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Novel Substrates for Tyrosinase Serve as Precursors of 6-Hydroxydopamine and 6-Hydroxy-Dopa†

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ABSTRACT: Tyrosinase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) hydroxylates 2,4-dihydroxyphenylethylamine and the corresponding amino acid 2,4-dihydroxyphenylalanine in the 5-position. The products are commonly referred to as "6-hydroxydopamine" and "6-hydroxy-Dopa", respectively; they are well-known neurotoxins that are taken up into catecholamine neurons and destroy these neurons. The enzymatic products of the tyrosinase reaction were identified by their retention times on analyses performed by high-performance liquid chromatography (HPLC) with electrochemical detection and by comparison of their electrochemical properties with known standards. In addition, standards were available in the phenylethylamine series for 2,3,4- and 2,4,6-trihydroxyphenylethylamine. These compounds could be separated from 6-hydroxydopamine by HPLC and were not seen as products of the enzymatic reaction with 2,4-dihydroxyphenylethylamine. These results are of interest, since they provide a potential approach to the selective chemotherapy of malignant melanoma, a tyrosinase-rich tumor.

6-Hydroxydopamine (2,4,5-trihydroxyphenylethylamine) and 6-hydroxy-Dopa (2,4,5-trihydroxyphenylalanine) are well-known neurotoxins that destroy catecholamine neurons in both the central and peripheral nervous systems (Kostrzewa & Jacobowitz, 1974). Both compounds autoxidize at rapid rates at neutral pH (Graham et al., 1978). The autoxidation of 6-hydroxydopamine has been well studied and results in the formation of hydrogen peroxide, superoxide, and the hydroxyl radical (Heikkila & Cohen, 1972, 1973; Cohen & Heikkila, 1974; Cohen et al., 1976). Reactive *o*- and *p*-quinones are also products of the autoxidative reactions. Neurotoxicity has been related to the products of autoxidation, particularly the hydroxyl radical (Cohen et al., 1976; Graham et al., 1978).

Tyrosinase is a copper-containing enzyme that catalyzes the formation of melanin from tyrosine in mammals, plants, fungi, and bacteria (Lerch, 1981). In insects, tyrosinase is involved in cuticle formation (Lerch, 1981). L-*p*-Tyrosine is the natural substrate for tyrosinase; it is hydroxylated to form L-3,4-Dopa. L-3,4-Dopa is then enzymatically oxidized to its quinone, which undergoes a series of enzymatic and nonenzymatic reactions to form deeply colored melanin pigments.

It was shown previously that mushroom tyrosinase can catalyze an unusual reaction, namely, hydroxylation of L-3,4-Dopa in the 5-position to form 5-hydroxy-Dopa (Hansson et al., 1980). Thus, it appears that the 3- and 5-positions, both meta to the side chain, are candidates for enzymatic hydroxylation by tyrosinase. We report here the enzymatic synthesis of 2,4,5-trihydroxyphenylethylamine and 2,4,5-trihydroxyphenylalanine from 2,4-dihydroxyphenylethylamine and 2,4-dihydroxyphenylalanine, respectively, via hydroxylation by tyrosinase in the 5-position.

Materials and Methods

Mushroom tyrosinase and standards of 6-hydroxydopamine and 6-hydroxy-Dopa were obtained from Sigma. B-16 mouse melanoma tissue was used as a source of mammalian tyrosinase; the tissue was obtained from Mason Research Institute and stored frozen over liquid nitrogen until use.

For studies with mushroom tyrosinase, a solution of 1 mM substrate (2,4-dihydroxyphenylalanine or 2,4-dihydroxyphenylethylamine) in 50 mM phosphate buffer, pH 6.8, containing 1 mM ascorbate, 50 μM L-3,4-Dopa, and 100 μM DTPA[1] was incubated on a shaker bath at 22 °C. The as-

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[1] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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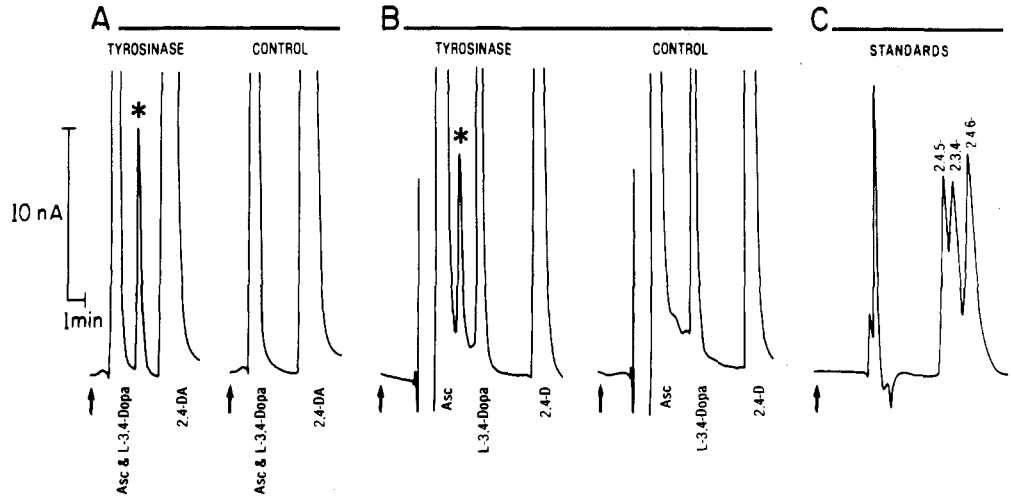

FIGURE 1: HPLC chromatograms illustrating the enzymatic synthesis of 2,4,5-trihydroxyphenylethylamine (6-hydroxydopamine), panel A, and 2,4,5-trihydroxyphenylalanine (6-hydroxy-Dopa), panel B, from 2,4-dihydroxyphenylethylamine and 2,4-dihydroxyphenylalanine, respectively. The reaction conditions and HPLC conditions for panels A and B are described under Materials and Methods. Panel C shows a separation of 2,4,5-, 2,3,4-, and 2,4,6-trihydroxyphenylethylamine in the buffer system used in panel A but at a slower flow rate of 1 mL/min. The point of sample injection is marked by an arrow. The product in panels A and B is marked by an asterisk. Mushroom tyrosinase was used. Abbreviations: Asc, ascorbate; 2,4-DA, 2,4-dihydroxyphenylethylamine; 2,4-D, 2,4-dihydroxyphenylalanine.

corbate served to maintain the product in the reduced state. L-3,4-Dopa served as cosubstrate (or activator) for tyrosinase (Pomerantz & Warner, 1967). DTPA is a chelating agent that serves to suppress metal-catalyzed autoxidation of the product, L-3,4-Dopa, and ascorbate. At zero time, mushroom tyrosinase was added to a final concentration of 0.06 mg/mL (i.e., 0.5 μM of the tetrameric form). Controls received an equal volume of either buffer or heat-inactivated enzyme in place of active enzyme. At 2, 4, and 6 min, aliquots of 0.45 mL were removed and added to 50 μL of 4 N perchloric acid with immediate mixing to quench the enzymatic reaction. Controls were sampled at 0 and 8 min. All samples were centrifuged at 8700g for 1 min (Beckman microfuge) and stored on ice.

Analysis of products was performed by HPLC (Waters) with electrochemical detection (Bioanalytical Systems) at an oxidizing potential of +0.9 V vs. a Ag/AgCl reference electrode (50-nA full scale, 20-μL injection). For analysis of 6-hydroxydopamine, a mobile phase consisting of 50 mM acetate buffer, pH 5.1, containing 1 mM EDTA, 2.5 mM sodium octyl sulfate (as a paired ion), and 20% methanol was used at a flow rate of 2.0 mL/min over a C-18 reverse-phase column (Waters, 10-μm bead, 25-cm length). For analysis of 6-hydroxy-Dopa, a mobile phase consisting of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA was used at a flow rate of 1.5 mL/min over a C-18 reverse-phase column (Altex, 5-μm bead, 25-cm length). Reaction rates (product accumulation) were calculated from a least-squares fit of the data at 2, 4, and 6 min.

Analysis of half-wave potentials was performed by repeated injection of the 6-min sample and standards at various electrode potentials. A freshly polished glassy carbon electrode was employed to provide increased efficiency and sensitivity. Peak height was used to monitor the extent of oxidation.

For studies with B-16 melanoma tyrosinase, a 10% homogenate of tissue was prepared in 50 mM phosphate buffer, pH 6.8, containing 0.1% sodium cholate. A glass homogenizing tube with a motor-driven Teflon pestle was used. The homogenate was stirred for 30 min in an ice bath and used immediately. The equivalent of 5 mg/mL of tissue (wet weight) was added to experimental flasks. Sodium cholate (0.1%) was present in the reaction mixture; other components were present as before. Controls received an equal volume of either buffer or heat-inactivated enzyme in place of active enzyme. Samples were removed at 0, 2, 4, 6, 10, and 15 min and quenched as above. Controls were sampled at 0, 8, and 17 min.

HPLC analysis of 6-hydroxy-Dopa was performed at an electrode potential of +0.4 V (10-nA full scale, 20-μL injection). Since the B-16 tyrosinase was much more dilute than the mushroom enzyme, the amount of product formed was less. The 6-hydroxy-Dopa peak appeared on the trailing shoulder of the ascorbate peak. At a potential of +0.4 V, the ascorbate peak was diminished to a much greater extent than the 6-hydroxy-Dopa peak. This provided the increase in sensitivity needed to analyze the smaller amounts of product.

Results and Discussion

Figure 1 shows sample chromatograms demonstrating the formation of 2,4,5-trihydroxyphenylethylamine and 2,4,5-trihydroxyphenylalanine from 2,4-dihydroxyphenylethylamine and 2,4-dihydroxyphenylalanine, respectively, as catalyzed by mushroom tyrosinase. The products are marked by an asterisk. The reaction rates were 1.31 μM/min (±0.03 SD, N = 3) for the formation of 2,4,5-trihydroxyphenylethylamine and 0.51 μM/min (±0.08 SD, N = 7) for the formation of 2,4,5-trihydroxyphenylalanine. In Figure 1, panel A, 2,4,5-trihydroxyphenylethylamine is seen after a 6-min incubation of precursor (2,4-dihydroxyphenylethylamine) in the presence of tyrosinase (left side of panel) but not in absence of enzyme (right side of panel). The starting material, 2,4-dihydroxyphenylethylamine, is also seen. The ascorbate and L-3,4-Dopa, which were present in the reaction mixture, appeared as a single merged peak under the conditions used in panel A for HPLC analysis. In panel B, a similar experiment is shown with 2,4-dihydroxyphenylalanine, and 2,4,5-trihydroxyphenylalanine is the product. Enzymatic synthesis is seen in the left side of panel B, while the right side shows a nonenzymatic control. Heat-denatured enzyme was inactive with either substrate (not shown). Under the chromatographic conditions used to separate the amino acid precursor and product in panel B, L-3,4-Dopa and ascorbate migrated as separate peaks. The products formed from 2,4-dihydroxyphenylethylamine and 2,4-dihydroxyphenylalanine cochromatographed with authentic 2,4,5-trihydroxyphenylethylamine

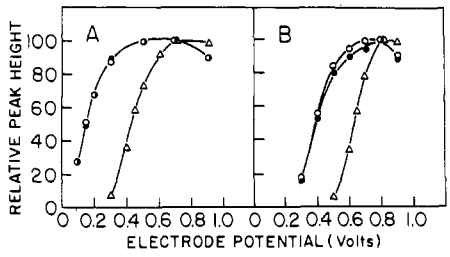

FIGURE 2: Electrochemical oxidation properties of standards and tyrosinase-generated products. Panel A shows the relative peak heights for tyrosinase-generated 6-hydroxydopamine (closed circles, 100 = 20.6 nA), authentic 6-hydroxydopamine (open circles, 100 = 23.4 nA), and 3,4-dopamine (open triangles, 100 = 28.4 nA) at different electrode potentials, pH 5.1. Panel B shows the relative peak heights for tyrosinase-generated 6-hydroxy-Dopa (closed circles, 100 = 19.9 nA), authentic 6-hydroxy-Dopa (open circles, 100 = 15.7 nA), and L-3,4-Dopa (open triangles, 100 = 16.0 nA) at different electrode potentials, pH 3.0. The maximum standard deviation in either panel for duplicate determinations at any point was 3.6 relative peak height units.

and 2,4,5-trihydroxyphenylalanine, respectively.

Because tyrosinase can hydroxylate in either the 3- or 5-position, two products might possibly be formed from the precursors. For example, with 2,4-dihydroxyphenylalanine, the possible products are 2,3,4- and 2,4,5-trihydroxyphenylalanine. For the phenylethylamine series, the appropriate alternate product, 2,3,4-trihydroxyphenylethylamine, was available. As shown in Figure 1, panel C, 2,3,4-trihydroxyphenylethylamine could be separated from 2,4,5-trihydroxyphenylethylamine at a slower flow rate. Under these conditions, 2,3,4-trihydroxyphenylethylamine was not detected as a product of the enzymatic reaction. Other theoretical products that might arise via hydroxylation in the 6-position are 2,4,6-trihydroxyphenylethylamine and 2,4,6-trihydroxyphenylalanine. A standard of 2,4,6-trihydroxyphenylethylamine was available. As shown in panel C of Figure 1, it also separated from 2,4,5-trihydroxyphenylethylamine. 2,4,6-Trihydroxyphenylethylamine was not detected as a product of the enzymatic reaction.

Figure 2 demonstrates that the electrochemical oxidation properties of each enzymatic product conform with those of authentic standards. To obtain these data, samples were injected repetitively for HPLC analysis with the detector set at various oxidation potentials (vs. a Ag/AgCl reference). The pH for analysis was that of the HPLC buffer. The half-wave potential of the product arising from 2,4-dihydroxyphenylethylamine (panel A) was 0.15 V at pH 5.1, as was that of authentic 2,4,5-trihydroxyphenylethylamine. The half-wave potential of the product arising from 2,4-dihydroxyphenylalanine (panel B) was 0.39 V at pH 3.0, similar to that of authentic 2,4,5-trihydroxyphenylalanine. 3,4-Dopamine (panel A) and L-3,4-Dopa (panel B) are included in Figure 2 for comparison (half-wave potentials = 0.43 V at pH 5.1 and 0.64 V at pH 3.0, respectively).

On the basis of cochromatography and similar electrochemical properties compared to known standards, as well as the elimination of the reasonable alternative products in the phenylethylamine series, the products arising from 2,4-dihydroxyphenylethylamine and 2,4-dihydroxyphenylalanine were identified as 2,4,5-trihydroxyphenylethylamine (6-hydroxydopamine) and 2,4,5-trihydroxyphenylalanine (6-hydroxy-Dopa), respectively.

In addition to being novel reactions, the production of the potent oxy-radical generating toxins, 6-hydroxydopamine and 6-hydroxy-Dopa, from nonautoxidizable precursors holds implications for the chemotherapy of malignant melanoma, a tyrosinase-containing tumor. 2,4-Dihydroxyphenylethylamine or 2,4-dihydroxyphenylalanine might serve as targeted "prodrugs" against malignant melanoma. The amino acid analogue, 2,4-dihydroxyphenylalanine, is of particular interest, since it is likely to be easily transported across the plasma membrane by amino acid carrier proteins. With this in mind, we determined the reaction rate of 2,4-dihydroxyphenylalanine with tyrosinase in an homogenate of B-16 melanoma tissue. The homogenate catalyzed the formation of 6-hydroxy-Dopa at a rate of 34 nM/min (±2 SD, $N = 4$). Heat-denatured enzyme was inactive. The homogenate was used at 200-fold dilution from the in vivo state, which implies a potential cellular rate of synthesis of 6-hydroxy-Dopa in the range of micromolar per minute. It was shown previously that micromolar levels of 6-hydroxy-Dopa added to cultures of melanoma cells produced potent inhibition of growth (Wick et al., 1979). Therefore, 2,4-dihydroxyphenylalanine is expected to produce toxic effects against melanoma cells. This idea is currently under investigation.

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