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PEROXIDATIC CONVERSION OF TYROSINE TO DOPACHROME*

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ABSTRACT

Peroxidatic conversion of tyrosine was assayed by measuring the formation of dopachrome (a melanin intermediate) at 475 nm. At a hydrogen peroxide concentration of 10^{-2} M, peroxidase converted tyrosine to dopachrome at a relatively slow rate without cofactor. With hydrogen peroxide concentrations of 10^{-3} M or lower, peroxidase required initial dopa or dihydroxyfumaric acid as cofactor for the conversion of tyrosine to dopachrome.

When dopa was used as cofactor, control experiments were carried out with tyrosine omitted. These indicated that tyrosine was actually converted to dopachrome, rather than acting as cofactor for the oxidation of dopa to dopachrome.

The ability of peroxidase to convert tyrosine to dopachrome supports the proposal that it has a role in mammalian melanogenesis.

Previous experiments in our laboratory [1] have demonstrated the conversion of tyrosine into melanin by purified human myeloperoxidase and horseradish peroxidase in the presence of dopa or dihydroxyfumarate (DHF), confirming the histochemical demonstration of peroxidase-mediated oxidation of tyrosine to melanin in a variety of cell types [2-11].

The ability to convert tyrosine to melanin appears to be a generic property of heme protein peroxidases. Hydroxylation of tyrosine to dopa was described by Mason [12] and is likely to be the first step in this synthesis. The ensuing conversion of dopa to melanin by peroxidase has been described by others [13] and proceeds by peroxidatic conversion of dopa to dopa quinone, followed by nonenzymatic cyclization of dopa quinone to dopachrome and further nonenzymatic oxidative steps. Dopachrome formation, therefore, is an indicator of enzymatic oxidation of tyrosine to melanin via dopa. This report describes peroxidatic conversion of tyrosine to dopachrome.

MATERIALS AND METHODS

Comparison of dopa and dihydroxyfumaric acid as cofactors for peroxidatic conversion of tyrosine to dopachrome. Dopachrome formation was recorded by measuring the absorption at 475 nm, using a Beckman DB-G recording spectrophotometer. In a typical experiment the reaction mixture consisted of 6.25 μmole of L tyrosine and 0.317 μmole of DL-dopa in 2.375 ml of 0.06 M acetate buffer, pH 5.6, 25 μmole of 7.5% hydrogen peroxide (final concentration 10^{-2} M), and 2 mg of horseradish peroxidase (type II, Sigma Chemical Co.) dissolved in 0.5 ml of distilled water. Temperature of reaction was 37°C. Reaction time varied from 15 to 85 min. Maximum millimolar concentration of dopachrome was calculated using the millimolar extinction coefficient of 3.7 [14]. In some experiments, 16.5 μmole of dihydroxyfumaric acid (DHF) were substituted for dopa as cofactor. When cofactor was used, a control was carried out in which tyrosine was omitted. Experiments were also carried out in the absence of cofactor, using either 10^{-2} M or 10^{-3} M hydrogen peroxide.

Effect of varying concentration of tyrosine and dopa. Dopachrome formation was recorded with tyrosine concentration constant and with dopa concentrations of 0.475 μmole, 0.317 μmole, 0.158 μmole, as well as with dopa concentration constant and tyrosine concentrations of 6.25 μmole, 3.12 μmole, and 1.56 μmole.

Dityrosine formation. Dityrosine formation was recorded by measuring absorption at 315 nm [15]. In a typical experiment the reaction mixture consisted of 6.25 μmole of L-tyrosine dissolved in 2.25 ml of 0.06 M acetate buffer, pH 5.6, 2.5 μmole of 0.75% hydrogen peroxide (final concentration 10^{-3} M), and 0.5 mg of horseradish peroxidase dissolved in 0.5 ml of distilled water.

RESULTS

Comparison of dopa and dihydroxyfumaric acid as cofactors for peroxidatic conversion of tyrosine to dopachrome. In the system containing peroxidase, tyrosine, dopa, and hydrogen peroxide, a biphasic absorption curve at 475 nm was obtained (Fig. 1, Curve A) having a steep initial phase which leveled off at 2.5 min. The maximum millimolar concentration of dopachrome was 1.75×10^{-1} (initial millimolar concentration of dopa was 1.16×10^{-1}). When tyrosine was omitted, a biphasic absorption curve resulted having a less steep initial phase and a lower maximum optical density (OD) (Fig. 1, Curve B). The background absorption of enzyme H_2O_2 complex at 475 nm was 0.06 OD (Fig. 1, Curve C).

With prolonged reaction (Fig. 2), the curve obtained in the absence of tyrosine declined more rapidly after reaching maximum than did the

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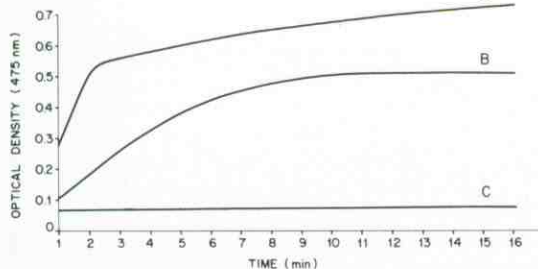

FIG. 1. Changes in the absorption at 475 nm by the action of horseradish peroxidase on (A) tyrosine, dopa, and hydrogen peroxide, (B) dopa and hydrogen peroxide, and (C) hydrogen peroxide.

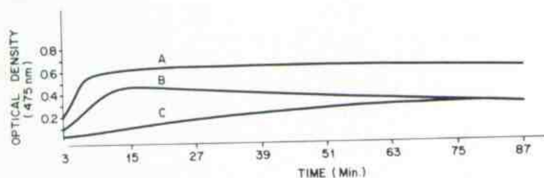

FIG. 2. Comparison of changes in absorption at 475 nm by the action of peroxidase on tyrosine without cofactor (C) with curves obtained in Fig. 1 (observation time extended to 85 min). Maximum dopachrome concentration of C equals differential of maxima of A and B.

curve obtained in its presence. When tyrosine was present without initial cofactor, a slow rise in dopachrome concentration was observed, which leveled off at 80 min; when the hydrogen peroxide concentration was reduced to 10^{-3} M, no dopachrome formation was observed in the absence of initial cofactor.

When DHF was substituted for dopa as cofactor, a biphasic absorption curve resulted with maximum OD of 0.94; when tyrosine was omitted, no dopachrome formation was observed.

Effect of varying concentration of tyrosine and dopa. Varying dopa concentration: When tyrosine concentration was constant, and the initial dopa concentration was varied (in the system illustrated by Figs. 1 and 2), the maximum dopachrome concentration was greater with higher dopa concentration in both tyrosine-plus-dopa and dopa-alone experiments. However, the maximum difference in dopachrome concentration of the coupled curves remained constant (Fig. 3). Varying tyrosine concentration: When the initial dopa concentration was constant and the tyrosine concentration was varied, the maximum difference in dopachrome concentration (tyrosine plus dopa) varied with the initial tyrosine concentration (Fig. 4).

Dityrosine formation. In the system containing peroxidase, tyrosine, and hydrogen peroxide (10^{-3} M), a biphasic absorption curve was obtained at 315 nm, indicative of dityrosine formation (Fig. 5). Under these conditions, as noted above, there was no increase in absorption at 475 nm. Even when the system was allowed to stand at room temperature for 3 days, there was no evidence of dopachrome or insoluble melanin formation.

Additional controls. When 10^{-3} M hydrogen peroxide was used in an experiment (Fig. 1) differential dopachrome with tyrosine was noted, but was less than with 10^{-2} M. Dopachrome and dityrosine formation were suppressed if boiled enzyme was used. When dopa was present in an experiment using boiled enzyme (30 min), a small amount of dopachrome formed by autooxidation (5% of enzymatic maximum OD). Formation of these products was suppressed when initial hydrogen peroxide was omitted and catalase was present.

In order to confirm that the increase in optical density at 475 nm represented dopachrome, scans were obtained in the visible region in systems containing tyrosine plus dopa and dopa alone. In all instances an absorption peak of 475 nm was

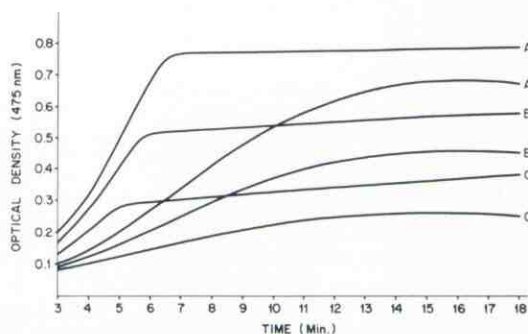

FIG. 3. Effect of varying dopa, with tyrosine constant, in the system described in Fig. 1. Curve A: L-tyrosine, 6.25 μmol plus DL-dopa in 20:1.5 ratio; Curve B: L-tyrosine, 6.25 μmole plus DL-dopa in 20:1 ratio; Curve C: L-tyrosine, 6.25 μmol plus DL-dopa in 20:0.5 ratio. Curves A′, B′, C′: corresponding concentrations of DL-dopa without tyrosine. Concentration of H_2O_2 throughout: 10^{-2} M. Differential maximum dopachrome concentration with each paired experiment was 0.12 O.D. ± 0.01.

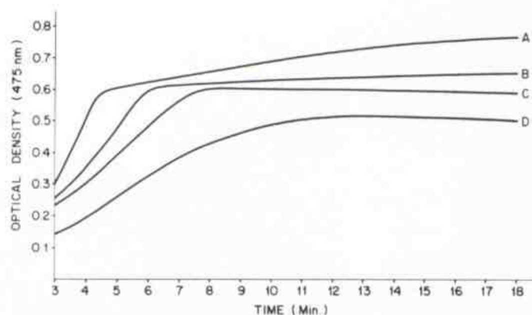

FIG. 4. Effect of varying tyrosine, with dopa constant, in the system described in Fig. 1. Curve D: DL-dopa 0.317 μmol (no tyrosine); Curve A: DL-dopa 0.317 μmole plus L-tyrosine (1:20 ratio); Curve B: DL-dopa 0.317 μmole plus L-tyrosine (1:10 ratio); Curve C: DL-dopa 0.317 μmole plus L-tyrosine (1:5 ratio). Concentration of H_2O_2 throughout: 10^{-2} M. Maximum differential dopachrome concentration is proportional to the tyrosine concentration.

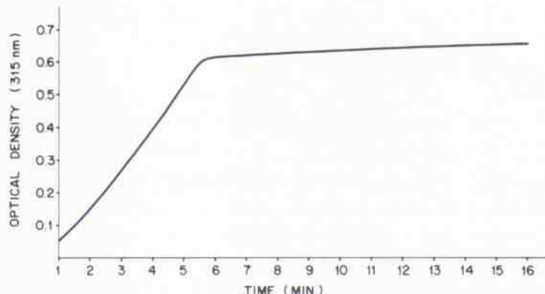

FIG. 5. Changes in the absorption at 315 nm (dityrosine) in the system containing peroxidase, L-tyrosine (6.25 μmole), and H_2O_2 (10^{-3} M). No increase in absorption at 475 nm was noted under these conditions.

obtained. (The interfering 420 absorption peak of the peroxidase-hydrogen peroxide complex was eliminated by suitable concentration of peroxidase and hydrogen peroxide in the blank cuvette.) Enzyme-hydrogen peroxide complex has insignificant absorption at 475 nm. Substitution of L-dopa for DL-dopa resulted in no significant difference in the results.

DISCUSSION

These experiments indicate that peroxidase can convert tyrosine to dopachrome (an obligatory melanin intermediate) in the presence of dopa or dihydroxyfumaric acid and hydrogen peroxide. When dopa was used as cofactor, observations consistently indicated that tyrosine acted as a substrate: Maximum differential dopachrome concentration was equal to maximum dopachrome concentration without initial dopa; and maximum differential dopachrome concentration varied with initial tyrosine concentration, but was independent of initial dopa concentration. Slow enzymatic conversion of tyrosine to dopachrome in the absence of starting cofactor and in the presence of high initial concentrations of hydrogen peroxide was probably due to the autooxidative formation of small amounts of dopa [16], with ensuing cofactor-dependent enzymatic hydroxylation of tyrosine. Similar results were obtained with isolated lactoperoxidase in recent experiments.

Our data contradict the conclusion of Bayse and Morrison [17] that in the system containing peroxidase, tyrosine, dopa, and hydrogen peroxide, tyrosine potentiates the conversion of dopa to dopachrome rather than acting as substrate. This conclusion was based on their assertion that total dopachrome formed in the reaction (when tyrosine was present) was equal to the initial dopa.

Stoichiometric calculations involving dopachrome are only approximations for several reasons: (a) dopachrome is an unstable compound (particularly at the alkaline pH [14] used by Bayse and Morrison [17]); (b) the extinction coefficient of dopachrome was derived indirectly at pH 5.6 [14], and correction for dopachrome instability would result in a value somewhat higher than the given figure. These would be compensating errors if the reaction were carried out at the pH used to derive the extinction coefficient of dopachrome. Since Bayse and Morrison [17] used a pH of 8.0, their figures for total dopachrome formed, both with and without tyrosine, would be too low.

Since we carried out the reactions at the pH used to calculate the extinction coefficient of dopachrome, our optical density maximum at 475 nm more closely approximated to total dopachrome formed. Our calculated maximum dopachrome concentration with dopa alone (Fig. 1) was 0.116 mM. This approximated the initial dopa concentration. Our calculated maximum dopachrome concentration with tyrosine and dopa (Curve A, Fig. 1) was 0.175 mM. This strongly supported our other data indicating that tyrosine was converted to dopachrome.

At alkaline pH, peroxidatic conversion of tyrosine to dopachrome is much more rapid. However, we used acid pH to reduce the instability of dopachrome. At acid pH, relatively high enzyme and H_2O_2 concentration must be used.

Bayse and Morrison [17] suggested that our histochemical demonstration of peroxidatic conversion of tyrosine to melanin may have been based solely on formation of tyrosine polymers (rather than peroxidatic conversion of tyrosine to melanin via dopa and dopachrome); Bayse et al [15] confirmed the finding of Gross and Sizer [18] that peroxidase can convert tyrosine to dityrosine. Peroxidatic formation of dityrosine was also found in our present study. However, this did not interfere with measurement of peroxidatic conversion of tyrosine to dopachrome, since dityrosine has no significant absorption at 475 nm. When dityrosine was formed under conditions excluding the formation of dopachrome, no insoluble melanin formed, even over a period of 3 days. Therefore, there is no evidence that melanin can form by peroxidatic polymerization of tyrosine.

Bayse and Morrison [17] suggested that the histochemical peroxide dependency of melanin synthesis in mammalian cells reflects peroxide requirement for polymerization of melanin rather than peroxidatic catalysis. Contrary to Bayse and Morrison, Swan and Wright [19] did not conclude that peroxide is required for polymerization of melanin. Aerobic dopa oxidase ("tyrosinase") can form insoluble melanin in the absence of hydrogen peroxide.

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