Human cataractous lenses vary in colour from pale yellow to deep brown, the nucleus of the lens being more deeply coloured than the cortex. A. Pirie (unpublished work) has shown that the brown colour is inseparable from an insoluble protein that is different from the well-recognized water-insoluble protein albuminoid, as it is insoluble not only in water but also in 7M-urea. van Heyningen & Pirie (1967) and Rees & Pirie (1967) found that the cataractous lens of the naphthalene-fed rabbit contained brown proteins that appeared to be compounds of lens proteins with 1,2-naphthaquinone, which was formed in the eye by enzymic processes. From this it seemed possible that the colour of the brown human cataractous lens might be due to compounds of lens proteins with quinones that either occur naturally in the eye or could be produced there. One such quinone-forming mechanism is oxidation of tyrosine either by tyrosinase or by the iron–ascorbic acid–EDTA system (Lissitzky, Rolland, Reynaud & Lasry, 1962) to give dopa,* which is readily autoxidized to dopa-quinone.

Tyrosine, ascorbic acid and iron are present in the lens (van Heyningen, 1968). Tyrosinase is present in the ciliary processes and the iris (Bowness & Morton, 1953), and Bernheimer (1964) found dopa and catecholamines in calf iris. de Berardinis & de Rosa (1951) found increased quantities of iron in human cataractous lenses, particularly in deeply coloured ones.

The present paper describes the preparation and

properties of compounds of lens proteins with products of tyrosine oxidation.

MATERIALS AND METHODS

Materials. Pronase and tyrosinase (mushroom) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Tyrosinase (ciliary body) was prepared by the method of Bowness & Morton (1953). [^{14}C]Tyrosine was obtained from The Radiochemical Centre, Amersham, Bucks. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset.

Cattle lens extracts. Cattle lenses, removed within 2 hr. of death, were ground with sand in a mortar and extracted with four times their weight of water. The slurry was spun at 10000g and the supernatant was dialysed against several changes of water at neutral pH for 48 hr. at 4°. This extract was either used at once or kept at –30°.

Separation of α -crystallin. Cattle lens extracts prepared as described above were brought to pH 5.0 and the precipitated α -crystallin was allowed to sediment for some hours at 4° and was then spun down at 10000g. The precipitate was dissolved at neutrality and reprecipitated at pH 5. It was again dissolved at neutrality and then the solution of α -crystallin was either used at once or kept at –30°.

Separation of β - + γ -crystallin. The soluble proteins of the lens remaining after precipitation of the α -crystallin were dialysed at neutrality against water at 4°. Any precipitate was spun off and discarded and the supernatant, which was a mixture of β - and γ -crystallins, was used at once or stored at –30°.

Reaction of lens proteins with tyrosine plus tyrosinase. Extract (20 ml., containing about 1 g. of protein) was brought to pH 7 and mixed with a suspension of 50–100 mg. of tyrosine containing 2–5 mg. of dopa; 1000 units of mushroom tyrosinase and two drops of CHCl_3 were added and

* Abbreviation: dopa, 3,4-dihydroxyphenylalanine.

the flasks were incubated at 20° or 30° for 24 hr. Controls without enzyme or with boiled enzyme were incubated in parallel.

Reaction of lens proteins with [U-¹⁴C]tyrosine plus tyrosinase. Protein (100 mg. in 2 ml.) was added to 1 mg. of tyrosine and 0.1 mg. of dopa dissolved in 0.1 N-HCl, 5 μ C of [U-¹⁴C]tyrosine was added, and the volume was brought to 2.2 ml. with phosphate buffer to give a final concentration of 50 mM-buffer, pH 7.3. Tyrosinase (mushroom) (200 units) and two drops of CHCl₃ were added and the flask was incubated at 30° overnight. A control without enzyme was incubated in parallel.

Reaction of lens proteins with tyrosine plus ferrous sulphate-ascorbic acid-EDTA. Protein (100 mg. in 2 ml.), 10 mg. of ascorbic acid, FeSO₄ to a final concentration of 0.8 mM and EDTA to a final concentration of 10 mM were mixed with 5 mg. of tyrosine. Phosphate buffer, pH 6.8, to a final concentration of 30 mM was added, and the volume was made to 6 ml. with water. Controls in parallel without tyrosine and without tyrosine and FeSO₄ were left at 20° with shaking overnight. The CHCl₃ was added as preservative.

Reaction of β - + γ -crystallin with [U-¹⁴C]tyrosine and a ferrous sulphate-ascorbic acid-EDTA oxidizing system. This reaction was carried out as with non-radioactive tyrosine, but the final FeSO₄ concentration was decreased to 0.1 mM and [U-¹⁴C]tyrosine (3 μ C) was added. A control without FeSO₄ was incubated in parallel.

Reaction of cysteine with dopa in the presence of tyrosinase. Dopa (5 ml. of a 20 mM solution) was mixed with 4 ml. of 40 mM-cysteine at neutrality, and 1000 units of tyrosinase (mushroom) in phosphate buffer, pH 6.8 (final concentration 10 mM), was added. The mixture was incubated at 20° overnight.

Hydrolysis of proteins. This was carried out with 6 N-HCl by the method described by Moore & Stein (1963).

Paper chromatography of acid hydrolysates. This was carried out on Whatman no. 1 or no. 52 paper, with 2% acetic acid as solvent 1 followed by butan-1-ol-acetic acid-water (40:9:20, by vol.) as solvent 2. Forrester solvent, acetic acid-conc. HCl-water (30:3:10, by vol.), was also used.

Paper electrophoresis. This was carried out in a ridge-pole apparatus on Whatman no. 52 paper with a buffer of formic acid-acetic acid-water, pH 1.6 [125 ml. of 90% (v/v) formic acid and 375 ml. of acetic acid in 2.5 l.], at 11 v/cm. for 3 hr.

Electrophoresis was followed by chromatography in solvent 2.

Examination of chromatograms. Phenols were detected by dipping the chromatograms through an aqueous solution of 0.3% FeCl₃-3% (w/v) K₃Fe(CN)₆ (Hathaway, 1960). The iodine-azide spray [0.1 N-I₂ in 0.12 N-KI to which NaN₃ (3%, w/v) was added just before use (Pierpoint, 1966)] was used to detect C-S or thiol groups. Amino acids were detected by the ninhydrin reagent of Wiggins & Williams (1952).

Thiol groups. Thiol groups were determined by the method of Ellman (1959).

Spectra. Absorption spectra were recorded on an Optica CF 4R spectrophotometer. Fluorescence spectra were recorded on an Aminco-Bowman spectrofluorimeter. Fluorescence was detected by using a Turner fluorimeter.

RESULTS

Reaction of lens protein with tyrosine and tyrosinase. The reaction mixture that contained enzyme quickly turned yellow and then brown, and was black after overnight incubation. The control mixture without enzyme remained colourless. Both were then dialysed against water at 4° for 48-72 hr. Nearly all the colour remained in the sac. Experiments of this general nature were done with tyrosinase from ciliary processes or from mushroom. The tyrosine-hydroxylating system of ferrous sulphate, ascorbic acid and EDTA was also used; in this system the colour changed to brown rather than black.

The colour could not be separated from the treated lens proteins. Evidence that a compound had been formed was given by the electrophoretic behaviour of the reaction mixture. When the mixture was subjected to electrophoresis at pH 1.6 (see the Materials and Methods section) almost all the brown colour migrated towards the cathode, and this brown band stained for protein. At this pH phenols and melanin formed by the oxidation of tyrosine by tyrosinase remained at the origin. Further evidence of the reaction came from the investigation

Table 1. *Loss of thiol by lens proteins in the presence of a tyrosinase system*

Soluble proteins from cattle lens were used after dialysis. The temperature was 30° and the pH 7.2.

Expt. no.	Concn. of protein (%, w/v)	Enzyme	Substrate	Time (hr.)	Concn. of thiol groups (μ moles/ml.)	
					Control	Enzyme-treated
1	2	Ciliary-body tyrosinase (5 units/ml.)	Dopa (4 mM)	0	1.35	1.38
				4	1.16	0.55
				21	0.95	0.17
2	6	Mushroom tyrosinase (50 units/ml.)	Tyrosine (saturated) + dopa (50 μ M)	4	3.6	1.9
				24	4.0	0.8
3	5	Mushroom tyrosinase (50 units/ml.)	Tyrosine (saturated) + dopa (25 μ M)	0	4.7	4.7
				19	4.05	2.2

Table 2. *Relative fluorescence of lens proteins treated with tyrosine-oxidizing systems*

The preparation of the proteins is described in the Materials and Methods section. Spectra were recorded on an Aminco-Bowman spectrofluorimeter.

Preparation	Concn. (mg./ml.) and conditions	Activation wavelength (m μ)	Emission wavelength (m μ)	Relative fluorescence (%)
β -+ γ -Crystallins treated with tyrosine+tyrosinase	0.5, in 3% (w/v) NaOH	360	460	70
β -+ γ -Crystallins treated with tyrosinase	10, pH 10.5	375	460	52
β -+ γ -Crystallins treated with FeSO ₄ -ascorbate-EDTA	10, pH 10.5	375	460	31
β -+ γ -Crystallins, untreated	10, pH 10.5	375	460	5

of thiol groups in the treated lens proteins. Table 1 shows that there was progressive loss of thiol as the reaction proceeded. A similar loss of thiol took place during reaction of lens proteins with 1,2-naphthoquinone (Rees & Pirie, 1967).

No difference could be detected between the ultraviolet-absorption spectrum of the tyrosine-tyrosinase-treated proteins and that of the normal lens proteins, but the tyrosine-tyrosinase-treated protein showed a marked increase in fluorescence.

Fluorescence of tyrosine-tyrosinase-treated bovine lens proteins. The proteins of the mammalian lens show a sky-blue fluorescence. This was both increased and changed to a more greenish hue after treatment with systems oxidizing tyrosine. Table 2 shows that the treated β -+ γ -crystallins fluoresced strongly at 460m μ when activated at 360m μ . Treated α -crystallin had a much lower fluorescence and normal lens proteins showed hardly any fluorescence at this wavelength. Table 2 shows that fluorescence at 460m μ also developed if the proteins were treated with tyrosinase alone or with the ferrous sulphate-ascorbic acid oxidizing system. Thus oxidation of the tyrosine residues in the protein itself led to some fluorescence at 460m μ , but this was not as marked as when extra tyrosine was added.

Hydrolysis and analysis of the treated lens proteins. Lens proteins treated with tyrosine and one or other of the two oxidizing systems were hydrolysed to see whether characteristic products could be identified. Pronase digestion was found to be unsuitable as it left an insoluble residue. Acid hydrolysis (Moore & Stein, 1963) was therefore used. The analytical and detection methods are described in the Materials and Methods section. Acid hydrolysates were chromatographed in solvent 1 followed by solvent 2. No extra ninhydrin-positive spots were found. A strong phenolic spot was found with R_F 0.6 in solvent 1 and 0.0-0.1 in solvent 2. This spot was present neither in hydrolysates of untreated lens proteins nor in hydrolysates of α -crystallin treated with tyrosine and tyrosinase; it was derived from the treated β -+ γ -crystallins of the lens.

Fig. 1. Absorption spectra of the compound of cysteine and dopa made from a reaction mixture of dopa, cysteine and tyrosinase, and of the compound isolated from the acid hydrolysate of lens proteins treated with tyrosine+tyrosinase. Aqueous solutions, pH 7, were used. ----, 'Cysteine-dopa'; —, phenol from lens proteins.

Properties of phenolic material from treated lens proteins. After chromatography, the phenolic material gave a weakly positive ninhydrin reaction, a slowly developing reaction with alkaline silver nitrate and a positive reaction with diazotized sulphanilic acid and with the iodine-azide reagent for C-S and thiol compounds (Pierpoint, 1966). But dopa was found to be positive with this reagent, and hence the test is not specific. The nitroprusside reaction for thiol was negative with the lens material. The reaction with ferric chloride-potassium ferri-cyanide was as quick as with diphenols, the blue colour appearing before that of tyrosine. No reaction was given with leucomethylene blue, indicating absence of quinone groups.

The absorption spectrum of the phenolic material, after elution from a chromatogram, gave a peak at 292m μ and a shoulder at 254m μ , the ratio of intensities being near 1:1.6 (Fig. 1). This spectrum was similar to that of the compound of GSH and

dopa described by Bouchilloux & Kodja (1960). Mason & Peterson (1965) found that a mixture of cysteine, dopa and tyrosinase slowly developed an absorption peak at $310\text{m}\mu$, whereas Roston (1960) found one quickly developed at $250\text{m}\mu$.

These properties make it possible that the phenolic material obtained by acid hydrolysis of lens proteins treated with tyrosine together with tyrosine-oxidizing systems contained both cysteine and dopa. A mixture of cysteine and dopa was therefore treated with tyrosinase (see the Materials and Methods section) to determine whether a compound corresponding to the phenol from lens was formed.

Properties of product of reaction between cysteine and dopa. The reaction mixture was analysed by repeated chromatography in solvent 2. Three phenol-positive bands appeared, and the eluate from one of them had the same R_F in both solvent 1 and solvent 2 as the phenol from treated lens protein. The phenol reaction was as quick as with diphenols. The material was weakly positive with ninhydrin and gave a positive reaction with alkaline silver nitrate but none with sodium nitroprusside. A slow positive reaction was given with the iodine-azide reagent. Analysis (by Weiler and Strauss, Oxford) gave C, 36.1; H, 5.3; N, 7.5; S, 8.2; residue, 7.7%. After correction for the residue the percentages are C, 39.2; H, 5.7; N, 8.2; S, 8.9%. A compound of cysteine and dopa formed by a C-S or C-N link would have C, 45.5; H, 5.0; N, 8.8; S, 10.1%.

The absorption spectrum (Fig. 1) is similar to that of the phenolic material from treated lens proteins. A chromatographic comparison of the two materials was therefore carried out.

Comparison of 'cysteine-dopa' with the phenol from treated lens proteins. Table 3 shows that these preparations had the same R_F in three solvents. When superimposed they gave a single spot on a chromatogram. This similarity was confirmed by experiments in which [^{14}C]tyrosine was used to prepare the lens phenol (see the Materials and

Methods section). The acid hydrolysate of lens proteins treated with [^{14}C]tyrosine and tyrosinase or ferrous sulphate-ascorbic acid showed a radioactive spot corresponding to that given by the synthesized 'cysteine-dopa' compound. A spot of the acid hydrolysate was mixed with one of 'cysteine-dopa' and chromatographed in solvent 1 followed by solvent 2 which was allowed to drip. A radioactive spot with R_F 0.63 in solvent 1 was partly superimposed on a strongly positive phenol spot (R_F 0.61). The radioactive spot travelled 0.6 in. in solvent 2 and the phenol spot 0.55 in. There was not therefore complete correspondence of position, but it seems possible that complexity of the acid hydrolysate prevented this.

DISCUSSION

This work started from the idea that the brown colour of some human cataracts might be due to the presence of compounds formed with oxidizing tyrosine, analogous to the brown 1,2-naphthaquinone-proteins that are formed in the lens of the naphthalene-fed rabbit (van Heyningen & Pirie, 1967). The present experiments show that brown or black compounds of lens proteins with tyrosine oxidation products are readily formed. The clearest similarity between all these brown proteins lies in their fluorescence spectra. Brown proteins from human cataractous lens (A. Pirie, unpublished work), the 1,2-naphthaquinone-proteins of rabbit lens, bovine lens proteins treated with tyrosine and tyrosinase, and tropocollagen treated with tyrosinase (Dabbous, 1966) all have new peaks of fluorescence between 440 and $470\text{m}\mu$ when activated near $350\text{m}\mu$. The phenol isolated from tyrosine-tyrosinase-treated lens proteins fluoresces at $490\text{m}\mu$ when activated at $380\text{m}\mu$. The phenol synthesized from dopa and cysteine fluoresces at $500\text{m}\mu$ when activated at $370\text{m}\mu$. It is probable that thiol is only one of the groups in protein that reacts with oxidizing tyrosine. The thiol compound may be easier to detect than other products because it is stable to acid hydrolysis.

The three fluorescent proteins that were prepared *in vitro* were all treated with a quinone or quinone-forming mechanism. There is no evidence that such a mechanism is operative in the formation of the brown fluorescent proteins in human cataractous lenses, although the constituents of one of the tyrosine-oxidizing systems are contained in the lens and dopa is present in the adjacent iris.

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Table 3. *Chromatographic comparison of the phenol from lens with the compound of cysteine with dopa*

For experimental details see the Materials and Methods section.

Solvent	R_F		
	'Cysteine-dopa'	Lens phenol	'Cysteine-dopa' + lens phenol
Forrestal solvent	0.48	0.47	0.48
Solvent 1	0.6, 0.61	0.66, 0.61	0.6, 0.61
Solvent 2	0.0, 0.02	0.04	0.0, 0.0
Solvent 2 (drip)	3.0 in.	3.3 in.	3.1 in.

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