Acta Derm Venereol (Stockh) 1986; 66: 369–374

Oxidation of Dopa in the Skin of Black and Albino Mice

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Carstam R, Hansson C, Rorsman H, Rosengren E. Oxidation of dopa in the skin of black and albino mice. Acta Derm Venereol (Stockh) 1986; 66: 369–374.

The dopa oxidase activity of tyrosinase in the skin from albino and black mice was assayed using a technique based on the formation of two diastereomers of 5-S-cysteinyldopa when incubating tissue extracts with both L-dopa and D-dopa as substrates in the presence of cysteine. The amounts of 5-S-L-cysteinyl-L-dopa and 5-S-L-cysteinyl-D-dopa formed were then determined. In the extract from black mouse skin the L-L-diastereomer was produced in more than ten times the amount of the L-D-diastereomer. This stereospecific dopaoxidation is indicative of the presence of tyrosinase and corresponds well with earlier determinations of the rates of oxidation for human tyrosinase, using L-dopa and D-dopa as substrates. Stereospecific dopa oxidation was absent in albino skin, and the nonspecific dopaoxidation was two orders of magnitude less than the dopa oxidation in black skin. The study demonstrates the lack of tyrosinase activity in albino skin, and quantifies the nonspecific dopa oxidation. The lack of tyrosinase activity in the eluates from albinotic skin was found not to be due to the presence of a tyrosinase inhibitor. *Key words: Tyrosinase; Melanin; 5-S-cysteinyldopa.* (Received April 16, 1986.)

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The initial steps in the melanin formation are catalysed by the bi-functional enzyme tyrosinase. The enzyme functions as an oxygenase, with tyrosine as its substrate, producing dopa, and this compound also serves as a cosubstrate. Dopa is then the substrate for the enzyme in its oxidase function, yielding dopaquinone. These reactions and their interrelationship have been outlined in a recent study (1). In higher organisms the enzyme is stereospecific for the L-forms of the substrates (2).

In vivo, dopaquinone is transformed into indolines and indoles, or, in the presence of cysteine, undergoes a nucleophilic addition of cysteine to form cysteinyldopas. These two groups of compounds are then further oxidized and polymerized to form melanins (3).

Several methods for the assay of tyrosinase have been described. The earlier ones used manometric measurement of oxygen consumption (4), measurement of dopachrome formation by measuring absorbance at 475 nm (5), or the formation of labelled melanins using tyrosine-C^{14} (6). The method in current use utilizes tritiated tyrosine, measuring the production of labelled H_2O (7). Recently methods using immunological (8, 9) and electrometric (10) techniques have been published.

For several years we have been studying the melanin synthesis, focusing initially on the presence of 5-S-cysteinyldopa (5-S-CD) as a marker for melanin production of normal melanocytes, but also as a marker for the presence of malignant melanoma tissue (11). Many findings have shown that 5-S-CD is a metabolite of all pigment producing cells, and the former strict division into eumelanin and phaeomelanin has now given way to the concept of mixed melanins (12). For the determination of the concentration of 5-S-CD in blood and urine we utilize HPLC with electrochemical detection as earlier described (13), and use 5-S-L-cysteinyl-D-dopa as an internal standard (14).

5-S-CD has been found to be a marker for facultative pigmentation, but cannot be used as a determinant for the constitutional pigmentation (15). In fact 5-S-CD has been shown in plasma and urine from several types of human and mouse albinos (16, 17, 18, 19). This

means that there must be a non-specific, i.e. not tyrosinase-catalysed oxidation of dopa, in a compartment, where cysteine or glutathione is present, forming 5-S-cysteinyldopa either directly, or through the degradation of glutathionedopa (20).

We have studied the function of tyrosinase, initially mushroom tyrosinase, but in recent years predominantly tyrosinase from cultured melanoma cells. In these studies we have described the stereospecificity of human melanoma tyrosinase for L-tyrosine and L-dopa, and the function of dopa as a co-substrate, reducing the active centre of tyrosinase. This reaction is a necessary step for the tyrosine hydroxylation. For the assay of tyrosinase activity we have used the measurement of dopa oxidation expressed as 5-S-CD formed in an incubation system with tyrosinase, dopa, and cysteine (1).

In several species, there exist albinos, individuals with varying degrees of hypopigmentation, where melanin formation is impaired, either through the lack of enzyme, or by some other, undefined block in the melanin formation pathway.

In studies of black and albino mouse skin we have now ascertained the absence of stereospecific dopa oxidase activity in the skin of NMRI albino mice.

MATERIALS AND METHODS

Tyrosinase preparation

The dorsal skin of five 8–11 day-old black (C57 Bl/6J) and four albino (NMRI) mice of both sexes was used in each experiment. The animals were killed by cervical dislocation. The skins were shaved and excised in one piece. They were carefully cleaned from adipose tissue, minced finely with scissors, weighed and cooled on dry ice. The samples were then placed in a steel cylinder chilled with liquid nitrogen, and the tissue specimens cracked by applying a pressure of 10 000 kp for three seconds. As soon as the nitrogen had boiled away, the residual powder was transferred to a 100 ml Erlenmeyer flask containing 5 ml 0.01 M phosphate buffer, pH 7.4. The flask was cooled to −20°C and 50 ml of acetone at −20°C was added. The suspension was kept at this temperature for 15 min, and frequently stirred during the first five minutes. It was then allowed to settle and the acetone was decanted. The residue was sucked as dry as possible on a filter paper using a Buchner funnel. The resulting cake was transferred to a tube containing a 0.5% Triton X-100 solution in 10 ml 0.01 M phosphate buffer, pH 7.4. The mixture was then treated with a Polytron model PT 10–35 homogenizer (Kinematica, Kriens-Luzern, Switzerland) for three seconds and the resulting suspension centrifuged at 50 000 g for 30 min. The supernatant fraction was applied to a column of Con A-Sepharose (Pharmacia Fine Chemicals) kept at 0°C, 0.5×5 cm. After washing the column with 5 ml 4 mM potassium phosphate buffer, pH 7.0, containing 1 M KCl and 0.5% Triton X-100 and then with 5 ml 4 mM phosphate buffer, pH 7.0, containing 0.5% Triton X-100, it was allowed to stand for 30 min at room temperature. The adsorbed tyrosinase was then eluted with 8 ml 0.5 M methyl alpha-D-mannoside in 4 mM potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100.

Tyrosinase assay

The measurement of tyrosinase activity was based on the determination of 5-S-L-cysteinyl-L-dopa formed in the presence of D, L-dopa and L-cysteine (14). 0.3 ml of the eluate obtained from the preparation procedure described above was added to 0.7 ml of a solution containing L-dopa, D-dopa, L-cysteine and phosphate buffer. (The final concentrations were 1 mM of L- and D-dopa, 3 mM of cysteine and 0.25 M of the phosphate buffer.) The mixture was incubated at 37°C for five min under gentle air bubbling. The reaction was interrupted by the addition of 9 ml 0.4 M perchloric acid. After centrifugation the content of cysteinyldopas in the supernatant was determined by means of HPLC and electrochemical detection.

Cysteinyldopa determinations

The HPLC-system consisted of a LKB 2150 HPLC pump, a sampling-valve injector Rheodyne model 7120 (Rheodyne, Berkley, Calif., USA), equipped with a 100 µl loop; the column 125×4.6 mm, packed by the upward slurry method with Nucleosil C_{18}, 5 µm. (Machery, Nagel & Co., Duren, GFR); mobile phase 6 g methanesulphonic acid, and 3 g phosphoric acid per litre of MilliQ purified water. pH was adjusted to 3.0. Flow-rate 1.5 ml/h. An external standard containing a known amount of 5-S-L-cysteinyl-L-dopa was injected immidiately prior to each determination.

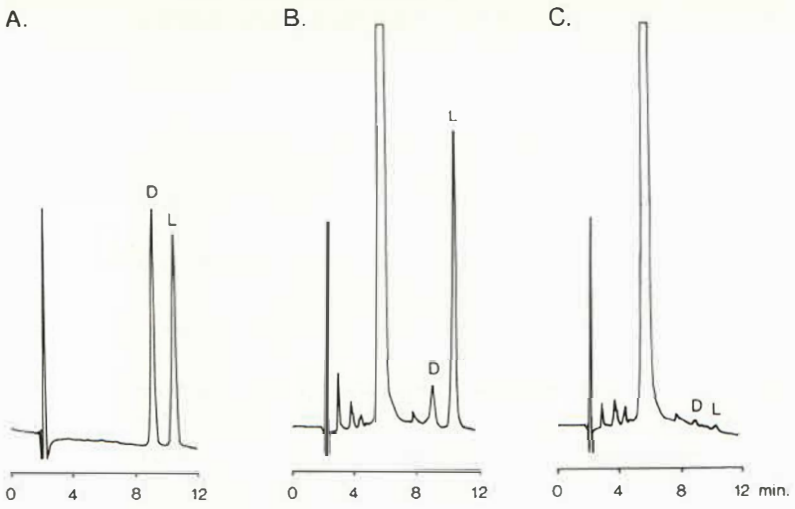

Fig. 1. HPLC chromatograms. (A) Equal quantities of 5-S-L-cysteinyl-D-dopa (D), and 5-S-L-cysteinyl-L-dopa (L). (B) Incubate with eluate from black mouse skin. (C) Incubate with eluate from albino mouse skin.

Detection: A model LC-10 amperometric detector (Bioanalytical Systems Inc., West Lafayette, Ind., USA) was used. The detector was operated at 0.75 V vs. an Ag/AgCl (3 M NaCl) reference electrode. the working electrode was prepared from carbon paste, CPO (Bioanalytical Systems Inc., West Lafayette, Ind., USA). Chromatography integrator: SP 4270 (Scandia Metric AB, Solna, Sweden).

The possible occurrence of a tyrosinase inhibitor in the eluate of albino skin was investigated by mixing equal quantities of Con-A eluates from albino and black skin. Dopa oxidase activities in the eluates from the albino skin, from the black skin, and in the mixed eluates were then determined as described above.

RESULTS AND COMMENTS

In black mouse skin, a mean amount of 33 nmole 5-S-L-cysteinyl-L-dopa per minute and g wet weight was formed, and 2.7 nmole 5-S-L-cysteinyl-D-dopa. In albino skin the mean values were 0.36 and 0.35 nmole, repectively.

Table I. *Diastereomers of 5-S-cysteinyldopa formed from L-dopa and D-dopa and L-cysteine incubated with tyrosinase fraction from mouse skin*

nmole/min and g wet weight

	5-S-L-cysteinyl-L-dopa	5-S-L-cysteinyl-D-dopa
Black	28	2.3
	39	2.8
	61	4.7
	17	2.4
	21	1.6
Mean	33	2.7
Albino	0.13	0.09
	0.38	0.38
	0.44	0.47
	0.47	0.44
Mean	0.36	0.35

These values indicate that the dopa oxidase activity found in the black mouse skin was stereospecific for L-dopa, with the yield of 5-S-L-cysteinyl-L-dopa being one order of magnitude higher than that of 5-S-L-cysteinyl-D-dopa. This tallies with our earlier findings on oxidation rates for L- and D-dopa (1).

When preparations from albino mouse skin were used, the values for 5-S-cysteinyldopas formed showed that the dopa oxidase activity in albino skin is not stereospecific. The nonspecific dopa oxidation was two orders of magnitude less than the enzymatic oxidation in black mouse skin.

The incubating system was devised as above in order to take into account the different reactions known to occur in melanin formation, and merits some comments.

The rise of cysteinyldopa was linear with tyrosinase concentration, and with time, for at least 5 min.

Cysteinyldopa does not seem to be a substrate for human tyrosinase (1). The oxidation of cysteinyldopa to cysteinyldopaquinone by dopaquinone is negligible, since, in the presence of excess cysteine, all dopaquinone formed is added to cysteine in a much faster reaction (21). In our system one eventually finds a slow, steady rise in absorption of visible light, indicating some cysteinyldopaquinone formation, but this does not take place in the short incubation time used (21).

When cysteine and dopa are incubated with tyrosinase, in addition to 5-S-cysteinyldopa, 2-S-cysteinyldopa, some 2,5-S,S-dicysteinyldopa, and minute amounts of 6-S-cysteinyldopa are also formed. The ratio between these isomers is constant (3), which means that for assaying the molar oxidation of dopa we need only to measure 5-S-cysteinyldopa. Under our conditions, 80% of the dopa consumed, can be retrieved as 5-S-cysteinyldopa (21).

The location of the block in melanin formation in different types of albinism is debated. Hearing postulated an inhibitor of tyrosinase activity. When coincubating subcellular fractions from the eyes of black and albino (Balb/c) mice he found that the albino fraction had an inhibitory effect on the tyrosinase from black mice (22).

Pomerantz & Li found measurable amounts of tyrosinase activity in the skin of albino (AKR/J) mice (23). In Tamate's recent study no tyrosinase was found in albino mice, while the dopa oxidase activity, measured as dopachrome formation indicated by the change of absorbancy at 475 nm differed by two orders of magnitude between albino and black mice (9).

When we coincubated fractions from black and albino mice, we could not detect any inhibition of tyrosinase activity, measured as dopa oxidase activity. The amounts of 5-S-CDs formed in this incubation were proportional to the amount of homogenate from the black mouse. Our results show that with our homogenization and purification procedures, there is no specific dopa oxidase activity in albino mouse skin. In addition, there is in this same fraction from albino mouse no inhibitor acting on the dopa oxidase activity in black mouse skin.

Albinism is defined as an inherited deficiency of pigment in skin, hair, and eyes: Oculacutaneous Albinism (OCA), or eyes only: Ocular Albinism (OA). In addition, albinos have optic and otic tract anomalities with nystagmus, photophobia, foveal hypoplasia, and abnormal routings of optic and otic fibers. In albinoidism, the hypopigmentation resembles that of albinism, but the optic neuronal pathways are normal. The classification of albinism is based on a combination of clinical and genetical features, ultrastructural differences, and tyrosinase activity in hair bulb incubation tests. Eleven different types of OCA and five types of OA are recognized, as are nine types of albinoidism (24). In studies of the tyrosinase activity of albinotic subjects the hair bulb test (25) has been highly useful. Adequate genetic counselling calls for a specific and sensitive method for the determina-

tion of tyrosinase activity. The method used by us in this and some previous studies has recently been applied to tyrosinase obtained from hair bulbs, and has been found to correlate well with the tritiated water assay (26).

The great advantage of the tyrosinase assay here described is its built-in control of nonspecific oxidation. The ratio between cysteinyldopa enzymatically formed from L-dopa and D-dopa should be constant in a defined system when nonspecific oxidation is absent or negligible. Another advantage of the method is the use of nonradioactive material. The skin sample is treated in a comparatively gentle manner, minimizing the risk for chemical degradation of tyrosinase prior to assay.

The specificity, sensitivity, and relative simplicity of the method used in this study will certainly be of value in further investigation of tyrosinase in albinism and other melanin disorders.

ACKNOWLEDGEMENTS

This investigaiton was supported by grants from the Swedish Cancer Society (Project 626-B86-14XB), the Swedish Medical Research Council, the Walter, Ellen and Lennart Hesselman Foundation for Scientific Research, the Edvard Welander Foundation for Scientific Research, and the donation funds of the Faculty of Medicine, University of Lund.

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