IDENTIFICATION OF CYSTEINYLDOPA-DERIVED UNITS IN EUMELANINS FROM MAMMALIAN EYES

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1. Introduction

It is generally agreed that black and brown eumelanins from mammalian hair, skin and eye are irregular polymers consisting mainly of various 5,6-dihydroxyindole units derived biogenetically from tyrosine [1]. However, all the eumelanins so far isolated have been found to contain a certain amount of sulphur, which is retained even after removal of accompanying proteins by prolonged acid hydrolysis [2]. This fact, coupled with the known way of deposition of the pigment polymer on the protein matrix of melanosomes, have led to the view that the sulphur content of eumelanins arises probably from covalent SH-bindings of structural proteins to 5,6-indole quinone chromophoric units [3,4]. This interpretation has been questioned [5] following the isolation from human melanomas of an unusual type of eumelanin containing as much as 5.8% of sulphur that could be explained as deriving from proteins. When combined with the occurrence in melanoma tissues of large amount of 5-*S*-cysteinyldopa [6,7], and related metabolites [8], this finding suggested that the isolated pigment might arise via intermeshing between eumelanin and phaeomelanin pathways with subsequent co-polymerization involving both indole and cysteinyldopa intermediates [9].

Supporting evidence for the proposed mechanism has recently [10] been provided by in vitro biosynthetic experiments showing that enzymic oxidation of dopa in presence of varying amounts of 5-*S*-cysteinyldopa leads to pigments whose chemical and physical properties are intermediates between those of pure eumelanin- and phaeomelanin-type polymers.

In order to get conclusive evidence for the process of intermeshing in melanogenesis, we have now examined by chemical degradations the presence of cysteinyldopa-derived units in some typical emeulanins isolated from mammalian eyes.

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2. Materials and methods

2.1. *Source of melanins*

Ocular pigmented material from the following species have been investigated: rabbit (Fauve de Bourgogne and Wild-type), cattle (Frisian and Pie rouge), pig (Large White) and human (kindly provided by C. Pierre and M. Chabanon, Department of Ophthalmology, Hôpital Ed. Herriot). Collected eyes were pooled and frozen in liquid nitrogen before dissection of choroïds and ciliary processes.

2.2. *Isolation of eumelanins*

Pigmented material was homogenized in chilled distilled water, and the melanin isolated as in [5]. After centrifugation at 30 000 × *g* for 30 min, pellets were suspended in 0.1 M phosphate buffer (pH 7.5) and treated with pronase (B grade, Sigma, 1 mg/ml) and collagenase (130 units/mg, Calbiochem, 1 mg/ml), for 48 h at room temperature. After 3 days, the protein-free pigment was collected by centrifugation, washed with distilled water (3 times) and eventually treated with 6 M HCl at room temperature for 12 h to remove any accompanying protein residues. The pigment was then collected by centrifugation, washed with water and acetone, and dried in vacuo over P_2O_5.

2.3. *HI degradation*

A suspension of purified melanin (20 mg) in 3 ml freshly distilled hydriodic acid containing 5 mg red phosphorus, was heated under reflux for 24 h. After cooling, the reaction mixture was filtered and the hydriodic acid evaporated under reduced pressure. The residue was taken up in 2 ml water, filtered and the filtrate evaporated in a vacuum dessicator over KOH pellets. Paper chromatography of the reaction mixture on Whatman 3 MM (*n*-butanol–acetic acid–water 60:15:25 (by vol.) aqueous Fast red B salt as spraying reagent) showed the presence of 3-hydroxy-4-amino-phenylalanine and β-6-(4-hydroxy-benzothiazolyl)-alanine. The identification of these products was confirmed by a direct comparison of their characteristic spectral (UV) and chromatographic properties with those of authentic samples [10].

2.4. *Alkaline fusion*

A mixture of purified melanin (20 mg), NaOH (100 mg), $Na_2S_2O_4$ (15 mg) and water (0.3 ml) was heated in a platinum crucible at 280–300°C for 10 min. After cooling the fused mass was worked-up as in [12] and analyzed for the presence of 5,6-dihydroxyindole by paper chromatography in *n*-butanol–acetic acid–water (60:15:25, by vol.) using as spraying reagent 3% ethanolic $FeCl_3$ and aqueous Fast red B salt (Fluka).

3. Results and discussion

The melanins used here were isolated from the choroids and ciliary processes of rabbit, cattle, pig and man using a general procedure which involves successive enzymic and chemical hydrolysis to remove any accompanying protein. Physical and chemical properties of the purified melanins are summarized in table 1.

It can be seen that all the pigments examined resemble eumelanins with respect to colour and solubility, except that from human eyes which is reddish brown in colour and partially soluble in dilute alkali. Such a difference seems related to the sulphur content of the melanin which is much higher (~4%) than that of the analogous pigments from other mammals.

Unfortunately, the human eyes used as starting material for the extraction of the pigment were pooled from various individuals, thus preventing a possible correlation between the type of pigmentation, e.g., of the hair, and chemical composition of the ocular melanin. It is significant, however, that no substantial difference is observed in the properties of the melanins from 2 strains of rabbit or cattle having eumelanic and phaeomelanic hair, respectively. This suggests that in an individual species there may be no relationship between ocular and hair melanins, as also observed in [13].

Table 1
Properties of the melanins isolated from mammalian eyes

Source	Pigment colour	Solubility in 0.1 N NaOH	Elementary analysis		Degradation products		
			N%	S%	a		b
Rabbit							
– Fauve de Bourgogne[c]	Black	Nil	9.2	1.2	+	+	++++
– Wild-type[d]	Black	Nil	9.0	1.4	+	+	++++
Cattle							
– Frisian[d]	Black	Nil	9.3	1.5	+	+	++++
– Pie rouge[c]	Black	Nil	9.5	1.6	+	+	++++
Pig	Black	Nil	9.4	1.6	+	+	++++
Human	Brown	Low	10.1	3.9	++	++	+++

[a] 57% HI and red phosphorus; [b] alkaline fusion; [c] phaeomelanic hair; [d] eumelanic hair

When subjected to alkali fusion, all the ocular melanins give the expected 5,6-dihydroxyindole, typical of eumelanin-type polymer. However, on reductive hydrolysis with HI and red phosphorus they also afford, albeit in lower yields, degradation products typical of phaeomelanic pigments, such as 3-hydroxy-4-aminophenylalanine and 4-hydroxybenzothiazolyl-alanine. This finding provides evidence that the sulphur content of these pigments arises, as suggested [5], from the incorporation of cysteinyldopa units resulting from a certain degree of intermeshing between the eumelanin and phaeomelanin pathways.

Such a process of intermeshing seems to be a quite general feature of melanogenesis in mammals, as evidenced by the constant presence of cysteinyldopa in all active melanocytes [7] as well as by the polymorphism of melanosomes found in normal and pathological pigment producing cells. In this context it should be emphasized that the current classification of melanins in two distinct groups, eumelanins and phaeomelanins, may be valuable only when applied to pigments from organisms which are genotypically well defined with respect to colour mutants, such as the a black or a yellow mouse. In most mammals and expecially in man, lack of selective breeding between individuals with the same colour mutants has resulted in a considerable variation of the pigmentary expression of melanocytes reflecting the degree of intermeshing between the two original eu- and phaeomelanin pathways.

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