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# Dopa Oxidation and Tyrosine Oxygenation by Human Melanoma Tyrosinase

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A tyrosinase purified from cultured human melanoma cells was studied for dopa oxidation and tyrosine oxygenation. $K_m$ for oxidation of L-dopa was 0.5 mM, and for D-dopa 3 mM. L-tyrosine was oxygenated only in the presence of a cosubstrate. L-Dopa was superior to D-dopa, dopamine, L- and D-α-methyldopa, dopac, and 5,6-dihydroxyindole-2-carboxylic acid as cosubstrate. Ascorbic acid, 5-S-cysteinyldopa, and 5-OH-dopa did not function as cosubstrates. The rate of tyrosine hydroxylation was much lower than that of dopa oxidation. Tyrosine inhibits dopa oxidation, and dopa in high concentrations inhibits tyrosine hydroxylation. The cosubstrate function of dopa, the substrate functions of dopa and tyrosine, and the mutual inhibition of tyrosinase by these compounds are explained in three equations. *Key words: Copper; Melanin; 5-S-Cysteinyldopa.* (Received April 16, 1983.)

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The principles for the action of mammalian tyrosinases were established more than three decades ago by Lerner et al. (1). They showed that a tyrosinase preparation from Harding-Passey mouse melanoma catalysed dopa-formation from tyrosine in the presence of dopa, and that copper was essential for the activity of the enzyme (2). Pomerantz et al. made comprehensive studies on hamster melanoma tyrosinase, and described the kinetics of the enzyme in its monophenolase and diphenolase functions (3–6). They found L-dopa to be the best cosubstrate for the catalysis of tyrosine hydroxylation by tyrosinase. In recent years Hearing and collaborators have performed extensive investigations on tyrosinase isolated from normal and malignant mouse melanocytes (7–9). They determined the steric requirements for cosubstrate function, and compared tyrosine hydroxylation and melanin formation in prolonged experiments.

The above-mentioned investigations on mammalian tyrosinases and studies on mushroom and Neurospora tyrosinase (10–13) have led to better understanding of the mechanism of tyrosinase action. Two reviews from 1971 and 1981 describe the progress in this area (14, 15).

Tyrosinase has also been prepared from human melanomas (16–18), and a particulate form ($T_3$) has been solubilized. The electrophoretic mobility of the soluble enzyme ($T_1$) can be changed to another position ($T_2$) by treatment with neuraminidase. The result supports a dynamic conversion of $T_3 \rightarrow T_1 \rightarrow T_2$ (19). We have previously described the presence of tyrosinase in the medium of cultured human melanoma cells (20). Tyrosinase from such cells has now been purified, and some data on the oxidation of dopa and the hydroxylation of tyrosine by this enzyme will be presented.

## MATERIAL AND METHODS

*Chemicals.* L-Tyrosine (Sigma), L-dopa (Merck), D-dopa (Sigma), dopamine (Sigma), dopac (Fluka AG), 5-OH-dopa (Hoffman-La Roche), L-α-methyldopa (Dumex Ltd), D-α-methyldopa (Dumex Ltd),

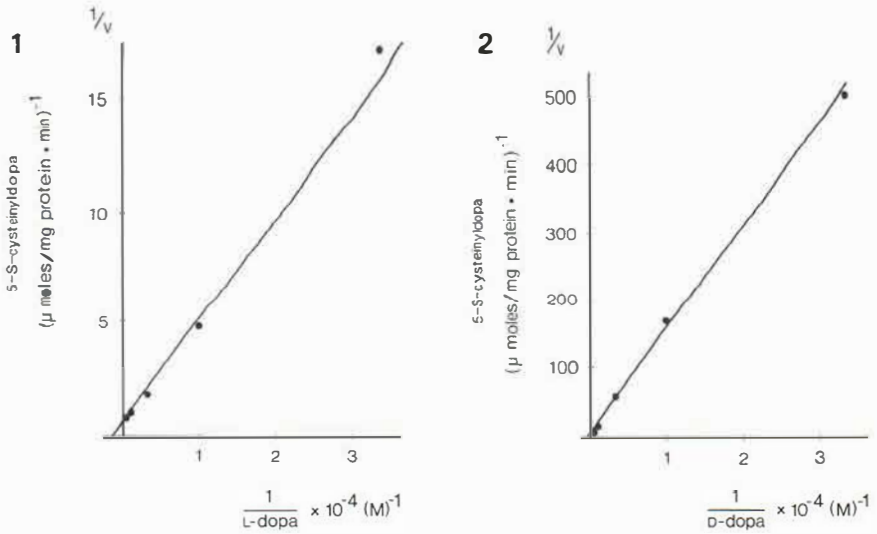


*Fig. 1.* Lineweaver-Burk plot for oxidation of L-dopa by human tyrosinase. Incubates contained human tyrosinase (80 μg protein), L-dopa as substrate, 1 mM cysteine, and 0.5 M sodium phosphate buffer, pH 7.4. Dopa oxidation was measured as the formation of 5-S-cysteinyldopa. Incubations were for 3 min at 37°.

*Fig. 2.* Lineweaver-Burk plot for oxidation of D-dopa by human tyrosinase. Incubates contained human tyrosinase (80 μg protein), D-dopa as substrate, 1 mM cysteine, and 0.5 M sodium phosphate buffer, pH 7.4.

ascorbic acid (Analar). 3,5-$^3$H-tyrosine (Amersham). 5-S-L-cysteinyl-L-dopa was prepared enzymatically, and purified as recently described (21). 5,6-Dihydroxyindole-2-carboxylic acid was prepared from 5,6-dibenzoxyindole-2-carboxylic acid (Sigma) by the method of Benigni and Minnis (22).

*Cultures* of a human pigment-producing melanoma cell line (IGR 1) were obtained from Dr Christian Aubert, Marseille, and kept since March 1982 in culture at the Tornblad Institute, University of Lund, by methods previously described (23). The medium used was minimal essential medium MEM +15% fetal calf serum (Flow).

All purification procedures were carried out at 0°C. Cells, 0.4 g, were suspended in 10 ml of 0.01 M phosphate buffer, pH 7.8, and disrupted by sonication. The suspension was centrifuged at 35 000 g for 2 h to remove as much melanin as possible. Triton X-100 was added to the supernatant to a final concentration of 0.1 %, and the suspension was then centrifuged at 105 000 g for 2 h. The supernatant was passed through a hydroxylapatite (Bio Gel HTP) column (0.9×10 cm). Elution was performed with a linear 0.01–0.3 M gradient of sodium phosphate, pH 7.8, in a total volume of 500 ml. The buffer contained 0.1% of Triton X-100. The effluent was collected in 8 ml fractions, and the tyrosinase activity of the fractions was determined by the dopachrome method (6).

Fractions containing tyrosinase activity were combined and purified further by affinity chromatography on Concanavalin A-Sepharose (Pharamacia Fine Chemicals, Uppsala, Sweden). This gel was washed with a solution containing 10 mM Tris-HCl, pH 7.2, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ prior to use. The column (0.5×5 cm) was equilibrated with 4 mM potassium phosphate buffer, pH 7.0, also containing 1 M KCl. After applying the sample the column was washed with 5 ml of the phosphate buffer containing 1 M KCl, and then with 5 ml of buffer alone. These steps were carried out at 4°C. Elution was performed at room temperature with 6 ml 1 M α-methyl-D-mannoside in the buffer. All solutions used for washing and elution of the Sepharose column contained 0.1 % Brij 35. Fractions of 2 ml were collected. The protein content was analysed by absorbance at 280 nm. The degree of purification was more than 100 times by this procedure. Details of the purification will be published elsewhere.

*Liquid Chromatographic (HPLC) analysis of dopa and 5-S-cysteinyldopa*

A Varian model 5000 (Varian, Palo Alto, Calif., USA) solvent delivery system was used. Samples were injected with a valve injector Rheodyne model 7120 (Rheodyne, Berkeley, Calif., USA) equipped with a 100-μl-loop. A model LC-10 amperometric detector (Bioanalytical Systems Inc.,

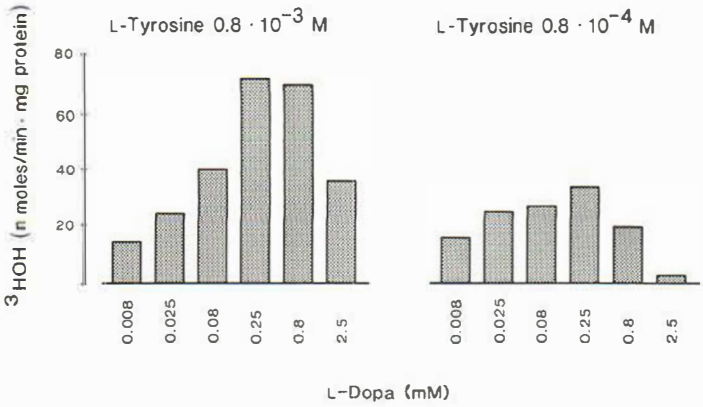


*Fig. 3.* Hydroxylation of L-tyrosine by human tyrosinase in the presence of different concentrations of L-dopa. The experiments were performed as described by Pomerantz (6), and tyrosine hydroxylation was measured as the formation of $^3$HOH from $^3$H-labelled L-tyrosine.

West Lafayette, Ind., USA) was used. The detector potential was +0.75 V vs. the Ag/AgCl (3 M NaCl) reference electrode.

The working electrode was prepared from carbon paste (CPO) material. Columns were packed in 250×4.6 mm stainless steel tubes with a chemically bonded 5 μm $C_{18}$ material (Nucleosil $C_{18}$, Machery, Nagel and Co., Düren, GFR). The mobile phase contained 6.0 g of methane sulphonic acid and 3.0 g of orthophosphoric acid per litre of MilliQ purified water. pH was adjusted to 3.00 with sodium hydroxide.

*Dopa oxidation* was studied by incubating L- or D-dopa of different concentrations with 0.5 M sodium phosphate buffer, pH 7.4, 1 mM cysteine, and 80 μg tyrosinase in a final volume of 1 ml. After 3 min incubation at 37° under air bubbling, perchloric acid was added to a concentration of 0.4 M, and the quantity of 5-S-cysteinyldopa was determined by HPLC (21). The reaction was linear with time under these conditions.

*Tyrosine hydroxylation.* The possible cosubstrate function of different compounds was examined by incubating 0.5 M sodium phosphate buffer, pH 7.4, 1 mM L-tyrosine, and 80 μg tyrosinase in a final volume of 1 ml for 5 or 10 min at 37° with 1 mM of the compounds to be examined. The reaction was linear with time under these conditions. Samples of 0.1 ml were withdrawn from the incubate and added to 0.9 ml of 0.4 M perchloric acid. Dopa was determined by direct injection of the samples on HPLC. The substances examined for cosubstrate function were L-dopa, D-dopa, L-α-methyldopa, D-α-methyldopa, dopamine, dopac, 5-OH-dopa, 5-S-cysteinyldopa, and 5,6-dihydroxyindole-2-carboxylic acid.

*Cosubstrate function of L- and D-dopa.* Solutions of (3,5-$^3$H)-tyrosine were incubated with L- and D-dopa (0.008–2.5 mM) in the presence of approximately 50 μg tyrosinase as described by Pomerantz (6). The quantity of tritiated water formed was determined.

*Inhibition of dopa oxidation by tyrosine.* The mixtures for the assays consisted of: 0.1 or 1 mM L-tyrosine; 0.5 or 3 mM L- or D-dopa; 1 mM cysteine; 0.5 M sodium phosphate buffer, pH 7.4; and 21 or 80 μg tyrosinase in a total volume of 1 ml. Incubations were performed for 3 min at 37° under constant air bubbling. They were stopped by adding 9 ml 0.4 M perchloric acid, and the formation of 5-S-cysteinyldopa was measured by HPLC.

## RESULTS

### *Dopa oxidation*

The rate of oxidation of L- and D-dopa by the melanoma tyrosinase is illustrated in Figs. 1 and 2, which show Lineweaver-Burk plots of 5-S-cysteinyldopa formed at different dopa concentrations. $K_m$ for L-dopa was 0.5 mM and for D-dopa 3 mM. $V_{max}$ was for L-dopa 1.5 and for D-dopa 0.2 μmol 5-S-cysteinyldopa formed per min and mg protein. 5-S-Cysteinyldopa represented about 80% of the quantity of dopaquinone formed under our experimental conditions.

### *Tyrosine hydroxylation*

No hydroxylation of L-tyrosine occurred in the absence of cosubstrate, but L-tyrosine was hydroxylated in the presence of L- and D-dopa, L- and D-α-methyldopa, dopamine, dopac,

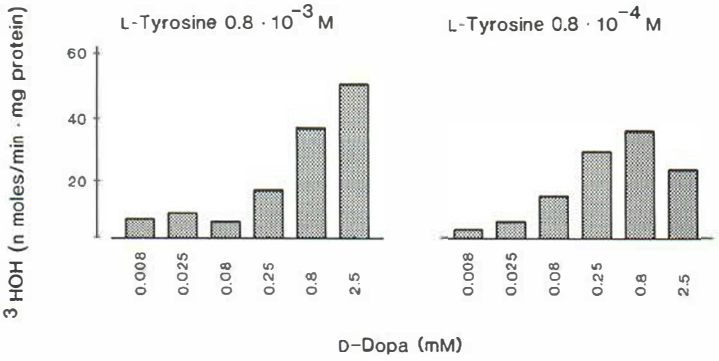


*Fig. 4.* Hydroxylation of L-tyrosine by human tyrosinase in the presence of different concentrations of D-dopa. The experiments were performed as described by Pomerantz (6), and tyrosine hydroxylation was measured as the formation of $^3HOH$ from $^3H$-labelled L-tyrosine.

and 5,6-dihydroxy-indole-2-carboxylic acid. L-Dopa was the most effective cosubstrate. Addition of 1 mM ascorbic acid, 5-OH-dopa, or 5-S-cysteinyldopa did not initiate hydroxylation of tyrosine. Catalase (50 μg/ml) was used in these experiments to prevent oxygenation by possibly formed $H_2O_2$.

The hydroxylation of L-tyrosine in the presence of L-dopa is illustrated in Fig. 3. The maximum rate of hydroxylation was obtained at a dopa concentration of 0.25 mM, which is close to the $K_m$ for L-dopa in the L-dopa oxidation reaction. L-Dopa at higher concentrations inhibited tyrosine hydroxylation, and this inhibition was more pronounced at the lower concentration of tyrosine.

D-Dopa also initiated the hydroxylation of L-tyrosine (Fig. 4). At 0.8 mM L-tyrosine the highest rate of hydroxylation was found with the highest concentration of D-dopa tested (2.5 mM), which is close to the observed $K_m$ for the D-dopa oxidase function of tyrosinase. At 0.08 mM L-tyrosine the highest rate of hydroxylation was found at 0.8 mM D-dopa, while higher concentrations of D-dopa inhibited tyrosine hydroxylation.

The experiments on tyrosine hydroxylation at different concentrations of L- and D-dopa showed that this reaction was inhibited when the concentration of dopa was high in comparison with the tyrosine concentration. It could also be shown that tyrosine had an inhibitory effect on dopa oxidation (Fig. 5). This inhibition was most pronounced at dopa concentrations close to $K_m$ for the dopa oxidase function of tyrosinase, and it was related to the concentration of tyrosine.

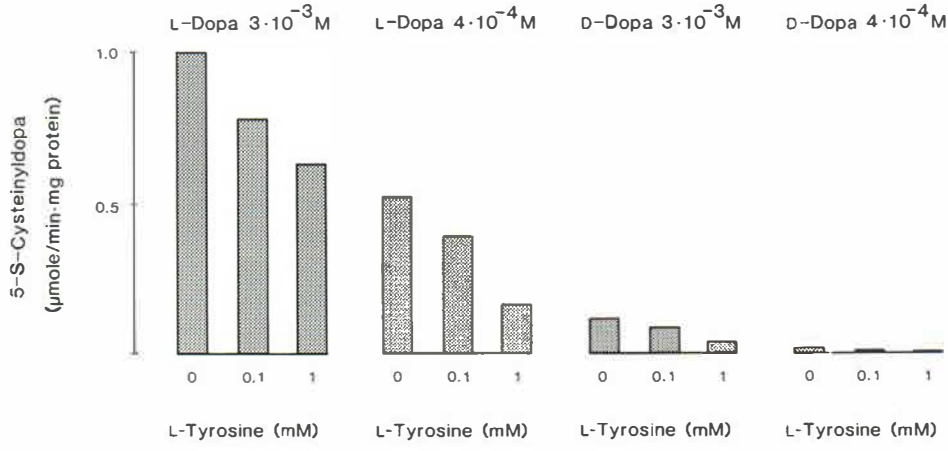


*Fig. 5.* Inhibition by L-tyrosine of the oxidation of L- and D-dopa catalysed by human tyrosinase.

## DISCUSSION

The diphenolase activity of tyrosinase purified from cultured human melanoma cells was studied by determination of 5-S-cysteinyldopa formed in the presence of excess cysteine. In such a system all dopaquinone formed combines with cysteine to give several isomeric products that can be identified by HPLC. About 80% of the total amount of the products formed under our reaction conditions is 5-S-cysteinyldopa. The other quantitatively important product is 2-S-cysteinyldopa. Monocysteinyldopas, which are formed instantly from dopaquinone, can be quantitated rapidly and sensitively by HPLC analysis (21). Thus, the diphenolase reaction can be measured by a direct method. Previously used methods measure either ascorbic acid oxidation by dopaquinone, or dopachrome formation (6).

The tyrosinase isolated from human melanoma had $K_m$ values for dopa in the dopa oxidation reaction that were close to those reported by Pomerantz (6) for hamster melanoma tyrosinase, and to values given by Miyazaki & Seiji (24) and Jimbow et al. (25) for mouse melanoma tyrosinases. The $K_m$ (1 mM) for L-dopa oxidation reported by Hearing & Ekel (7) for tyrosinase from normal C57 B1 skin is not comparable, since these workers used an indirect method based on determination of acid-insoluble oxidation products formed from $^{14}C$-labelled dopa. From the constants calculated for the oxidation of L-dopa and D-dopa (Figs. 1 and 2) it is evident that the L-isomer is oxidized at a higher rate, and that this isomer has a much higher affinity for the enzyme than has D-dopa.

L-Dopa was clearly superior to D-dopa as a cosubstrate for tyrosine hydroxylation. The values of Figs. 3 and 4 show that the rate of tyrosine hydroxylation was dependent not only on the concentration of L- or D-dopa, but also on the concentration of L- and D-dopa relative to that of tyrosine. It should be noted that the interpretation of the experiments using D-dopa as a cosubstrate of tyrosinase is complicated by the fact that L-dopa is formed in the hydroxylation reaction, and will then act as a cosubstrate with a lower $K_m$ than that for D-dopa. Thus, an accurate calculation of the relative efficacy of D-dopa as a cosubstrate will be difficult.

The kinetics for tyrosine hydroxylation by our human enzyme preparation seem to be similar to those of tyrosinase from hamster melanoma cells (6). However, we have not calculated any $K_m$ for tyrosine for reasons given below. The prolonged incubation time used by Hearing & Ekel (7) in their studies on mouse skin tyrosinase precludes a comparison with our findings on the human enzyme.

The rate of dopa oxidation found in this study was more than 10 times higher than that of tyrosine hydroxylation. This is in contrast to the results of Nishioka (19), who reported approximately the same rates of dopa oxidation and tyrosine hydroxylation by a human tyrosinase. A recently reported hydroxylation reaction of tyrosine catalysed by an enzyme preparation from normal human skin apparently differs in nature from the tyrosinase reaction, because dopa was not required as a cosubstrate for the skin enzyme (26). Interpretation of the results obtained with this enzyme is made difficult by the prolonged incubation times used without adequate control of any nonspecific formation of dopa.

One molecule of human tyrosinase seems to contain 2 atoms of copper (19). The fact that tyrosine hydroxylation occurred only in the presence of dopa suggests that the copper atoms were in the cupric state in our enzyme preparation and that tyrosinase expresses its oxygenase function only after reduction of the copper atoms to the cuprous state.

The reasoning on the function of dopa as cosubstrate and substrate for tyrosinase, and of tyrosine as substrate, is simplified by the following equations.

Abbreviations:

E =tyrosinase with copper valency not specified

$E_{ox}$ =tyrosinase with oxidized copper

$E_{red}$ =tyrosinase with reduced copper
d =dopa
dq =dopaquinone
t =tyrosine

I. *a*. $E_{ox}+d \rightarrow E_{ox}d$
*b*. $E_{ox}d \rightarrow E_{red}+dq+2H^+$

II. *a*. $E_{red}+O_2+d \rightarrow EO_2d$
*b*. $EO_2d+2H^+ \rightarrow E_{ox}+dq+2H_2O$

II′. *a*. $E_{red}+O_2+t \rightarrow EO_2t$
*b*. $EO_2t+2H^+ \rightarrow E_{ox}+d+H_2O$

These equations describe in full the function of tyrosinase in its dopa oxidase (I+II) and its coupled dopa oxidase-tyrosine hydroxylase (I+II′) functions.

Calculation of the detailed kinetics of tyrosinase is difficult because the active site of tyrosinase reacts with dopa, oxygen, and tyrosine. Analysis, too, is complicated by the fact that tyrosinase deals with dopa in two different ways, dopa molecules being oxidized either as cosubstrate in reaction I or as substrate in reaction II.

Equation I describes the reduction of the active centre of tyrosinase, which is an obligate step in the function of human tyrosinase. This reduction requires a reductant with high stereospecificity; L-dopa, as the natural reducing compound, is oxidized to dopaquinone. Equations II and II′ describe alternative reactions. According to equations I and II the enzyme functions as dopa oxidase only. Equations I and II′ describe the tyrosine hydroxylase function of tyrosinase with dopa as a cosubstrate.

A comparison of $V_{max}$ for the oxidation of L-dopa (1.5 μmole 5-S-cysteinyldopa/min×mg protein, corresponding to 1.8 μmole dopaquinone/min×mg protein; Fig. 1) with the maximum rate of tyrosine hydroxylation (71 nmoles/min×mg protein; Fig. 3) shows that tyrosine hydroxylation according to equation II′ is much slower than dopa oxidation according to equations I and II. The relative amounts of dopa and tyrosine available will decide to what extent the enzyme functions according to equations I and II or I and II′. If the relative concentration of dopa is great compared to tyrosine, reaction II will dominate over II′ and the dopa oxidase reaction will prevail; on the other hand, II′, i.e. tyrosine hydroxylase, will prevail if the concentration of tyrosine is relatively high. The data given in Figs. 1 and 2 reflect the sum of dopa oxidation according to equations I and II, i.e. the same amount of dopa is oxidized as cosubstrate and as substrate. If the dopa levels are far below $K_m$ and tyrosine concentrations are high, dopa will be formed from tyrosine while dopa oxidation will be largely restricted to the reaction according to equation I (which step is mandatory for the function of the enzyme).

$K_m$ for tyrosine oxygenation will vary with the concentration of dopa. At a low dopa concentration a Lineweaver-Burk plot of tyrosine hydroxylation will not give a straight line since tyrosine at high concentrations seems to block the dopa-dependent reduction of the enzyme. Tyrosine cannot reduce the copper atoms which remain oxidized, because tyrosine also prevents the access of dopa to the active centre of the enzyme. Dopa deoxygenates the enzyme according to equation II, becoming oxidized to dopaquinone with the formation of water. By this reaction tyrosine oxygenation according to equation II′ is inhibited. There is thus a mutual inhibition of tyrosinase by dopa and tyrosine. $V_{max}$ for tyrosine hydroxylation can be estimated from Lineweaver-Burk plots, but the rates calculated from such plots will never be reached because of the mutual inhibition of dopa and tyrosine. We have therefore refrained from such calculations.

The kinetic observations made on tyrosine oxygenation and on oxidation of L- and D-

dopa will be of great value designing methods for demonstration of tyrosinase in serum and tissues, where nonspecific tyrosine hydroxylation and dopa oxidation have created considerable problems (27). The stereospecificity of human tyrosinase for L-dopa, which at low concentrations is oxidized at 30 times higher rate than D-dopa, provides an excellent tool for analysis of tyrosinase activity. The nonspecific oxidation of dopa that is not stereospecific can easily be monitored by the oxidation of D-dopa. The recently developed method for analysis of diastereomers of 5-S-cysteinyldopa will be helpful in this work (21).

The inability of human tyrosinase to oxidize and use 5-S-cysteinyldopa and 5-OH-dopa as cosubstrates illustrates the high specificity of the human enzyme in contrast to mushroom tyrosinase (28, 29).

The finding that 5-S-cysteinyldopa, the quantitatively dominant catecholic amino acid of the melanocyte, does not act as a cosubstrate for human tyrosinase simplifies the picture of melanization in man. Dopaquinone formed by the oxidation of dopa becomes all important as oxidant of the compounds in the cysteinyldopa pathway. The fact that 5,6-dihydroxyindole-2-carboxylic acid can function as cosubstrate for tyrosinase in model experiments may be of little significance in vivo, since dopa is much better as cosubstrate and since 5,6-dihydroxyindoles are oxidized by dopaquinone (30).

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