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A further step in the kinetic characterisation of the tyrosinase enzymatic system

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Tyrosinase is a cuproprotein that hydroxylates monophenols to *o*-diphenols, which it then oxidises to *o*-quinones, using molecular oxygen. Based on kinetic studies of the steady state and measuring product formation during the action of the enzyme on *o*-diphenols, we determine the Michaelis constant and the maximum velocity, respectively. Similarly, we determine these kinetic constants for the enzyme acting on monophenols. From these constants obtained for a monophenol/*o*-diphenol pair, it is possible to calculate a new constant, the Michaelis constant of the enzyme for an *o*-diphenol acting on the corresponding monophenol, by means of an equation that relates the above-mentioned kinetic constants. Furthermore, it is also possible to establish the relation between the Michaelis constants for the oxygen in the presence of monophenol and in the presence of *o*-diphenol from the relation between the maximum velocities of the monophenol and *o*-diphenol experimentally determined by measuring aminochrome. From applying the equations described above to the kinetic data of the many tyrosinases described in the literature, we find that the Michaelis constant for the *o*-diphenol in the presence of monophenol is much lower than that obtained when the enzyme acts on *o*-diphenol alone. The Michaelis constant for oxygen in the presence of monophenol is also much lower than that obtained in the presence of its *o*-diphenol.

KEY WORDS: biological source, characterisation, kinetic constant, reaction mechanism, Tyrosinase

1. Introduction

Tyrosinase or polyphenoloxidase (PPO) is a copper enzyme widely distributed throughout the phylogenetic scale. It catalyses the hydroxylation of

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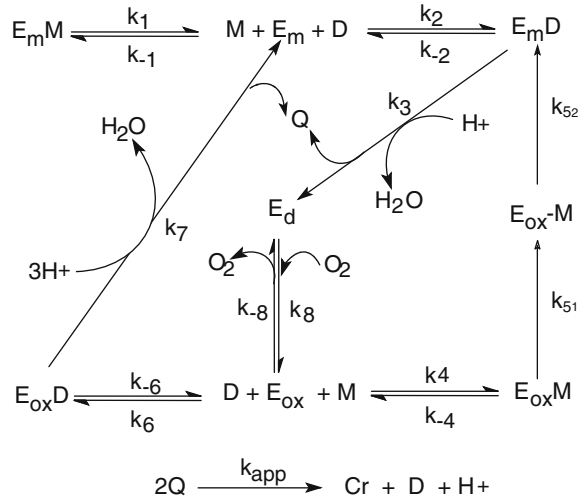

Scheme 1. Proposed kinetic reaction mechanism of tyrosinase acting on M and D, with the non-enzymatic reactions corresponding the evolution of Q. E_d is converted to E_{ox} by binding oxygen (O_2). E_{ox} can either react with a monophenol, M or with an *o*-diphenol, D. In the case of E_{ox} reacting with M, this evolves to D and may be oxidised to Q, generating E_d, or be released into the medium, generating, in turn, E_m. If E_{ox} reacts with D, the corresponding Q is generated. E_m can oxidise D, generating Q and be converted to E_d, and E_m may bind to M generating the dead-end complex, E_mM. Spontaneous endocyclization of Q gives rise to dopachrome (Cr) and D (mechanism previously proposed by us) [29].

monophenol (M) to *o*-diphenols (D) and their subsequent oxidation to *o*-quinones (Q), in both cases by molecular oxygen [1, 2]. The catalytic action of tyrosinase on M is possible because there is a small proportion of oxytyrosinase (E_{ox}, 2–30%) in the native enzyme [3].

The kinetic behaviour of tyrosinase is very complex due to the contemporaneous occurrence of the enzymatic oxidation of M and D to Q, on the one hand, and the coupled non-enzymatic reactions of Q, on the other [3, 4]. Taking the above into account, we proposed a detailed kinetic mechanism and deduced the analytical expressions for the steady-state rates for the monophenolase and diphenolase activities of tyrosinase, scheme 1 [5, 6]. The validity of this mechanism has been substantiated for enzymes extracted from a variety of fruits and vegetables [6–8].

From steady state kinetic studies of the enzyme acting on D, we have determined $V_{\max}^{D,Cr}$ (maximum velocity for the enzyme acting on *o*-diphenols) and K_m^D (the Michaelis constant for D) for a large number of tyrosinases from the whole phylogenetic scale [6–24]. As regards the enzyme acting on M, we have also obtained a group of values $V_{\max}^{M,Cr}$ (the maximum velocity in the presence of monophenol) and K_m^M (the Michaelis constant for the enzyme in the presence of monophenol) [6–26].

As regards D, the kinetic characterisation was completed by determining $K_m^{O_2(D)}$ (the Michaelis constant for oxygen in the presence of D), either by using the initial velocity method or, for small values, the integrated Michaelis equation [27, 28]. When the enzyme acts on M, the $K_m^{O_2(M)}$ values are usually very small so that the Michaelis constant for the oxygen cannot be determined experimentally using the integrated Michaelis constant, as has previously been demonstrated [28], because the steady state breaks down in the sequence of reactions of the mechanism in scheme 1. In such cases, the only way to determine $K_m^{O_2(M)}$ is by means of the analytical expression relating the kinetic parameters and constants for monophenol and D [28, 29].

When tyrosinase acts on M, it is known to show a lag phase (τ), which, as we have demonstrated previously [5, 6], is the time needed for the system to accumulate a given quantity of D in the medium. In the presence of M, therefore, the enzyme shows saturation by D different from K_m^D, and which we shall refer to as $K_m^{D(M)}$ (the Michaelis constant for D in the presence of its M); by estimating this kinetic constant, the kinetics of the system can be completed.

In this contribution, we shall demonstrate how it is possible to amplify the kinetic characterisation of any tyrosinase from the steady state kinetic constants ($V_{\max}^{D,Cr}$, K_m^D, $V_{\max}^{M,Cr}$, K_m^M), taking into account the analytical expressions deduced in the kinetic analysis of this mechanism in previous studies, [11, 30–32].

More specifically, we propose calculating $K_m^{D(M)}$ and the ratio between the Michaelis constants $K_m^{O_2(M)}/K_m^{O_2(D)}$ for oxygen, based on the data published in the bibliography for tyrosinases from several sources. We shall discuss the values obtained in order to draw general conclusions concerning the kinetic behaviour of the enzyme and the fulfilment of scheme 1 across the whole phylogenetic scale. In addition, in the case of mushroom tyrosinase, for which we know the K_m^{M,O_2} and K_m^{D,O_2}, the kinetic characterisation is completed from quantitative relations, which have a kinetic meaning, which can be used to obtain information on the system under study.

2. Material and methods

NMR Assays. ^{13}C NMR spectra of the different M and D tested were obtained in a Varian Unity spectrometer of 300 MHz.

3. Results and discussion

The action mechanism of tyrosinase on D is that depicted in scheme 1, with $[M]_0 = 0$. The analytical expression for the steady state rate of product accumulation (dopachrome, Cr, is the physiological substrate L-Dopa) [11, 29–31] is:

$$V_{ss}^{D,Cr} = \frac{V_{max}^{D,Cr}\,[D]_0\,[O_2]_0}{K_m^D K_s^{O_2} + K_m^{O_2(D)}\,[D]_0 + K_m^D\,[O_2]_0 + [D]_0\,[O_2]_0}. \tag{1}$$

Taking into account kinetic considerations developed in previous studies [11, 29–31]:

$$V_{max}^{D,Cr} = k_{cat}^D\,[E]_0 = k_7\,[E]_0\,, \tag{2}$$

$$k_{cat}^D = k_7, \tag{3}$$

$$K_m^D = k_7/k_6, \tag{4}$$

$$K_m^{O_2(D)} = k_7/k_8, \tag{5}$$

$$K_s^{O_2} = k_{-8}/k_8. \tag{6}$$

From the measurements of enzymatic activity in the steady state ($V_{ss}^{D,Cr}$) and by non-linear regression fitting to equation (1), we can determine $V_{max}^{D,Cr}$ and K_m^D. If the enzymatic activity can be estimated by measuring the consumption of oxygen (V_{ss}^{D,O_2}), we can determine V_{max}^{D,O_2} and K_m^D; now, according to the stoichiometry of scheme 1, $V_{ss}^{D,Cr} = V_{ss}^{D,O_2}$ and so $V_{max}^{D,Cr} = V_{max}^{D,O_2}$. The data for the tyrosinases from several biological sources are presented in tables 1 and 2.

The action of the enzyme on M corresponds to the mechanism described in scheme 1. Following the methodology and kinetic considerations expressed in previous studies [11, 29–31], the analytical expression for the enzyme's steady state action rate for the accumulation of product can be obtained.

$$V_{ss}^{M,Cr} = \frac{V_{max}^{M,Cr}\,[M]_0\,[O_2]_0}{K_m^M K_s^{O_2} + K_m^{O_2(M)}\,[M]_0 + K_m^M\,[O_2]_0 + [M]_0\,[O_2]_0} \tag{7}$$

with

$$V_{max}^{M,Cr} = k_{cat}^M\,[E]_0 = \frac{k_{5_1}k_{5_2}}{k_{5_1} + k_5}\,[E]_0\,, \tag{8}$$

$$k_{cat}^M = \frac{k_{5_1}k_{5_2}}{k_{5_1} + k_{5_2}}, \tag{9}$$

$$K_m^M = \frac{k_{cat}^M}{k_4}, \tag{10}$$

$$K_m^{O_2(M)} = \frac{3k_{cat}^M}{2k_8}. \tag{11}$$

Table 1
Kinetic constants characterising the reaction of tyrosinases from different biological sources on monophenols, diphenols and oxygen.

Tyrosinase source*	Monophenol/Diphenol	k_{cat}^{M} (s^{-1})	K_m^M (mM)	k_{cat}^{D} (s^{-1})	K_m^D (mM)	$K_m^{D(M)}$ (μM)	$K_m^{O_2(M)}/K_m^{O_2(D)}$	R
S.glaucescens [9]	4HPPA/DHPPA	134.0	0.48	1830.0	1.05	38.4	0.11	0.08
	4HPAA/DHPAA	48.8	1.16	142.0	1.16	199.3	0.52	0.17
	LTyr/LDopa	13.2	0.41	1445.0	5.77	26.3	0.01	0.06
N. crassa [10]	4HPAA/DHPAA	250.0	0.18	850.0	4.40	647.0	0.44	3.54
	LTyr/LDopa	320.0	0.59	1070.0	1.04	155.5	0.45	0.26
	DTyr/DDopa	160.0	0.32	340.0	0.38	89.4	0.71	0.28
	Tyramine/Dopamine	160.0	0.67	1350.0	0.28	16.5	0.18	0.02
A.bisporus [11,13]	P/Cat	12.7	0.70	877.6	0.30	2.1	0.02	0.003
	Tyramine/Dopamine	25.9	0.51	439.0	2.20	64.8	0.09	0.13
	LTyr/ LDopa	7.9	0.21	107.4	0.80	29.4	0.11	0.14
	DTyr/DDopa	8.0	1.86	107.4	4.50	167.5	0.11	0.09
	DLTyr/DLDopa	8.1	0.90	107.0	1.40	52.9	0.11	0.06
	4HPPA/DHPPA	66.7	0.44	553.5	1.89	113.8	0.18	0.26
	4HPAA/DHPAA	44.3	1.91	631.6	5.10	178.8	0.11	0.09
	LTyrMeE/LDopaMeE	3.4	0.38	35.5	0.57	27.2	0.14	0.07
	LαMeTyr/LαMeDopa	0.6	1.20	44.3	6.80	46.0	0.02	0.04
	DLαMeTyr/DLαMeDopa	0.6	1.45	44.3	8.00	54.1	0.02	0.04

*The enzymes used in these studies were purified from *S. glaucescens* [9], *N.crassa* [10] and *A. bisporus* [11,13]. Kinetic data without errors in the original references [9,10]. 4HPPA: 4-hydroxyphenyl propionic acid; DHPPA: 3,4-dihydroxyphenyl propionic acid; 4HPAA: 4-hydroxyphenyl acetic acid; DHPAA: 3,4-dihydroxyphenyl acetic acid; LTyr: L-tyrosine; DTyr: D-Tyrosine; P: phenol; Cat: catechol; LTyrMeE: L-Tyrosinemethylester; LDopaMeE: L-dopametylester; LαMeTyr: L-α-methyltyrosine; LαMeDopa: L-α-methyldopa; DLαMeTyr: DL-α-methyltyrosine; DLαMeDopa: DL-α-methyldopa.

Table 2
Kinetic parameters and constants characterising the reaction of tyrosinases from different biological sources on monophenols, diphenols and oxygen.

Tyrosinase source*	Monophenol/Diphenol	V_{max}^{M} (μM/min)	K_m^M (mM)	V_{max}^{D} (μM/min)	K_m^D (mM)	$K_m^{D(M)}$ (μM)	$K_m^{O_2(M)}/K_m^{O_2(D)}$	R
Apple *cv.verdedoncella* [8]	Tyramine/Dopamine	0.2	1.70	36.2	1.50	4.1	0.008	0.002
	4HPAA/DHPAA	1.8	8.30	1890.0	39.80	19.0	0.001	0,002
	4HPPA/DHPPA	3.8	2.80	716.0	3.30	9.0	0.008	0.003
Pear *cv blanquilla* [8]	Tyramine/Dopamine	0.3	1.70	70.3	1.50	3.2	0.006	0.002
	4HPAA/DHPAA	1.9	2.20	2500.0	53.10	20.2	0.001	0.009
	4HPPA/DHPPA	4.1	0.50	1500.0	8.80	12.0	0.004	0.024
Tuber Melanosporum [14]	LTyr/ LDopa	9.0[d]	0.29	139.0[d]	0.34	11.0	0.097	0.038
Mouse-will type [15]	LTyr/ LDopa	0.9[e]	0.08	38.8[e]	0.46	5.3	0.035	0.067
Mouse-H389L [15]	LTyr/ LDopa	0.9[e]	0.11	38.9[e]	1.83	20.6	0.034	0.195
Mouse-Q378H [15]	LTyr/ LDopa	1.5[e]	0.08	32.7[e]	22.20	499.0	0.067	6.572
B16 Mouse Melanoma-H[b][16]	LTyr/ LDopa	5.7[e]	0.12	343.0[e]	0.51	4.2	0.017	0.035
B16 Mouse Melanoma-L[c][16]	LTyr/ LDopa	0.3[e]	0.23	46.0[e]	1.90	6.6	0.007	0.029
B16 Mouse Melanoma [17]	4-S-CAP/4-S-CAC	6.4[g]	0.32	101.0	0.40	12.6	0.095	0.040
Strawberry *cv tioga* [19]	4PHPPA/DHPPA	0.7	0.30	96.0	1.50	54.0	0.011	0.018
Gerbil eye [12]	LTyr/ LDopa	0.5[f]	62.00[h]	26.6[f]	0.41	3.8	0.028	0.062
Avocado *cv Haas* [20]	4PHPPA/DHPPA	0.6	0.30	30.0	3.40	34	0.030	0.113
Musa acuminata [21]	Tyramine /Dopamine	2.1	0.75	7.3	0.57	82.0	0.431	0.109
Musa acuminata [22]	Tyramine/Dopamine+SDS	2.1	0.75	71.4	0.57	8.4	0.044	0.011
Salanum melongena [23]	4-MeP/4-MeCat	47.9	0.38	14000.0	2.70	4.6	0.005	0.012
Pycnoporus sanguineus CBS 614.73 [24]	LTyr/ LDopa	54.0[b]	1.00	112.0[b]	0.90	216.0	0.723	0.217
Agaricus bisporus [34]	GHB/GDHB	2.1	0.30	210.0	7.80	39.0	0.015	0.130

*Kinetic data without errors in the original references ([12,14,15,21–23]). [a]U/ml/min. [b]U/mg. [c]U/mg^{-1}. [d]mU/mg^{-1}. [e]mU/eye. [f]nmol/min/mg. [g]μM. GHB: γ-L-glutaminyl-4-hydroybenzene; GDBH: γ-L-glutaminyl-3, 4-dihydroxybenzene.

Since the ratio, R, between $[D]_{ss}$ (the quantity of D that accumulates in the medium during the evolution of Q) and $[M]_0$ [6, 29–31], can be established in the steady state:

$$\frac{[D]_{ss}}{[M]_0} = R = \frac{V_{max}^{M,Cr} K_m^D}{2V_{max}^{D,Cr} K_m^M} = \frac{k_{cat}^M k_{cat}^D}{2k_{cat}^D K_m^M} = \frac{k_4}{2k_6} \tag{12}$$

equation (7) can be expressed as a function of $[D]_{ss}$ in the form:

$$V_{ss}^{M,Cr} = \frac{V_{max}^{M,Cr} [D]_{ss} [O_2]_0}{K_m^{D(M)} + K_m^{O_2(M)} [D]_{ss} + K_m^{D(M)} [O_2]_0 + [D]_{ss} [O_2]_0} \tag{13}$$

with

$$K_m^{D(M)} = \frac{k_{cat}^M}{2k_6}, \tag{14}$$

where $V_{max}^{M,Cr}$ and $K_m^{O_2(M)}$ are given by equations (8) and (11), respectively. From the measurements made of the enzymatic activity in its steady state, $V_{ss}^{M,Cr}$, and by fitting these data by non-linear regression according to equation (7), we can obtain $V_{max}^{M,Cr}$ and K_m^M. If the enzymatic activity can be estimated by measuring the consumption of oxygen (V_{ss}^{M,O_2}), we can determine V_{max}^{M,O_2} and K_m^M; now, according to the stoichiometry of scheme 1, $V_{ss}^{M,Cr} = (2/3) V_{ss}^{M,O_2}$ and so $V_{max}^{M,Cr} = (2/3) V_{max}^{M,O_2}$. The data for the tyrosinases from several biological sources are presented in tables 1 and 2.

From the analytical expressions for $V_{max}^{D,Cr}$, K_m^D, $V_{max}^{M,Cr}$ and K_m^M, equations (2), (4), (8) and (10), respectively, the following expressions can be established [27–31]:

$$K_m^{D(M)} = \frac{V_{max}^{M,Cr} K_m^D}{2V_{max}^{D,Cr}} \tag{15}$$

and

$$\frac{K_m^{O_2(M)}}{K_m^{O_2(D)}} = \frac{3}{2} \frac{V_{max}^{M,Cr}}{V_{max}^{Cr,D}} = \frac{V_{max}^{M,O_2}}{V_{max}^{D,O_2}}. \tag{16}$$

Or, if oxygen consumption has been measured the following equations result from V_{max}^{M,O_2}, K_m^M, V_{max}^{D,O_2} and K_m^D;

$$K_m^{D(M)} = \frac{V_{max}^{M,O_2} K_m^D}{3V_{max}^{D,O_2}}, \tag{17}$$

$$\frac{K_m^{O_2(M)}}{K_m^{O_2(D)}} = \frac{V_{max}^{M,O_2}}{V_{max}^{D,O_2}}. \tag{18}$$

Bearing in mind the data shown in tables 1 and 2 and taking into account equations (15) and (16), the values of $K_m^{D(M)}$ and the ratio $K_m^{O_2(M)}/K_m^{O_2(D)}$ can be calculated (shown in the same tables). The following conclusions arise from these values:

(a) According to tables 1 and 2, the values of $V_{\max}^{D,Cr}$ are always greater than the values of $V_{\max}^{M,Cr}$: $V_{\max}^{D,Cr} > V_{\max}^{M,Cr}$. If we take into account the analytical expressions of $V_{\max}^{D,Cr}$ (equation (2)) and $V_{\max}^{M,Cr}$ (equation (8)), it can be seen that the enzyme oxidises the D more easily than the M. In the first case (D), the critical steps would be (scheme 2): non-covalent binding of D to E_{ox}, nucleophilic attack of the oxygen from OH of the C-4 on the copper, simultaneous transfer of the H^+ to the peroxide, nucleophilic attack of the oxygen from OH of the C-3 on the copper followed by oxidation/reduction of the D to Q. However, in the case of M, the critical steps would be: non-covalent binding of M to E_{ox}, nucleophilic attack of the oxygen from OH of the C-4 on the copper and simultaneous transfer of the H^+ to the peroxide, electrophilic attack of the peroxide in position C-3 of the M and subsequent oxidation/reduction to Q or the release of D. From an electronic point of view, in table 3, we show the values of the chemical displacements of the C-4 of some D and their respective M, from which values it is concluded that D are better nucleophiles than M, which means that $k_{cat}^D > k_{cat}^M$ (equations (3) and (9), respectively).

(b) In general, for all the tyrosinases studied $K_m^D \geqslant K_m^M$ (see tables 1 and 2). According to their analytical expressions (equations (4) and (10)), the values of $k_7 > k_{5_1}k_{5_2}/k_{5_1} + k_{5_2}$, although $k_6 > k_4$, according to the values of δ_4 for D and M (see table 3). However, the catalytic steps are much faster in the former case, which influences k_{cat} and the fact that $K_m^D \geqslant K_m^M$ (tables 1 and 2).

(c) For all the tyrosinases mentioned in this work $K_m^{D(M)} < K_m^D$, which is confirmed by the analytical expressions of equations (3), (4) and (14) since $k_{cat}^M < k_{cat}^D$ and the denominator in $K_m^{D(M)}$ is double that in K_m^D. These two expressions for the Michaelis constants, K_m^D and $K_m^{D(M)}$, do

Scheme 2. Structural mechanism proposed to explain the kinetic reaction mechanism of tyrosinase acting on M. E_m, *met*tyrosinase, E_{ox}, *oxy*tyrosinase, E_d, *deoxy*tyrosinase, M, monophenol, D, *o*-diphenol, B, acid-base catalyst. *Dead-end pathway*: E_mM_0, interaction complex between E_m and M, E_mM_1, nucleophilic attack complex from M to E_m. *Oxidase cycle*: E_mD_0, interaction complex between E_m and D, E_mD_1, axial nucleophilic attack complex from OH of C-4 to E_m, E_mD_2, diaxial binding complex of D with E_m, $E_{ox}D_0$, interaction complex of D with E_{ox}, $E_{ox}D_1$, axial binding complex of D with E_{ox}, $E_{ox}D_2$ diaxial binding complex of D with E_{ox}. *Hydroxylase cycle*: $E_{ox}M_0$, interaction complex of M with E_{ox}, $E_{ox}M_1$, axial nucleophilic attack complex from M to E_{ox}, E_mD_3, axial-equatorial hydroxylation complex from M to D, E_mD_4, rearrangement complex with C-4 bond break.

not mean that the enzyme has two binding sites for D: in our mechanism, schemes 1 and 2, there is only one site for the binding of substrate and enzyme for M and D, unlike in [33], where two separate sites are

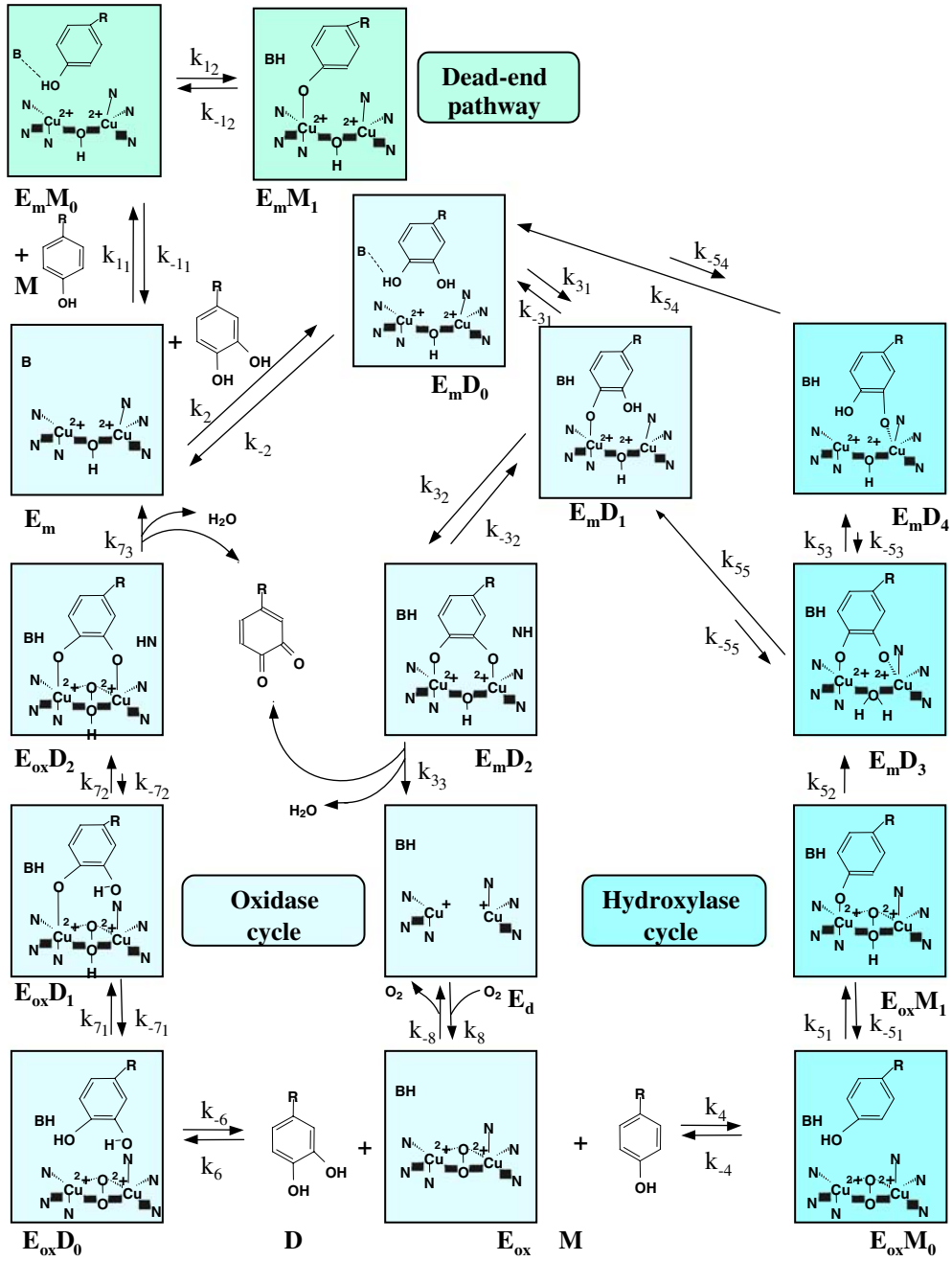

Table 3
Values of $\delta_3 y \delta_4$ for C-3 and C-4, respectively, of the benzene ring of monophenols and *o*-diphenols[a].

Monophenol	δ_3 (ppm)	δ_4 (ppm)	Diphenol	δ_3 (ppm)	δ_4 (ppm)
Tyramine	118.49	157.28	Dopamine	146.79	145.58
LTyr (D and DL)	118.08	158.86	LDopa (D and DL)	146.92	146.06
LTyrMeE	118.53	157.75	LDopaMeE	146.90	146.18
LαMeTyr (DL)	118.11	159.10	LαMeDopa(DL)	146.74	146.19
P	118.13	158.15	Cat	146.59	146.59
4HPAA	118.05	156.49	DHPAA	146.43	144.96
4HPPA	118.02	156.13	DHPPA	146.51	144.61
GHB[b]	120.11	160.58	GDHB[b]	146.45	143.51

[a]Reference [31].
[b]Reference [34].

proposed. It should be remembered that these kinetic constants are not binding constants but Michaelis constants with a kinetic meaning. Thus, the enzyme acting on M is saturated by low concentrations of D [29–31], and it is this D (D_{ss}), which accumulates in the medium through the action of the enzyme on M and, especially, as a result of the evolution of Q during the lag phase.

(d) Tables 1 and 2 show the values of the $K_m^{O_2(M)}/K_m^{O_2(D)}$ ratio for several monophenol/diphenol pairs. Note that the values are always < 1, indicating that, although it is always the E_d form that binds the O_2 in the catalytic cycle, with a fixed constant, $K_s^{O_2}$ [33], the Michaelis constants for oxygen in the presence of M are lower than in the presence of D, for all the tyrosinases included in the study. The analytical expressions $K_m^{O_2(D)}$ and $K_m^{O_2(M)}$ (equations (5) and (11), respectively) explain this difference in values since $k_{cat}^M < k_{cat}^D$. From a physiological point of view this relation is interesting since it indicates that the enzyme at low concentrations of oxygen is normally saturated kinetically, which is seen when the absolute values of $K_m^{O_2(D)}$ and $K_m^{O_2(M)}$ are determined [27, 28, 34]. For example, in the case of mushroom tyrosinase, the values of $K_m^{O_2(M)}$ were in the range (0.22 ± 0.02)–$(12.0 \pm 2.5)\,\mu$M for LTyrMeE and 4HA, respectively [27]. These values were obtained from equation (16), which is the only way to obtain $K_m^{O_2(M)}$ when the value is very low [27, 28].

(e) Calculation of constant R. This constant is described analytically according to equation (12). As seen in tables 1 and 2, the constant can be obtained from the kinetic parameters and constants of the steady state and indicates the $[D]_{ss}$ that must accumulate in the system for the steady state to be reached for a given $[M]$. For the enzyme's action on monophenols to be accurately measured, this concentration of D_{ss}

must be added at the beginning of the reaction. Note the low values of this constant, especially in the case of vegetal enzymes [8, 19]; furthermore, the value is related with the constant $K_m^{D(M)}$, equations (12) and (14).

3.1. Mushroom tyrosinase

Table 4 represents a complete characterisation of mushroom tyrosinase acting on three M/D pairs: (i) its physiological substrates γ-L-glutaminyl-4-hydroxybenzene/γ-L-glutaminyl-3,4-dihydroxybenzene, (ii) the physiological mammalian pair L-tyrosine/L-Dopa and (iii) on tyramine/dopamine.

The table shows how it is possible to calculate $K_m^{D(M)}$, R and the ratio $K_m^{M,O_2}/K_m^{D,O_2}$ from $V_{\max}^{MóD(Cr)}$ and $K_m^{MóD}$, the kinetic constants determined from steady state data by measuring the product and based on developing the kinetic analysis of this mechanism (scheme 1) [30, 31, 33], Note that $V_{\max}^{D,Cr} > V_{\max}^{M,Cr}$; $K_m^D > K_m^M$; $K_m^{D(M)} \ll K_m^D$ and that the dimensionless constants, R and $K_m^{M,O_2}/K_m^{D,O_2}$, are $\ll 1$. If the concentration of enzyme is known, as is the case with the data of table 4, it is possible to continue with the kinetic characterisation. From the values of $V_{\max}$, the k_{cat} can be calculated and, as mentioned above, $k_{\text{cat}}^D > k_{\text{cat}}^M$. In a previous step and according to the analytical expressions, it is possible to calculate k_4 and k_6, the constants for monophenol and diphenol binding to the form E_{ox}; as can be seen, the D bind to the enzyme better than the monophenols.

By measuring oxygen consumption, it is possible to determine $V_{\max}^{M,O_2}$ and $V_{\max}^{D,O_2}$ (see table 4), from which it can be seen that $V_{\max}^{D,O_2} = V_{\max}^{D,Cr}$ and $V_{\max}^{M,O_2} = 1.5V_{\max}^{M,Cr}$. In the case of D, the determination of $V_{\text{ss}}^{O_2}$ or the application of the integrated equation to the consumption of oxygen makes it possible to obtain K_m^{D,O_2}; by applying equation (18), K_m^{M,O_2} can be obtained immediately. If we normalise the values of $V_{\max}^{MóD,O_2}$ with respect to the concentration of enzyme, the ratios $V_{\max}^{M,O_2}/K_m^{O_2}$ and $V_{\max}^{D,O_2}/K_m^{D,O_2}$ should be equal according to equations (2) and (5) and equations (8) and (11), respectively, corresponding to k_8 (the binding constant of O_2 to the form E_d). The fact that the data shown in table 4 show slight deviations may be due to the fact that the data for the L-tyrosine/L-dopa pair were obtained at 20°C, while those for the tyramine/dopamine were obtained at 14.5°C [27]. The difference is even greater with the data from Espin et al. [34], which is to be expected because the MBTH method was used, while DMF was used to solubilise the chromophoric adducts of the Q [34]. As has been demonstrated during the course of this work, the $K_m^{M,O_2}/K_m^{M,O_2}$ ratio is low. The value of R (constant for one enzyme acting on one M/D pair) is shown in table 4 and is low in all cases. Lastly, as regards $K_1 = k_{-1}/k_1$ (the dissociation constant of the dead-end complex, E_mM), its value, as discussed in [30], must be lower than K_m^M since M, as occurs with D, binds better to E_m than to

Table 4
Kinetic parameters and constants characterising the reactions of tyrosinase from mushroom on monophenols, diphenols, and oxygen.

Kinetic constant	GHB[a]	GDHB[a]	LTyr[a]	LDopa[a]	Tyramine[b]	Dopamine[b]	LTyr[b]	LDopa[b]
V_{max}^{Cr}(nM/s)	0.85 ± 0.04	85.40 ± 2.96	1.26 ± 0.06	37.23 ± 1.70	7.30 ± 0.80	106.30 ± 0.80	4.51 ± 0.30	50.10 ± 0.80
$V_{max}^{O_2}$(nM/s)	1.30 ± 0.08	81.38 ± 3.32	1.91 ± 0.08	36.74 ± 2.07	10.80 ± 0.50	106.80 ± 0.50	6.85 ± 0.10	51.00 ± 0.10
K_m(mM)	0.30 ± 0.03	7.80 ± 0.41	0.50 ± 0.04	1.50 ± 0.07	1.51 ± 0.15	0.88 ± 0.07	0.50 ± 0.09	0.42 ± 0.05
$K_m^{O_2}$(μM)	1.50 ± 0.12	100.20 ± 8.22	1.98 ± 0.74	38.20 ± 3.10	5.02 ± 0.10	48.20 ± 0.20	1.26 ± 0.05	9.50 ± 0.08
k_{cat}^{M} (s^{-1})	1.42 ± 0.07		2.10 ± 0.09		12.17 ± 1.33		7.52 ± 0.50	
$k_4 = k_{cat}^{M}/K_m^{M}$ ($M^{-1}s^{-1}$)	$(4.72 \pm 0.52)10^3$		$(4.20 \pm 0.38)10^3$		$(8.05 \pm 1.19)10^3$		$(1.50 \pm 0.29)10^4$	
k_{cat}^{D} (s^{-1})		142.33 ± 4.93		62.10 ± 2.83		177.17 ± 1.33		83.49 ± 1.33
$k_6 = k_{cat}^{D}/K_m^{D}$ ($M^{-1}s^{-1}$)		$(1.82 \pm 1.15)10^4$		$(4.14 \pm 0.27)10^4$		$(2.01 \pm 0.16)10^5$		$(1.98 \pm 0.29)10^5$
K_m^{D} (μM)		38.82 ± 3.05		25.38 ± 2.05		30.21 ± 4.09		18.90 ± 2.59
$V_{max}^{O_2}/K_m^{O_2}$ (s^{-1})	$(8.67 \pm 0.87)10^{-4}$	$(8.12 \pm 0.74)10^{-4}$	$(5.10 \pm 1.90)10^{-4}$	$(9.62 \pm 0.95)10^{-4}$	$(1.45 \pm 0.33)10^{-4}$	$(22.00 \pm 0.19)10^4$	$(5.43 \pm 0.23)10^{-3}$	$(5.27 \pm 0.95)10^{-3}$
$k_{cat}/K_m^{O_2} = k_8$ ($M^{-1}s^{-1}$)	$(0.94 \pm 0.09)10^6$	$(1.42 \pm 0.13)10^6$	$(1.06 \pm 0.39)10^6$	$(1.62 \pm 0.19)10^6$	$(2.43 \pm 0.27)10^{-6}$	$(3.68 \pm 0.03)10^6$	$(5.96 \pm 0.96)10^6$	$(8.78 \pm 0.15)10^6$
R	$(13 \pm 2)10^{-2}$		$(5.08 \pm 0.58)10^{-2}$		$(2.00 \pm 0.34)10^{-2}$		$(3.78 \pm 0.85)10^{-2}$	
$K_m^{O_2,M}/K_m^{O_2,D}$	$(1.50 \pm 0.17)10^{-2}$		$(5.18 \pm 1.98)10^{-2}$		0.10 ± 0.01		0.13 ± 0.01	
K_1 (μM)					300 ± 30		34 ± 11	

[a] Reference [34].
[b] Reference [27].

E_{ox} ($k_1 > k_4$), since a base (histidine) helps in the deprotonation of the M and favours the nucleophilic attack of the oxygen on copper. This constant, K_1, has only been determined in transition phase studies of the pairs L-tyrosine/L-dopa, tyramine/dopamine and L-tyrosine methyl esther/L-dopa methyl esther [30, 35]. Therefore, with the data shown in table 4, the action of a tyrosinase acting on a monophenol/diphenol pair is characterised.

In summary, from this study it is established that tyrosinases from a variety of biological sources catalyse the hydroxylation of M and the oxidation of D through the mechanism described in schemes 1 and 2. In addition, we establish a method for calculating a new constant, $K_m^{D(M)}$, from the steady state kinetic parameters and constants, $V_{max}^{M,Cr}$, K_m^M, $V_{max}^{D,Cr}$ and K_m^D. Moreover, from the steady state kinetic constants it is possible to determine the ratio between the Michaelis constants with respect to oxygen. In this way, we extend the kinetic characterisation of the tyrosinase enzymatic system in different biological sources.

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