SHORT REPORTS

Production of 6-Hydroxydopa by Human Tyrosinase

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A tyrosinase obtained from cultured human melanoma cells was found to oxygenate 2,4-dihydroxyphenylalanine to the strongly cytotoxic amino acid 6-hydroxydopa (2,4,5-trihydroxyphenylalanine). The oxygenation was dependent on the presence of a reducing co-substrate such as dopa or dopamine. The rate of oxygenation of 2,4-dihydroxyphenyl-D,L-alanine was similar to that of L-tyrosine, the normal substrate of tyrosinase. The enzymatic reaction demonstrated may prove of value in the chemotherapy of human melanoma. *Key words: 2,4-Dihydroxyphenylalanine; Dopa; Melanocyte; Melanoma chemotherapy.* (Received October 6, 1984.)

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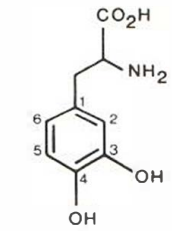
Dopa and other precursors of melanin have cytotoxic properties (1, 2, 3). Similar compounds such as 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine) are neurotoxins that destroy certain catecholamine neurons and produce "chemical denervation" (4). The corresponding amino acid 6-hydroxydopa (2,4,5-trihydroxyphenylalanine) also shows a selective toxicity to melanoma cells (5) which is much higher than that of the natural melanin precursor dopa (3), but the general toxicity of 6-hydroxydopa has precluded its use in melanoma therapy. The recent observation that mushroom tyrosinase or a homogenate of B-16 melanoma tissue can catalyse the formation of 6-hydroxydopa from 2,4-dihydroxyphenylalanine suggests that the melanocytotoxic effect of 6-hydroxydopa could be obtained without general toxicity through the selective production of 6-OH-dopa in melanocytes containing tyrosinase (6). We therefore undertook the present investigation on the possible oxygenation of 2,4-dihydroxyphenylalanine by human tyrosinase.

MATERIAL AND METHODS

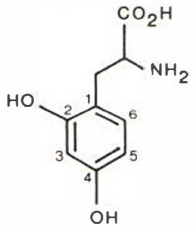
Chemicals. L-Tyrosine (Sigma), L-dopa (Merck), D-dopa (Sigma), dopamine (Sigma), L-6-OH-dopa (Labkemi AB, Gothenburg), ascorbic acid (BDH), superoxide dismutase (Sigma), catalase (Sigma). 2.4-dihydroxyphenyl-D,L-alanine was the gift of Dr Gerald Cohen, Department of Neurology, Mount Sinai School of Medicine, New York.

Human tyrosinase was prepared from medium of a pigment-producing melanoma cell line (IGR 1) obtained from Dr Christian Aubert, Marseille, and kept since March 1982 in culture at the Tornblad Institute, University of Lund. The medium used was minimal essential medium (MEM) +15% fetal calf serum (Flow) in which cells had grown for 3 days. The medium was treated with ammonium sulphate for 30 min on ice, and the precipitate obtained between 30 and 50% saturation was dissolved in 10 mM phosphate buffer, pH 7.4. The solution was dialysed for 48 h against 2×5 l of 10 mM phosphate buffer, pH 7.4 in cold. The dialysed protein was then applied on an ion exchange column (DEAE Sephacel), volume 20 ml, equilibrated with 1 l 10 mM phosphate buffer, pH 7.8. The column was eluted with an increasing NaCl gradient (10 mM phosphate, 0.3 M NaCl pH 7.8) by using a gradient mixer (4M-1 Pharmacia Fine Chemicals AB). Samples were collected in 6 ml fractions, and the tyrosinase activity was determined by oxidation of D,L-dopa 10^{-3} M in the presence of 3×10^{-3} M L-cysteine to 5-S-cysteinyldopa analysed by HPLC and electrochemical detection. Protein content was measured by absorption at 280 nm. Fractions containing high tyrosinase activity were used for further purification on concanavalin-A sepharose (Pharmacia). The column was equilibrated with 90 ml of phosphate buffer containing 1 M KCl, pH 7.0. After sample application the column was washed with 2×5 ml 4 mM phosphate buffer, in 1 M KCl, pH 7.0, and then with 2×5 ml 4 mM phosphate buffer at pH 7.0. Elution was performed at room temperature in 2 ml-fractions with 1 M α-methyl-D-

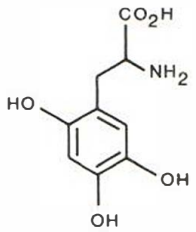


(3,4-dihydroxyphenyl) alanine

= dopa



(2,4-dihydroxyphenyl) alanine



(2,4,5-trihydroxyphenyl) alanine

= 6-OH-dopa

Fig. 1. Structures of the amino acids discussed. 6-OH-Dopa is given as a derivative of dopa even though in the present study it is formed from 2,4-dihydroxyphenylalanine.

mannoside in 4 mM phosphate buffer, pH 7.0. The tyrosinase activity and protein content were determined in these fractions. Fractions number 1 and 2 with the highest tyrosinase activity were analysed for oxygenation of 2,4-dihydroxyphenylalanine.

Liquid chromatographic (HPLC) analysis of dopa and 6-OH-dopa. 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., West Lafayette, Ind., USA) was used. The detector potential was +0.75 V vs. the Ag/AgCl reference electrode.

The working electrode was prepared from carbon paste (CPO) material. Columns were packed in 250×4.6 mm stainless steel tubes with chemically bonded 5µm 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 ortho phosphoric acid per 1 of MilliQ purified water. The pH was adjusted to 1.75 or 2.50 with sodium hydroxide.

All *incubation* volumes were 1 ml and contained 100 µl of the tyrosinase solution, 25 µg of superoxide dismutase, and 10 µg of catalase in 0.2 M phosphate buffer, pH 7.4. The concentration of 2,4-dihydroxyphenyl-D,L-alanine was 10 mM. Ascorbic acid (3 mM) was added as reducing agent. Incubations were performed for 5 min at 37°C under constant air bubbling. They were stopped by

Table I. *Formation of 6-OH-dopa (nmol/5 min) from 2,4-dihydroxyphenyl-D,L-alanine by human tyrosinase*

All values are mean values for 4 incubations. Variations less than ±10%

Cosubstrate	6-OH-dopa
None	0
L-Dopa	9.6
Dopamine	1.8

adding 100 µl of incubate to 900 µl 0.4 M perchloric acid, and the formation of 6-OH-dopa was measured by HPLC.

The importance of co-substrate for oxygenation of 2,4-dihydroxyphenylalanine was examined by performing incubations with and without addition of 10 µg 3,4-dihydroxyphenyl-L-alanine (0.05 mM).

The rate of formation of 6-OH-dopa from 2.4-dihydroxyphenylalanine was compared with the rate of formation of dopa from L-tyrosine. Incubations were performed as above, but in these experiments dopamine (0.05 mM) was used as co-substrate instead of dopa. Concentrations of 2,4-dihydroxyphenylalanine and of tyrosine were 10 mM.

RESULTS

There was no measurable formation of 6-OH-dopa without added co-substrate. With dopa as co-substrate 9.6 nmol of 6-OH-dopa was formed in 5 min, and with dopamine as co-substrate 1.8 nmol was formed (Table I). The formation of dopa from tyrosine with dopamine as co-substrate was 1.3 nmol per 5 min. Thus the rates of oxygenation of L-tyrosine and of 2,4-dihydroxyphenyl-D,L-alanine were of the same order of magnitude.

DISCUSSION

Oxygenation of 3,4-dihydroxyphenylalanine (dopa) in 5-position is a function of mushroom tyrosinase (7, 8), and oxygenation of 2,4-dihydroxyphenylalanine in 5-position has also been shown to occur (6). The present results extend the observations of Morrison and Cohen to human tyrosinase. Like the ortho-oxygenation of tyrosine by human tyrosinase, the oxygenation of 2,4-dihydroxyphenylalanine is dependent on the presence of a co-substrate, e.g. dopa or dopamine (9).

In addition to the chemical interest of the new enzymatic reaction, the formation of 6-OH-dopa by human tyrosinase may prove useful in the chemotherapy of malignant melanoma, because the cytotoxic effects of 6-OH-dopa are very pronounced. The cytotoxic properties of 6-OH-dopa seem to be due to the susceptibility of this substance to oxidation (10). It is possible that the cytotoxicity is mediated by oxygen radicals or H_2O_2 formed at oxygenation of 6-OH-dopa (11). Another explanation of the cytotoxicity of 6-OH-dopa could be the reaction of the quinone formed by oxidation of 6-OH-dopa with nucleophilic groups of cellular macromolecules (4, 5, 12, 13).

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