5-OH-DOPA, PRODUCT OF AND SUBSTRATE FOR TYROSINASE

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Abstract. The formation of 5-OH-dopa on incubation of tyrosine or dopa with mushroom tyrosinase was studied. Dopa oxidase activity was defined by measuring the formation of 5-S- and 2-S-cysteinyldopa from dopa in the presence of excess amounts of cysteine. This procedure quantitating the immediate nucleophilic reaction products of dopaquinone constitutes a new method for assessing tyrosinase activity. The rate of 5-OH-dopa formation from dopa was similar to that of the formation of dopa from tyrosine. The rate of dopaquinone formation was one order of magnitude higher. When tyrosine was oxidized in the presence of ascorbic acid, 5-OH-dopa formation represented 14% of the original amount of tyrosine. At low concentrations of dopa, formation of 5-OH-dopa was proportinal to the dopa concentration. At high concentrations the relative quantity of 5-OH-dopa formed decreased. Tyrosine in high concentrations inhibited the formation of 5-OH-dopa from dopa. 5-OH-dopa proved to be a substrate for tyrosinase. The rate of oxidation of 5-OH-dopa was similar to that of dopa. The oxidation products of 5-OH-dopa were transformed into relatively stable fluorophores.

Key words: 5-OH-Dopa; Dopa; Tyrosinase; 5-S-Cysteinyldopa; Ascorbic acid; Cysteine

Tyrosinase is the enzyme responsible for melanin synthesis. Two functions of tyrosinase involving molecular oxygen have been extensively studied (2, 3, 7–10, 13–16, 19, 20). One is the introduction of oxygen into the ortho position of tyrosine, and the other the dehydrogenation of dopa to dopaquinone. A third function of tyrosinase has recently been demonstrated, namely the formation of 5-OH-dopa from dopa (5). With ascorbic acid in the incubate, considerable accumulation of 5-OH-dopa occurs even in the presence of cysteine, which substance contains a strongly nucleophilic SH-group prone to react with the electron-deficient dopaquinone molecule. This finding indicates that 5-OH-dopa is formed not by addition of the weakly nucleophilic water to dopaquinone, since such addition would be prevented by the strongly nucleophilic thiol, but through hydroxylation of dopa by the tyrosinase (6).

Thus, tyrosinase may have two different effects on dopa, one oxygenating and one dehydrogenating. This could provide a clue to the puzzling reaction mechanism of the enzyme, which remains an enigma in spite of intense research.

This paper provides further data supporting the view that 5-OH-dopa is produced by the oxygenase function of tyrosinase, and also describes 5-OH-dopa as a substrate for this enzyme.

MATERIAL AND METHODS

Material

The chemicals used were L-tyrosine (Sigma), L-dopa (Merck), L-cysteine (Merck), L-ascorbic acid (Merck), and 5-OH-dopa (Hoffmann-La Roche). The preparation of 2-S-cysteinyldopa and 5-S-cysteinyldopa is described elsewhere (1). The enzyme used was mushroom tyrosinase (2230 U/mg, Sigma).

Performance of analyses

All catecholic amino acids were determined by high-pressure liquid chromatography using a model 6000 A (Waters Ass., Milford, Mass.) high-pressure liquid chromatograph with a Model 7120 100 μl sample valve injector (Rheodyne, Berkeley, Calif.) and electrochemical detector Model LC-10 (Bioanalytical Systems, West Lafayette, Ind.). The detection potential was set at +0.75 V against an Ag/AgCl reference electrode. The electrode was prepared from CPO graphite material. The column packing material was Nucleosil C_{18} (5 μm, Macherey, Nagel & Co, Düren, G. F. R.). Columns were 250×4.6 mm. The mobile phase contained 6 g methane sulphonic acid and 2.9 phosphoric acid per litre water. The pH was adjusted to 1.75 with 5 M NaOH. Isocratic elution was used, and the flow rate was 1.5 ml per min.

Experiments

Definition of dopaquinone formation by the tyrosinase used

Methods recently developed for measurement of cysteinyldopas (4) have provided excellent means of recording the rate of formation of the highly fugitive oxidation product dopaquinone as cysteinyldopas, since dopaquinone reacts with cysteine 1 000 times faster than the intracyclization reaction leading to indol formation (18). In an initial series of experiments, incubates of L-
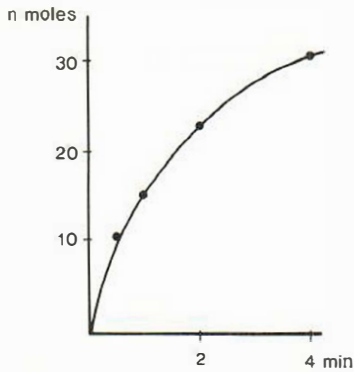
n moles

Fig. 1. Quantities of 2-S-cysteinyldopa + 5-S-cysteinyl-
dopa formed on incubation of L-dopa (10^{-3} M) and L-cys-
teine (10^{-2} M) with 10 μg tyrosinase in 1 ml 0.5 M phos-
phate buffer at 0°C.

µ moles

Fig. 3. Formation of L-dopa and 5-OH-dopa on incubation
of tyrosine (10^{-3} M) with 2 mg tyrosinase in the presence
of ascorbic acid (2×10^{-2} M). Incubation volyme 10 ml.

dopa, 10^{-3} M with 10 μg tyrosinase, and different amounts
of cysteine (3×10^{-2}, 10^{-2} and 3×10^{-3} M) in 1 ml 0.5 M
phosphate buffer (pH 6.5) were performed at 0°C under
constant air bubbling. Incubations were interrupted by
dilution with 25 ml 0.4 M perchloric acid, and the forma-
tion of 2-S- and 5-S-cysteinyldopas was measured after 30
sec and after 1, 2, and 4 min.

Under these conditions the oxidation of the monocys-
teinyldopas is absent or negligible, and the sum of 2-S- and
5-S-cysteinyldopas formed gives an almost exact measure
of dopaquinone formation. A third monocysteinyldopa,
6-S-cysteinyldopa, is also formed, but the yield is so low
that the amount of dopaquinone giving this product can be
disregarded.

Fig. 1 shows the sum of 2-S- and 5-S-cysteinyldopa
formed with 10^{-2} M cysteine after the different time inter-
vals. The rate of formation of cysteinyldopas declines
with time. This decline is more pronouced with higher
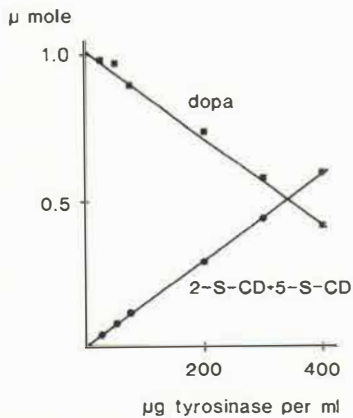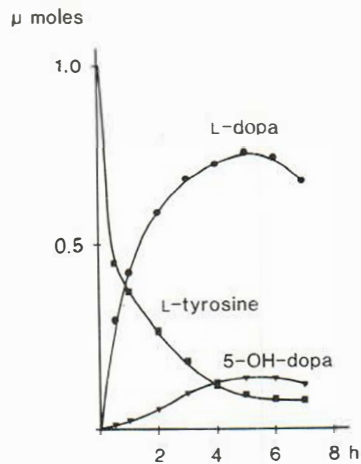concentration of cysteine. The advantage of performing

experiments at low temperature is evident, since this al-
lows approximation of the reaction curve to a straight line
when a short incubation time is used. Although a still
shorter incubation time would have been preferable, for
practical purposes we used an incubation time of 45 sec,
unless otherwise stated.

In a second experiment the rates of dopa consumption
and of cysteinyldopa formation were correlated to the
quantity of the enzyme, using the following amounts of
enzyme in the incubate: 10, 25, 50, 75, 200, 300, and 400
μg. The concentration of dopa was 10^{-3} M, and of cys-
teine. 10^{-2} M. Fig. 2 shows the results. The formation of
2-S- and 5-S-cysteinyldopa corresponds to the consump-
tion of dopa, and the rate is correlated to the quantity of
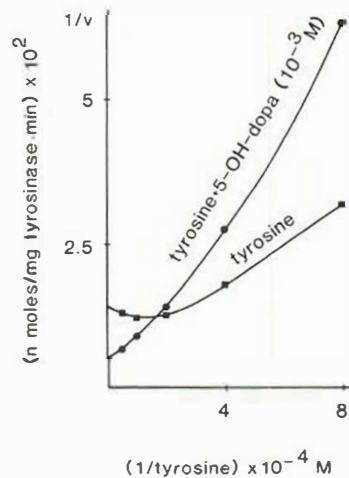tyrosinase. Under the conditions used, the dopaquinone

µ mole

µg tyrosinase per ml

Fig. 2. Decrease in dopa and increase in 2-S-cysteinyl-
dopa + 5-S-cysteinyldopa on incubation of dopa (10^{-3} M)
and cysteine (10^{-2} M) with varying amounts of tyrosinas
for 45 sec at 0°C.

(1/tyrosine) x 10^{-4} M

Fig. 4. Double.reciprocal plot of dopa formation on in-
cubation of different quantities of tyrosine alone and of
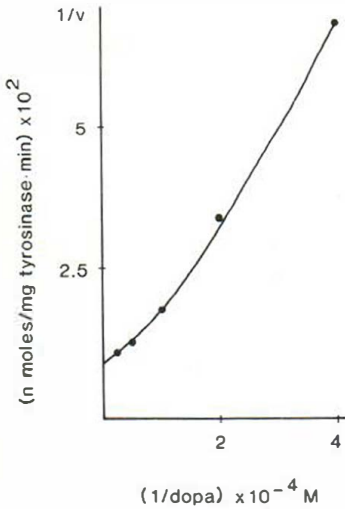tyrosine + 5-OH-dopa (10^{-3} M).

Fig. 5. Double reciprocal plot of 5-OH-dopa formation on incubation of various quantities of dopa with tyrosinase in the presence of ascorbic acid (10^{-2} M).

Fig. 7. Disappearance of 5-OH-dopa on incubation with tyrosinase. For comparison, the disappearance of dopa under identical conditions is given.

formation measured as the sum of 2-S- and 5-S-cysteinyl-dopa formed was 1.9 μmole per mg tyrosinase and minute, which corresponds to the quantity of dopa consumed.

Production of 5-OH-dopa on oxidation of tyrosine by tyrosinase

This experiment was performed at 25°C with 2 mg tyrosinase. The concentration of tyrosine was 10^{-3}M and of ascorbic acid, 2×10^{-2} M. Incubation was performed in 1 ml 0.5 M phosphate buffer at pH 6.5 with constant air bubbling. Samples of 0.1 ml were withdrawn from the incubate, and 9.9 ml 0.4 M perchloric acid was added.

There was rapid formation of dopa and a slower increase in 5-OH-dopa concentration (Fig. 3). The highest values for both products were observed after 5 hours' incubation. 76% of the original tyrosine quantity was then
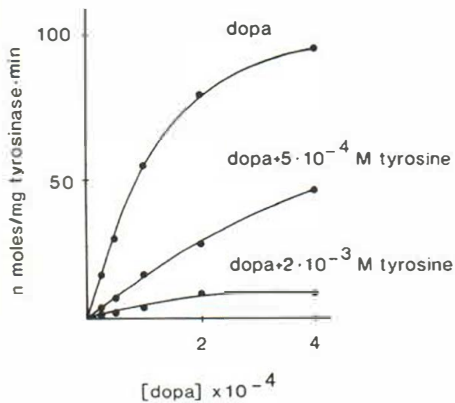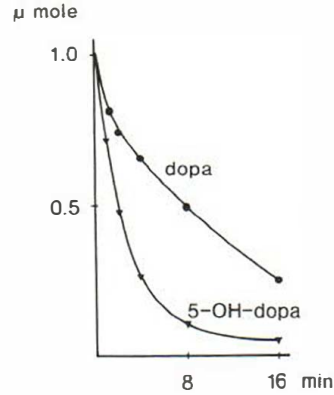
present as dopa, and the quantity of 5-OH-dopa represented 14% of the original amount of tyrosine.

The relative amounts of tyrosine and dopa available in the incubate are apparently of importance for the rate of 5-OH-dopa formation. No conclusion regarding the influence of 5-OH-dopa on the oxidation of tyrosine can be deduced from this experiment. This problem was analysed in the following experiment.

Influence of 5-OH-dopa on oxidation of tyrosine by tyrosinase

Various quantities of tyrosine were incubated in 1 ml 0.5 M phosphate buffer (pH 6.5) with 100 μg tyrosinase for 45 sec at 0° in the presence of 10^{-2} M ascorbic acid, and the dopa formation was measured. The experiments were performed with tyrosine alone as substrate, and with tyrosine in the presence of 5-OH-dopa 10^{-3} M. The results are shown as a double reciprocal plot (Fig. 4).

The curve for tyrosine alone shows the familiar inhibition by the substrate. This inhibition was reversed by 5-OH-dopa. At lower tyrosine concentrations 5-OH-dopa had an inhibitory effect on tyrosine oxidation.

V_{max} for tyrosine oxidation was about 150 nmol/mg tyrosinase and min, i.e. about 8% of V_{max} for dopaquinone formation from dopa.

Formation of 5-OH-dopa on oxidation of dopa by tyrosinase

The experiment on 5-OH-dopa formation during oxidation of tyrosine by tyrosinase in the presence of ascorbic acid (Fig. 3) gave only a rough idea of the rate of 5-OH-dopa formation from dopa, because of the complexity of the system. In the present experiment, various amounts of dopa were incubated with 50 μg tyrosinase in the presence of ascorbic acid 10^{-2} M for 45 sec at 0°C. The rates of 5-OH-dopa formation with the different amounts of dopa are seen in Fig. 5. The data do not give a straight line in a double-reciprocal plot, and rather complex kinetics are present in our system. The rates of 5-OH-dopa formation at the lower dopa concentrations are clearly proportional to the amounts of dopa. Relatively less 5-OH-dopa forms at the higher dopa concentrations.
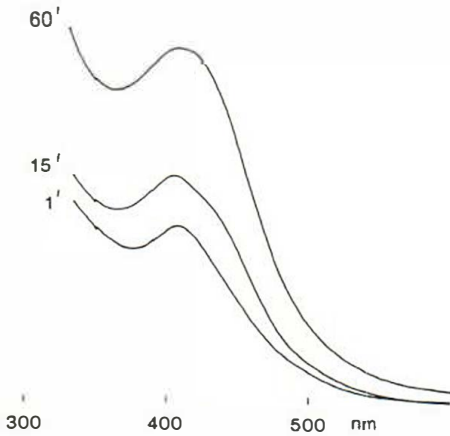

Fig. 6. Formation of 5-OH-dopa on incubation of dopa, alone or dopa + tyrosine with tyrosinase in the presence of ascorbic acid (10^{-2} M).

Fig. 8. Absorption spectra of oxidation products of 5-OH-dopa obtained during incubation with tyrosinase.

In a similar experiment, formation of 5-OH-dopa from dopa 10^{-3} M was studied also in the presence of tyrosine (2×10^{-3} M and 5×10^{-4}). At high concentrations, tyrosine strongly inhibited the formation of 5-OH-dopa from dopa. The inhibition seemed to be of a complex nature (Fig. 6).

V_{max} for formation of 5-OH-dopa from dopa was about 110 nmoles/mg tyrosinase/min.

The possibility that 5-OH-dopa may be formed nonspecifically by hydrogen peroxide, which might be produced in the reaction, was studied in an experiment in which 0.2 mg catalase was added to an incubate. No effect on 5-OH-dopa formation was observed.

Oxidation of 5-OH-dopa by tyrosinase

A 10^{-3} M 5-OH-dopa solution (1 ml phosphate buffer, pH 6.5) was incubated at 0°C with 400 μg tyrosinase, and the remaining quantity of 5-OH-dopa was determined after 1, 2, 4, 8, and 16 min (Fig. 7). For comparison a similar experiment was performed with dopa as substrate.

The faster decrease in 5-OH-dopa than dopa should not be taken as evidence that 5-OH-dopa is better substrate for tyrosinase, as considerable quantitites of dopaquinone are reduced to dopa by hydroxyindoline formed from dopa under the experimental conditions. In fact, the rate of oxidation of dopa may be greater than that of 5-OH-dopa, to judge from the experiment on dopa consumption and cysteinyldopa formation (Fig. 2).

1-ml solutions of 5-OH-dopa in concentrations varying from 6.25×10^{-5} M to 2×10^{-3} M were oxidized by 75 μg tyrosinase for 4 min at 25°C.

At concentrations below 10^{-3} M the rate of oxidation was proportional to the concentration of the substrate, but at higher concentrations of 5-OH-dopa, marked inhibition of oxidation occurred. Constants for 5-OH-oxidation by tyrosinase could therefore not be calculated.

When 5-OH-dopa was oxidized, compounds with maximum absorption at 405 nm were rapidly formed and were immediately tranformed into compounds which together had an absorption maximum at 415 nm (Fig. 8). The absorption at 415 nm increased with time, and a

fluorophore with excitation maximum at 425 nm and emission maximum at 500 nm appeared (uncorrected instrumental values). This fluorophore showed a pronounced stability to further oxidation (Fig. 9).

DISCUSSION

The formation of 5-OH-dopa by tyrosinase represents a function of this enzyme that, until recently, had remained obscure (5). However, previous studies have considered the formation of 6-hydroxydopa in melanogenesis (11, 17), and a methylated derivative of 6-hydroxydopa has actually been identified as a product of a tyrosinase-containing microorganism, *Microspira tyrosinatica* (12). We have found no evidence of formation of 6-OH-dopa under our conditions (5).

The definition of the activity of the enzyme used by measuring the formation of cysteinyldopas calls for some comments. Cysteine acts as a reducing substance and as a nucleophile reacting with dopaquinone. There seems to be no direct interaction between cysteine and the active site of the enzyme, because the L- and D-forms of cysteine produce cysteinyldopas at the same rate.

The hyperbolic progress curve of cysteinyldopa formation (Fig. 1) is probably a function mainly of inactivation of tyrosinase by cysteine radicals, since the constant of decay is of the same order of magnitude as that observed when tyrosinase is kept in a solution of cysteine (unpublished results). It is of interest to compare this curve with that of the
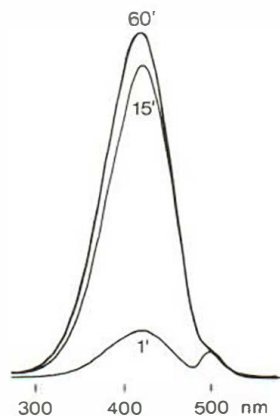

Fig. 9. Appearance of fluorophore on enzymatic oxidation of 5-OH-dopa by tyrosinase. Excitation maximum at 425 nm.

tyrosinase-catalysed oxidation of 5-OH-dopa (Fig. 7). The enzymatic oxidation of 5-OH-dopa at concentrations of 10^{-3} M and below proceeds closely in accordance with Michaelis-Menten's equation, and the curve does not seem to be influenced by enzyme inactivation (Fig. 1) or by re-reduction of oxidized products as for dopa alone (Fig. 7).

The finding of a new function of a thoroughly studied enzyme is remarkable. All our experiments were performed with mushroom tyrosinase. It will be most interesting to investigate whether the function leading to the formation of 5-OH-dopa is present also in tyrosinases of other origin.

It is difficult to predict if it will be possible to demonstrate 5-OH-dopa in tissues containing tyrosinase with 5-OH-dopa-forming capacity. Firstly, the relative cellular concentrations of tyrosine and of dopa at the site of the enzyme must be of the greatest importance for 5-OH-dopa production, since tyrosine strongly inhibits the formation of 5-OH-dopa from dopa by tyrosinase. Secondly, dopa has two different substrate functions, for hydroxylation leading to 5-OH-dopa, and for dehydrogenation leading to dopaquinone. Since V_{max} is much higher for dopaquinone formation, the metabolic pathway of dopa leading to the formation of 5-OH-dopa will always be less pronounced that that resulting in dopaquinone. Thirdly, 5-OH-dopa is itself a good substrate for tyrosinase. For this reason alone a substantial quantity of 5-OH-dopa in the cell can never be obtained. Fourthly, the oxidation potential of 5-OH is relatively low, and this fact will further contribute to the difficulties in demonstrating the 5-OH-dopa pathway by detection of the substance itself in biological tissues.

The oxidation products of 5-OH-dopa seem to be transformed into fluorescent compounds that are relatively stable and are not re-reduced to 5-OH-dopa. The fluorescent oxidation products might be more easily demonstrated in pigmented tissues than is 5-OH-dopa itself.

ACKNOWLEDGEMENTS

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