Oxygen Michaelis constants for tyrosinase

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The Michaelis constant of tyrosinase for oxygen in the presence of monophenols and *o*-diphenols, which generate a cyclizable *o*-quinone, has been studied. This constant depends on the nature of the monophenol and *o*-diphenol and is always lower in the presence of the former than of the latter. From the mechanism proposed for tyrosinase and from its kinetic analysis [Rodríguez-López, J. N., Tudela, J., Varón, R., García-Carmona, F. and

García-Cánovas, F. (1992) *J. Biol. Chem.* **267**, 3801–3810] a quantitative ratio has been established between the Michaelis constants for oxygen in the presence of monophenols and their *o*-diphenols. This ratio is used for the determination of the Michaelis constant for oxygen with monophenols when its value cannot be calculated experimentally.

INTRODUCTION

Tyrosinase is a copper-containing enzyme that catalyses the *ortho*-hydroxylation of monophenols (monophenolase reaction) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase reaction) [1]. The active site of tyrosinase consists of two copper atoms and three states, 'met', 'deoxy', and 'oxy' [2–5].

The Michaelis constant for oxygen of tyrosinase in the presence of *o*-diphenols has been widely studied. Ingraham determined the K_m for O_2 of French-prune tyrosinase in the presence of three *o*-diphenols [6]. In that study, a variation in the oxygen Michaelis constant according to the structure of the hydrogen donor was observed, and it was concluded that the enzyme combines with O_2 before it does so with the *o*-diphenol [6]. The same variation was observed by Duckworth and Coleman [7], but they did not agree with Ingraham [6] and proposed that the reaction might follow the opposite order, oxygen not binding first. These authors proposed two binding sites [7]. The kinetic analysis of *Neurospora crassa* tyrosinase showed random binding of *o*-diphenol and O_2 [8].

The complexity of the monophenol hydroxylation mechanism by tyrosinase has hindered the study of the oxygen Michaelis constant of this enzyme in the presence of monophenols, and the first mention found in the literature is by Lerch and Ettlinger [9]. They observed that the K_m and k_{cat} for the oxidation of L-3,4-dihydroxyphenylalanine (dopa) were strongly dependent on oxygen concentration, whereas those for L-tyrosine methyl ester were not [9]. These observations suggest that the enzyme was saturated by O_2 acting on monophenols and not on *o*-diphenols, indicating that the K_{m,O_2} was lower in the presence of the former than of the latter, although no mechanism for the action of this enzyme was proposed. Lerner and Mayer [10] attempted to study the kinetic constant for oxygen in the monophenolase reaction, but did not succeed, concluding that the results indicated that the mechanism differed from that for *o*-diphenol.

From the mechanism proposed for the monophenolase activity of tyrosinase [11] and its kinetic analysis [12], it was demonstrated that the *o*-diphenol accumulation in the steady state ($[D]_{ss}$) was linear with regard to monophenol concentration ($[T]_0$), and so $[D]_{ss} = R[T]_0$, where R is constant for each enzyme and each

monophenol/diphenol pair [12]. These results permitted the development of a method for measuring the monophenolase activity of tyrosinase. This consists of adding to the reaction medium the *o*-diphenol necessary ($[D]_{ss}$) to obtain the steady state. This method permits short assay times, with no lag period, no significant consumption of substrates, no suicide inactivation and no product breakdown. Therefore kinetic assays of the monophenolase activity can be carried out.

The purpose of the present paper is to report a kinetic study that permits us to obtain the Michaelis constants for oxygen with both monophenols and *o*-diphenols in the tyrosinase-catalysed reaction. The value of K_m for O_2 in the presence of monophenols is given for the first time in the literature, and the validity of the mechanism proposed for tyrosinase is confirmed.

NOTATION AND DEFINITIONS

Species

T	monophenol
D	<i>o</i> -diphenol
QH, Q	<i>o</i> -quinone- H^+ and <i>o</i> -quinone respectively
L	leukoaminechrome
DC	aminechrome
[X]	concentration of the species X during the course of the reaction
$[X]_{ss}$	concentration of the species X during the steady state of the reaction
$[X]_0$	initial concentration of the species X in the assay medium
E_{met}	met-tyrosinase or oxidized form of tyrosinase with Cu_2^{2+} in the active site
E_{deoxy}	deoxytyrosinase or reduced form of tyrosinase with Cu_2^{1+} in the active site
E_{oxy}	oxytyrosinase or oxidized form of tyrosinase with peroxide

Kinetic parameters

V_{DC}^T, V_{DC}^D	steady-state rate of the production of DC from T and D respectively
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Abbreviations used: tyrosine, L-tyrosine; dopa, L-3,4-dihydroxyphenylalanine; dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinone; tyramine, 4-hydroxyphenethylamine; dopamine, 3,4-dihydroxyphenethylamine; dopaminechrome, 2,3-dihydroindole-5,6-quinone.

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$V_{O_2}^T, V_{O_2}^D$ steady-state rate of the oxygen consumption from T and D respectively

Kinetic constants

k_i ($i = 1-8$) rate constants of the reaction mechanism of tyrosinase

$K_a = k_{+9}/k_{-9}$ dissociation constant of the deprotonation/protonation equilibrium between QH and Q

k_{+10} cyclization constant of Q into L

k_{+11} rate constant of the production of DC + D from L + QH

$k_{app.}$ apparent constant for the transformation of QH into D + DC + H⁺

K_1 dissociation constants of E_{met} towards T: $K_1 = k_{-1}/k_{+1}$

K_m^T, K_m^D Michaelis constants of tyrosinase towards T and D respectively

K_{oxy}^T, K_{oxy}^D Michaelis constants of E_{oxy} towards T

$V_{max.,DC}^T, V_{max.,DC}^D$ maximal steady-state rates of tyrosinase towards T and D respectively, determined by DC formation

$V_{max.,O_2}^T, V_{max.,O_2}^D$ maximal steady-state rates of tyrosinase towards T and D respectively, determined by oxygen consumption

K_m^T, K_m^D Michaelis constants of tyrosinase towards T and D respectively

K_{m,O_2}^T, K_{m,O_2}^D Michaelis constants of tyrosinase towards O₂ using T and D as substrates respectively.

MATERIALS AND METHODS

Reagents

Mushroom tyrosinase (monophenol,*o*-diphenol:O₂ oxidoreductase, EC 1.14.18.1; 3300 units/mg), tyrosine, dopa, tyramine hydrochloride and dopamine hydrochloride were purchased from Sigma. All other chemicals were of analytical grade and supplied by Merck. Mushroom tyrosinase was purified by the procedure of Duckworth and Coleman [7]. Protein concentration was determined by a modified Lowry method [13]. The enzyme concentration was calculated taking a value of M_r 120000.

Polarographic assays

O₂ evolution was measured by a Hansatech D.W. oxymeter based on the Clark-type electrode covered with a Teflon membrane, equipped with an Amel 863 digital X/Y recorder. The electrode calibration was carried out by the 4-*t*-butylcatechol/tyrosinase method [14]. The chamber of the polarograph (3.0 ml) contained 10 mM sodium phosphate buffer, pH 6.8, as assay medium. Different O₂ concentrations were obtained by mixing saturated aerobic and O₂-free solutions. The O₂-free solutions were obtained by flushing with nitrogen. To determine the kinetic parameters on monophenols, care was taken that recordings reached steady state. This aspect was solved by the addition to the reaction medium of a $[D]_0 \approx [D]_{ss}$. The experimental value of $[D]_{ss}$ was calculated by h.p.l.c. assays or by the addition of an excess of NaIO₄ when the system reached the steady state [12]. The value of $[D]_{ss}$ for the tyrosine/dopa pair at 20 °C was $[D]_{ss} = 0.042[T]_0$ ($R = 0.042$) and for the tyramine/dopamine pair at 14.5 °C $[D]_{ss} = 0.029[T]_0$ ($R = 0.029$). Tem-

perature was controlled using a Haake D1G circulating bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of ± 0.1 °C.

Spectrophotometric assays

Product accumulation was spectrophotometrically followed at the specific $\lambda_{max.}$ using a Perkin-Elmer Lambda-2 spectrophotometer, on-line interfaced with an Amstrad PC2086 microcomputer. Dopachrome was monitored at 475 nm (ϵ_{475} 3600 M⁻¹·cm⁻¹ [15]) and dopaminechrome at 480 nm. The ϵ_{480} for dopaminechrome (3300 M⁻¹·cm⁻¹) was determined by the instantaneous oxidation of known amounts of dopamine, in the presence of sodium periodate, at pH 6.8. The experimental conditions were the same as those used in the polarographic assays. Reference cuvettes contained all the components except the substrate, and the final volume was 3.0 ml.

Simulated assays

The kinetic behaviour of the reaction mechanism is described by a system of differential equations, whose numerical integration was carried out by using the predictor-corrector algorithm of Adams-Moulton, starting with the fourth-order Runge-Kutta method [16]. The algorithm was implemented and compiled in TurboBASIC 1.0 on an INVE PC-640A computer (IBM AT-compatible) with an Intel 80287 arithmetic coprocessor. The differential equations used for simulated assays and the simulation conditions are given in the Appendix.

Dopachrome formation

Fresh solutions of dopachrome were prepared by mixing a solution of dopa in 10 mM sodium phosphate buffer, pH 6.8, with mushroom tyrosinase (0.2 mg/ml) for 10 min. The mixture thus obtained was introduced through a Sephadex G-25 column equilibrated and eluted with 10 mM sodium acetate buffer, pH 3.0, to remove the enzyme. At pH 3.0, dopachrome was stable for at least 30 min.

Kinetic data analysis

The K_m and $V_{max.,DC}$ values of tyrosinase on monophenols and *o*-diphenols at saturating $[O_2]_0$ were calculated from triplicate spectrophotometric measurements of V_{DC} for each $[T]_0$ and $[D]_0$ respectively. By non-linear regression of V_{DC} versus $[T]_0$ and $[D]_0$, the kinetic constants K_m^T , $V_{max.,DC}^T$, K_m^D and $V_{max.,DC}^D$ were determined. Initial estimations of these kinetic constants were obtained from the Hanes-Woolf equation, a linear transformation of the Michaelis equation [17].

In the determination of the kinetic constants for the substrate oxygen, two different methods of procedure and analysis were used, depending on the monophenol/*o*-diphenol pair used as substrate. When dopamine or tyramine was used, the values of K_{m,O_2} and $V_{max.,O_2}$ were calculated from triplicate polarographic measurements of V_{O_2} versus $[O_2]_0$ at a saturating concentration of dopamine or tyramine. These data were fitted by non-linear regression [18] to eqns. (2) and (7) respectively, and the corresponding kinetic constants were determined. Initial estimations from the Hanes-Woolf equation [17] (initial-rate method). On the other hand, when dopa or tyrosine at saturating concentration were used as substrates of tyrosinase, the values of K_{m,O_2} and $V_{max.,O_2}$ were obtained from the curvature in ten plots of the oxygen consumption versus time. These data were fitted by non-linear regression to the integrated form [19] of their Michaelis

Scheme 1 Reaction mechanism for the diphenolase activity of tyrosinase coupled to non-enzymic reactions from *o*-quinone- H^+ up to aminechrome

equations (eqns. 2 and 7), using a computer program [20]. Initial estimations of these kinetic constants were obtained from the linearization of the integrated Michaelis equation [19] (the integrated method).

To provide analogy with experimental assays, both activities of tyrosinase were simulated and analysed by two different methods, using the same set of rate constants: (a) the initial-rate method, in which the kinetic constants for oxygen were calculated by non-linear regression fitting of V_{O_2} versus $[\text{O}_2]_0$ at saturating monophenol or *o*-diphenol; (b) the integrated method, which was simulated with the amount of oxygen varying during the whole assay time. In this, the kinetic constants were obtained by fitting the last portion of the curve to the integrated Michaelis equation, by non-linear regression.

RESULTS AND DISCUSSION

Kinetic analysis

The proposed mechanism for the diphenolase activity of tyrosinase is depicted in Scheme 1. This Scheme involves the three forms of the enzyme, as well as the chemical reactions coupled to the enzymic step. In a previous paper [12] a kinetic study of this pathway, applying the steady-state approach, was carried out. A steady-state rate for dopachrome accumulation in the diphenolase activity was obtained, where:

$$V_{\text{DC}}^{\text{D}} = \frac{\alpha_1 [\text{D}]_0 [\text{O}_2]_0 [\text{E}]_0 / (\beta_0 + \beta_1 [\text{D}]_0 + \beta_2 [\text{O}_2]_0 + \beta_3 [\text{D}]_0 [\text{O}_2]_0)}{1} \quad (1)$$

The coefficients α_1 and β_0 – β_3 are shown in the Appendix (eqn. 1A).

It is known that the ratio between oxygen consumption and dopachrome formation is 1.0 in the diphenolase activity of tyrosinase [12,21]. Therefore both rates have the same hyperbolic dependence on oxygen concentration at saturating $[\text{D}]_0$ values.

$$\begin{aligned}
 V_{\text{DC}}^{\text{D}} &= -V_{\text{O}_2}^{\text{D}} = V_{\text{max.,DC}}^{\text{D}} [\text{O}_2]_0 / (K_{\text{m,O}_2}^{\text{D}} + [\text{O}_2]_0) \\
 &= V_{\text{max.,O}_2}^{\text{D}} [\text{O}_2]_0 / (K_{\text{m,O}_2}^{\text{D}} + [\text{O}_2]_0)
 \end{aligned} \quad (2)$$

The analytical expression for the kinetic constants $V_{\text{max.,DC}}^{\text{D}}$, $V_{\text{max.,O}_2}^{\text{D}}$ and $K_{\text{m,O}_2}^{\text{D}}$ are (see the Appendix):

$$\begin{aligned}
 V_{\text{max.,DC}}^{\text{D}} &= V_{\text{max.,O}_2}^{\text{D}} = \alpha_1 [\text{E}]_0 / \beta_3 \\
 &= k_{+3} k_{+7} [\text{E}]_0 / (k_{+3} + k_{+7}) = k_{\text{cat}}^{\text{D}} [\text{E}]_0
 \end{aligned} \quad (3)$$

and

$$K_{\text{m,O}_2}^{\text{D}} = \beta_1 / \beta_3 = k_{+3} k_{+7} / [k_{+8} (k_{+3} + k_{+7})] = k_{\text{cat}}^{\text{D}} / k_{+8} \quad (4)$$

Scheme 2 Reaction mechanism for the monophenolase activity of tyrosinase coupled to non-enzymic reactions from *o*-quinone- H^+ up to aminechrome.

It can be seen that the analytical expression for $K_{\text{m,O}_2}^{\text{D}}$ (eqn. 4) could explain the widely observed variation in the Michaelis constant for O_2 depending on the structure of the *o*-diphenol [6,7].

For monophenolase activity, the mechanism is shown in Scheme 2. This mechanism considers the three forms of the enzyme and is based on the assumption that all the interactions between tyrosinase and its substrates (i.e. tyrosine, dopa and O_2) take place at the binuclear copper site of tyrosinase, without allosteric phenomena [4]. Moreover, this mechanism explains the most important characteristics described for the monophenolase activity of tyrosinase: (i) that the monophenolase activity is expressed together with the diphenolase activity [9]; (ii) that the monophenolase activity has the same chemical steps as found in diphenolase activity from *o*-dopaquinone- H^+ to dopachrome [11,22,23]; (iii) that a lag period is observed in the monophenolase

activity of tyrosinase when dopachrome formation or O_2 consumption is measured. This lag period can be eliminated by the addition of small amounts of dopa [12,24]; (iv) that the dopa accumulated in the steady state of the monophenolase activity ($[D]_{ss}$) fulfils the constant relationship $[D]_{ss} = R[T]_0$ [12].

We have recently established the turnover of the enzyme in the melanin-biosynthesis pathway in the steady state from tyrosine. The stoichiometry of the pathway implies that one molecule of enzyme must accomplish two turnovers in the hydroxylase cycle for each one in the oxidase cycle (Scheme 2) [12]. The stoichiometry predicts that $V_{O_2}^T/V_{DC}^T = 1.5$, and this ratio was verified experimentally. These results gave an analytical expression for the steady-state rate for the monophenolase activity of tyrosinase:

$$V_{DC}^T = \frac{\gamma_1 [D]_{ss} [O_2]_0 [E]_0}{\delta_0 + \delta_1 [D]_{ss} + \delta_2 [O_2]_0 + \delta_3 [D]_{ss} [O_2]_0 + \delta_4 [T]_0 [O_2]_0} \quad (5)$$

The coefficients γ_1 and δ_0 – δ_4 are given in the Appendix (eqn. 2A). From experimental and simulated data the linear relationship $[D]_{ss} = R[T]_0$ was obtained, which can be introduced into eqn. (5) to give:

$$V_{DC}^T = \frac{\gamma_1 R [T]_0 [O_2]_0 [E]_0}{\delta_0 + \delta_1 R [T]_0 + \delta_2 [O_2]_0 + \delta_3 R [T]_0 [O_2]_0 + \delta_4 [T]_0 [O_2]_0} \quad (6)$$

When $[T]_0 \rightarrow \infty$,

$$V_{DC}^T = V_{max,DC}^T [O_2]_0 / (K_{m,O_2}^T + [O_2]_0) \quad (7)$$

where:

$$V_{max,DC}^T = \gamma_1 R [E]_0 / (\delta_3 R + \delta_4) = \frac{2k_{+2}k_{+3}k_{+7}K_{oxy}^T K_1 R [E]_0}{(3k_{+2}k_{+7}K_{oxy}^T K_1 + k_{+2}k_{+3}K_{oxy}^T K_1)R + 3k_{-2}k_{+7}K_{oxy}^T + k_{+3}k_{+7}K_{oxy}^T + k_{+2}k_{+3}K_{oxy}^D K_1} = k_{cat}^T [E]_0 \quad (8)$$

and

$$K_{m,O_2}^T = \delta_1 R / (\delta_3 R + \delta_4) = \frac{3k_{+2}k_{+3}k_{+7}K_{oxy}^T K_1 R}{k_{+8}[(3k_{+2}k_{+7}K_{oxy}^T K_1 + k_{+2}k_{+3}K_{oxy}^T K_1)R + 3k_{-2}k_{+7}K_{oxy}^T + k_{+3}k_{+7}K_{oxy}^T + k_{+2}k_{+3}K_{oxy}^D K_1]} = (3/2)k_{cat}^T / k_{+8} \quad (9)$$

As mentioned above, $V_{O_2}^T = (3/2)V_{DC}^T$ and, therefore, $V_{max,O_2}^T = (3/2)V_{max,DC}^T$. Moreover, it can be seen that k_{cat}^T is not a true catalytic constant, since it depends on equilibrium constants and on the experimental R . From eqn. (9) it is possible to deduce that the K_{m,O_2}^T depends on the nature of the monophenol.

Experimental results

Determination of the kinetic constants of tyrosinase on monophenols and *o*-diphenols

When the oxygen concentration is saturating, eqns. (1) and (5) are useful to determine the kinetic constants of tyrosinase on *o*-diphenols and monophenols respectively. Spectrophotometric assays of aminochrome production have led to sets of V_{DC}^T versus $[D]_0$ and V_{DC}^T versus $[T]_0$ (results not shown). These data were fitted by non-linear regression and the corresponding kinetic constants determined (Table 1).

Determination of the kinetic constants of tyrosinase on O_2

When dopa at saturating concentration was oxidized in the presence of a high concentration of tyrosinase, all the O_2 was consumed in the first seconds of the reaction (Figure 1). The

Table 1 Experimental values of the kinetic constants for the oxidation of monophenols and *o*-diphenols catalysed by tyrosinase

Values for $V_{max,DC}$ and V_{max,O_2} are related at 0.6 nM tyrosinase. Values for the kinetic constants for the tyrosine/dopa pair were obtained at 20 °C, whereas those for the tyramine/dopamine pair were obtained at 14.5 °C. The values (regression parameter $\pm$ S.E.M.) were obtained as described in the Materials and methods and Results and discussion sections.

Substrate	K_m (mM)	K_{m,O_2} (μ M)	$V_{max,DC}$ (nM/s)	V_{max,O_2} (nM/s)
Tyrosine	0.5 ± 0.09	1.8 ± 0.01	4.51 ± 0.3	6.85 ± 0.1
Dopa	0.42 ± 0.05	9.5 ± 0.08	50.1 ± 0.8	51.0 ± 0.1
Tyramine	1.51 ± 0.15	5.02 ± 0.10	7.3 ± 0.8	10.8 ± 0.5
Dopamine	0.88 ± 0.07	48.2 ± 0.20	106.3 ± 0.8	106.8 ± 0.3

tracing was linear during the first 70 % of O_2 consumption, which indicated a low K_m value. When tyrosine was used as substrate under similar conditions, the tracing was linear during the first 92 % of oxygen consumption (Figure 1), indicating that the value of K_{m,O_2} in the presence of tyrosine was lower than that in the presence of dopa. Because of the low value of K_{m,O_2} for tyrosine, the integrated Michaelis equation should be applied. The values of K_{m,O_2} and V_{max,O_2} in the presence of tyrosine or dopa are shown in Table 1. In control experiments, a dopachrome concentration equal to that formed at the end of the reaction was added at the start of the reaction and no effect was observed (results not shown). Therefore this enzyme-catalysed reaction shows no inhibition by accumulating product.

Figure 1 O_2 consumption in the oxidation of dopa (trace a) and tyrosine (trace b) catalysed by tyrosinase at 20 °C

Reagents for trace a were 1.8 mM dopa and 24 nM tyrosinase, and for trace b they were 1.8 mM tyrosine, 75.6 μ M dopa and 96.2 nM tyrosinase. Other conditions were as detailed in the Materials and methods and the Results section.

Figure 2 O_2 consumption in the oxidation of dopamine (i) and tyramine (ii) catalysed by tyrosinase at 14.5 °C

Reagents for traces (i) were 15 mM dopamine and 26.3 nM tyrosinase and for traces (ii) they were 15 mM tyramine, 0.42 mM dopamine and 66 nM tyrosinase. The inset shows plots of $V_{O_2}^D$ and $V_{O_2}^T$ versus $[O_2]$, using 0.6 nM tyrosinase. ●, ■, Experimental data using saturating (15 mM) dopamine (●) or tyramine (■); —, data calculated using the final estimations from non-linear-regression fitting.

value for K_{m,O_2} in the presence of tyramine appears to be lower than in the presence of dopamine. Moreover, the curvature in these plots (Figure 2, main part) seem to indicate that the value for K_{m,O_2} with tyramine is higher than in the presence of tyrosine, and for this reason the K_{m,O_2} values for the tyramine/dopamine pair can be obtained by the initial-rate method (Figure 2, inset; Table 1).

Ratio between the kinetic constants of tyrosinase for monophenols and *o*-diphenols

From the analytical expression of the kinetic constants for *o*-diphenols (eqns. 3 and 4) and monophenols (eqns. 8 and 9) it is easy to deduce that:

$$(V_{max,DC}^T/K_{m,O_2}^T)/(V_{max,DC}^D/K_{m,O_2}^D) = (2/3) \quad (10)$$

and

$$(V_{max,O_2}^T/K_{m,O_2}^T)/(V_{max,O_2}^D/K_{m,O_2}^D) = 1 \quad (11)$$

The experimental results obtained in the above section were used to verify these ratios (Table 2). The results obtained for tyramine and dopamine fulfil these ratios 97% (Table 2). However, a deviation was observed when the kinetic constants obtained for tyrosine and dopa were analysed (Table 2). These last kinetic constants were calculated for the total consumption of $[O_2]$, analysed by the integrated Michaelis equation. To understand whether this experimental procedure was responsible for the above deviation, the monophenolase and diphenolase mechanisms were simulated by both the initial-rate and the integrated methods.

Table 2 Ratios obtained by fitting the experimental and simulated results to eqns. (10) and (11)

Results	Substrate pair	Ratio	
		Eqn. (10)	Eqn. (11)
Experimental	Tyrosine/dopa	$0.48 \pm 0.04^*$	0.71 ± 0.11
	Tyramine/dopamine	0.66 ± 0.09	0.97 ± 0.07
Simulated	Monophenol/ <i>o</i> -diphenol		
	Initial-rate method	0.68 ± 0.01	1.01 ± 0.01
	Integrated method	0.60 ± 0.01	0.89 ± 0.01

* Values represent the calculated data $\pm$ S.D., using the S.E.M. of the regression parameters obtained in Tables 1 and 3.

Simulation

Determination of kinetic constants

The values of K_{m,O_2} and V_{max,O_2} with monophenols and *o*-diphenols, using both methods of procedure and analysis (see the Materials and methods section and the Appendix) are given in Table 3. The simulated results obtained by the initial-rate method fulfil the ratios of eqns. (10) and (11) at 100% (Table 2). However, when the values were obtained by the integrated method, a similar deviation to that shown in the experimental results on dopa and tyrosine was observed (Table 2). It can be seen that the whole deviation referred to the K_{m,O_2}^T value, no effect on the other kinetic constants being observed (Table 3). Therefore these experimental and simulation deflections could be due to the

Table 3 Values of the kinetic constants for the oxidation of monophenols and *o*-diphenols by tyrosinase (10 nM) obtained by simulation of the proposed mechanisms (Schemes 1 and 2)

Method	Kinetic constant	Substrate... Monophenol†	<i>o</i> -Diphenol‡
Initial-rate*	K_{m,O_2} (μM)	1.79 ± 0.01	10.30 ± 0.02
	V_{max,O_2} (μM/s)	0.30 ± 0.01	1.70 ± 0.01
Integrated†	K_{m,O_2} (μM)	2.04 ± 0.01	10.30 ± 0.02
	V_{max,O_2} (μM/s)	0.30 ± 0.01	1.70 ± 0.01

* The simulation conditions for monophenolase activity were: $[T]_0$, 30 mM; $[D]_0$, 1.49 mM. The $[O_2]_0$ was varied from 0.1 to 20 μM. For the diphenolase activity, $[D]_0$ was 12 mM and $[O_2]_0$ was varied from 1.0 to 100 μM. The high $[T]_0$ (30 mM) and $[D]_0$ (12 mM) used were chosen to avoid any deviation caused by non-saturation in simulated assays.

† For the integrated method, the simulation conditions for monophenolase activity were: $[T]_0$, 30 mM; $[D]_0$, 1.49 mM; $[O_2]_0$, 20 μM. For diphenolase activity: $[D]_0$, 12 mM; $[O_2]_0$, 50 μM.

‡ The values for K_m^T (0.3 mM), K_m^D (0.12 mM), $V_{max,DC}^T$ (0.2 μM/s) and $V_{max,DC}^D$ (1.69 μM/s) were obtained by simulation of the monophenolase and diphenolase mechanisms at saturating $[O_2]_0$ (0.26 mM) and by varying $[T]_0$ and $[D]_0$.

Figure 3 Evolution with time of several rate ratios of the catalytic cycle

(a–c) Monophenolase activity (Scheme 2), $V_{\max, O_2}$ with 30 mM monophenol, 1.49 mM *o*-diphenol, 20 μ M O_2 and 1 μ M tyrosinase. (d) Diphenolase activity (Scheme 1), with 12 mM *o*-diphenol, 50 μ M O_2 and 1 μ M tyrosinase. The series of traces marked i was simulated with constant $[O_2]$ during the whole assay time, the differential equation for O_2 being omitted to carry out this simulation. In the series of traces marked ii the O_2 was completely consumed during the assay time.

fact that the kinetic mechanism for monophenolase activity deviates from the one proposed for carrying out the kinetic analysis (Schemes 1 and 2), at the low $[O_2]$ necessary for the integrated method.

Time-course simulation assays

Simulation permits us to know the ratio between the steps in the monophenolase and diphenolase mechanisms (Figure 3) and, therefore, to understand the reason for any deviation from the kinetic mechanism. In the steady state it was deduced that the stoichiometry of monophenolase activity implies that:

$$k_{+3}[E_{met}D] = (3/2)k_{+5}[E_{oxy}T] = 3k_{+7}[E_{oxy}D]$$

whereas for the diphenolase activity it was $k_{+3}[E_{met}D] = k_{+7}[E_{oxy}D]$. All of these ratios were accomplished when the

simulation was carried out at a constant $[O_2]$ during the whole assay time (Figure 3, traces i). However, when the $[O_2]$ was not constant during the whole assay time, the initial steady-state stoichiometry was broken in the monophenolase activity of tyrosinase (Figures 3a and 3b, traces ii). No effect on diphenolase activity was observed under the latter simulation conditions (Figure 3d, trace ii).

The diphenolase mechanism is characterized by only one oxidase cycle and all the enzyme participating in the turnover (Scheme 1). Under these circumstances the integrated Michaelis equation can be used [19]. On the other hand, in the monophenolase mechanism, this does not occur: there are two cycles (oxidase and hydroxylase) with three common intermediates ($E_{met}D$, E_{oxy} and E_{deoxy}) and a portion of the enzyme is scavenged from the catalytic turnover as dead-end complex $E_{met}T$ in the steady state (Scheme 2). When $[O_2]$ starts to be limiting, an accumulation of E_{deoxy} occurs, with a subsequent exit of the enzyme from the dead-end complex $E_{met}T$, in an attempt to maintain the steady-state stoichiometry of the pathway. The increase in the step governed by k_{+3} with respect to those governed by k_{+5} and k_{+7} (Figure 3) supports this hypothesis. Thus the breakdown in the steady state causes the kinetic mechanisms for monophenolase activity at low $[O_2]$ to deviate from that used for obtaining the kinetic equations (eqn. 7). Therefore the integrated Michaelis equation is not valid to obtain the K_{m, O_2}^T value (Table 1). Another contribution to the experimental deviation with tyrosine might be the impossibility of obtaining 100% saturation, owing to the poor solubility of this substrate.

Concluding remarks

The proposed mechanism for *o*-diphenols and its kinetic analysis explains the widely observed variation of the K_{m, O_2}^D according to the nature of the *o*-diphenol [6,7]. It has been proposed that this variation is only possible if the catechol substrate is the first to be bound by tyrosinase [7]. However, the analytical expression for K_{m, O_2}^D (eqn. 4) deduced in the present paper could explain how this variation is possible even if the O_2 is the first to be bound [6]. Moreover, a kinetic analysis of the monophenolase activity (eqn. 9) also explains the dependence of K_{m, O_2}^T on the nature of monophenol.

The values for K_{m, O_2} in the presence of *o*-diphenols and monophenols can be calculated by plots of V_{O_2} or V_{DC} versus $[O_2]_0$ at saturating *o*-diphenol and monophenol respectively. When the K_{m, O_2}^D is too low to be calculated by this procedure, this integrated Michaelis equation should be used (Table 1). However, this last method can never be used to determine K_{m, O_2}^T values. Therefore, when the value of K_{m, O_2}^T is very low, eqns. (10) and (11) should be used, since the other kinetic constants that appear in these equations are easily determined by spectrophotometric or polarographic methods. The K_{m, O_2}^T value calculated by regrouping eqn. (11) was $1.26 \pm 0.03 \mu$ M, this being the real value for the kinetic constant.

The above-described results support the proposed mechanism for the oxidation of monophenols and *o*-diphenols catalysed by tyrosinase [12]. Thus the contrast between experimental and simulation assays is of use in verifying the validity of the kinetic analysis.

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APPENDIX

Simulated assay conditions

The reaction mechanism of the diphenolase activity of tyrosinase (Scheme 1 of the main paper) is described by the following system of differential equations:

$$\begin{aligned}
 d[E_{\text{met}}]/dt &= k_{-2}[E_{\text{met}}D] + k_{+7}[E_{\text{oxy}}D] - k_{+2}[E_{\text{met}}][D] \\
 d[E_{\text{deoxy}}]/dt &= k_{+3}[E_{\text{met}}D] + k_{-8}[E_{\text{oxy}}] - k_{+8}[E_{\text{deoxy}}][O_2] \\
 d[E_{\text{met}}D]/dt &= k_{+2}[E_{\text{met}}][D] - (k_{-2} + k_{+3})[E_{\text{met}}D] \\
 d[E_{\text{oxy}}]/dt &= k_{-6}[E_{\text{oxy}}D] + k_{+8}[E_{\text{deoxy}}][O_2] - (k_{+6}[D] + k_{-8})[E_{\text{oxy}}] \\
 d[E_{\text{oxy}}D]/dt &= k_{+6}[E_{\text{oxy}}][D] - (k_{-6} + k_{+7})[E_{\text{oxy}}D] \\
 d[QH]/dt &= k_{+3}[E_{\text{met}}D] + k_{+7}[E_{\text{oxy}}D] - k_{\text{app.}}[QH] \\
 d[DC]/dt &= k_{\text{app.}}[QH]/2 \\
 d[O_2]/dt &= k_{-8}[E_{\text{oxy}}] - k_{+8}[E_{\text{deoxy}}][O_2]
 \end{aligned}$$

the initial conditions being: $[E]_0 = [E_{\text{met}}]_0 + [E_{\text{oxy}}]_0$, $[D] = [D]_0$, and $[E_{\text{met}}]_0/[E_{\text{oxy}}]_0 = 9:1$. $[D]$ was considered constant throughout the simulations, in accordance with experimental data. Other conditions are given in the main paper in the text and in the legends to Tables and Figures.

The reaction mechanism of the monophenolase activity of tyrosinase (Scheme 2 of the main paper) involves the following differential equations:

$$\begin{aligned}
 d[E_{\text{met}}]/dt &= k_{-1}[E_{\text{met}}T] + k_{-2}[E_{\text{met}}D] + k_{+7}[E_{\text{oxy}}D] - (k_{+1}[T] + k_{+2}[D])[E_{\text{met}}] \\
 d[E_{\text{deoxy}}]/dt &= k_{+3}[E_{\text{met}}D] + k_{-8}[E_{\text{oxy}}] - k_{+8}[O_2][E_{\text{deoxy}}] \\
 d[E_{\text{met}}D]/dt &= k_{+2}[E_{\text{met}}][D] + k_{+5}[E_{\text{oxy}}T] - (k_{-2} + k_{+3})[E_{\text{met}}D] \\
 d[E_{\text{met}}T]/dt &= k_{+1}[T][E_{\text{met}}] - k_{-1}[E_{\text{met}}T] \\
 d[E_{\text{oxy}}]/dt &= k_{-4}[E_{\text{oxy}}T] + k_{-6}[E_{\text{oxy}}D] + k_{+8}[E_{\text{deoxy}}][O_2] - (k_{+4}[T] + k_{+6}[D] + k_{-8})[E_{\text{oxy}}] \\
 d[E_{\text{oxy}}D]/dt &= k_{+6}[E_{\text{oxy}}][D] - (k_{-6} + k_{+7})[E_{\text{oxy}}D] \\
 d[E_{\text{oxy}}T]/dt &= k_{+4}[E_{\text{oxy}}][T] - (k_{-4} + k_{+5})[E_{\text{oxy}}T] \\
 d[QH]/dt &= k_{+3}[E_{\text{met}}D] + k_{+7}[E_{\text{oxy}}D] - k_{\text{app.}}[QH] \\
 d[DC]/dt &= k_{\text{app.}}[QH]/2 \\
 d[D]/dt &= k_{-2}[E_{\text{met}}D] + k_{-6}[E_{\text{oxy}}D] - (k_{+2}[E_{\text{met}}] + k_{+6}[E_{\text{oxy}}])[D] + (k_{\text{app.}}[QH]/2) \\
 d[O_2]/dt &= k_{-8}[E_{\text{oxy}}] - k_{+8}[E_{\text{deoxy}}][O_2]
 \end{aligned}$$

the initial conditions being: $[E]_0 = [E_{\text{met}}]_0 + [E_{\text{oxy}}]_0$, $[T] = [T]_0$, $[D] = [D]_0$ and $[E_{\text{met}}]_0/[E_{\text{oxy}}]_0 = 9:1$. $[T]$ was considered constant throughout the simulations, in accordance with experimental data. Other conditions are given in the main paper in the text and in the legends to Tables and Figures.

The values of the rate constants used for both simulation methods were chosen to approximately fulfil the value of the Michaelis constants of mushroom tyrosinase towards tyrosine, dopa and oxygen:

$$\begin{aligned}
 k_{+1} &= 9.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} & k_{+5} &= 2.98 \times 10^2 \text{ s}^{-1} \\
 k_{-1} &= 3.0 \times 10^3 \text{ s}^{-1} & k_{+6} &= 1.05 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1} \\
 k_{+2} &= 3.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} & k_{-6} &= 1.0 \times 10^3 \text{ s}^{-1} \\
 k_{-2} &= 3.0 \times 10^2 \text{ s}^{-1} & k_{+7} &= 4.0 \times 10^2 \text{ s}^{-1} \\
 k_{+3} &= 3.0 \times 10^2 \text{ s}^{-1} & k_{+8} &= 1.68 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} \\
 k_{+4} &= 1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} & k_{-8} &= 1.0 \times 10^3 \text{ s}^{-1} \\
 k_{-4} &= 1.0 \times 10^3 \text{ s}^{-1} & k_{\text{app.}} &= 1.0 \times 10^2 \text{ s}^{-1}
 \end{aligned}$$

Kinetic analysis

Eqn. (1) in the 'Kinetic analysis' sub-section of the main paper has the following coefficients:

$$\begin{aligned}
 \alpha_1 &= k_{+2}k_{+3}k_{+6}k_{+7}k_{+8} \\
 \beta_0 &= k_{+2}k_{+3}k_{-8}(k_{-6} + k_{+7}) \\
 \beta_1 &= k_{+2}k_{+3}k_{+6}k_{+7} \\
 \beta_2 &= k_{+8}[k_{+2}k_{+3}(k_{-6} + k_{+7}) + k_{+6}k_{+7}(k_{-2} + k_{+3})] \\
 \beta_3 &= k_{+2}k_{+6}k_{+8}(k_{+3} + k_{+7})
 \end{aligned} \tag{1A}$$

In the steady-state rate equation for the monophenolase activity of tyrosinase (eqn. 5) the coefficients are:

$$\begin{aligned}
 \gamma_1 &= 2k_{+2}k_{+3}k_{+6}k_{+7}k_{+8}(k_{-4} + k_{+5})K_1 \\
 \delta_0 &= k_{+2}k_{+3}k_{-8}(k_{-6} + k_{+7})(k_{-4} + k_{+5})K_1 \\
 \delta_1 &= 3k_{+2}k_{+3}k_{+6}k_{+7}(k_{-4} + k_{+5})K_1 \\
 \delta_2 &= k_{+8}(k_{-4} + k_{+5})K_1[k_{+2}k_{+3}(k_{-6} + k_{+7}) + k_{+6}k_{+7}(3k_{-2} + k_{+3})] \\
 \delta_3 &= k_{+2}k_{+6}k_{+8}(k_{-4} + k_{+5})(k_{+3} + 3k_{+7})K_1 \\
 \delta_4 &= k_{+6}k_{+7}k_{+8}(3k_{-2} + k_{+3})(k_{-4} + k_{+5}) + k_{+2}k_{+3}k_{+4}k_{+8}(k_{-6} + k_{+7})K_1
 \end{aligned} \tag{2A}$$