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# Kinetic study of monophenol and *o*-diphenol binding to *oxy*tyrosinase

F. García-Molina[a], M.J. Peñalver[a], L.G. Fenoll[a], J.N. Rodríguez-López[a], R. Varón[b], F. García-Cánovas[a,*], J. Tudela[a]

[a] *Grupo de Investigación de Enzimología (GENZ), Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología, Universidad de Murcia, A. Correos 4021, E-30080, Murcia, Spain*
[b] *Departamento de Química-Física, Escuela Politécnica Superior de Albacete, Universidad de Castilla La Mancha, E-02071 Albacete, Spain*

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**Abstract**

The complex reaction mechanism of tyrosinase involves three enzymatic forms, two overlapping catalytic cycles and a dead-end complex. The *deoxy*tyrosinase form binds oxygen with a high degree of affinity, $K_s^{O_2} = 46.6 \pm 2.4\,\mu M$. The *met*tyrosinase and *oxy*tyrosinase forms bind monophenols and *o*-diphenols, although the former is inactive on monophenols. Analytical expressions for the catalytic and Michaelis constants of tyrosinase towards phenols and *o*-diphenols have been derived. Thus, the Michaelis constant of tyrosinase towards monophenols ($K_m^M$) and *o*-diphenols ($K_m^D$) are related with the catalytic constants for monophenols ($k_{cat}^M$) and *o*-diphenols ($k_{cat}^D$), respectively, and with the binding rate constants of the *oxy*tyrosinase form with these substrates ($k_{+4}$ and $k_{+6}$, respectively), by means of the expressions $K_m^M = k_{cat}^M/k_{+4}$ and $K_m^D = k_{cat}^D/k_{+6}$. From these expressions, we calculate the values of the binding rate constant of *oxy*tyrosinase to the substrates (monophenols and *o*-diphenols) for tyrosinases from different biological sources, and reveal that the *o*-diphenols bind more rapidly to *oxy*tyrosinase than the monophenols. In addition, a new kinetic constant $K_m^{D(M)} = k_{cat}^M/2k_6$ (the Michaelis constant for *o*-diphenol in the monophenolase activity), is derived and determined. Thus, it has been shown that tyrosinase has apparently higher affinity towards *o*-diphenols in its monophenolase than in its diphenolase activity.


*Keywords:* Monophenol and *o*-diphenol; *Oxy*tyrosinase; Dead-end complex

## 1. Introduction

Tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) is a copper enzyme that is widely distributed throughout micro-organisms, plants and animals. It is of central importance in such processes as vertebrate pigmentation and the browning of fruits and vegetables [1]. Different tyrosinases obtained from several biological sources have similar structural and functional characteristics [2].

The active site of tyrosinase consists of two copper atoms and three states: "*met*", "*deoxy*" and "*oxy*" [3]. Structural models for the active site of these three forms have been proposed [4]. This enzyme catalyses the *ortho*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinone (diphenolase activity) [3,4]. The monophenolase activity is coupled to its diphenolase activity and to the non-enzymatic reactions from the corresponding *o*-quinones [5–7]. The kinetic characterisation of tyrosinase from different sources with respect to different monophenols and *o*-diphenols has been carried out, exhaustively studying its behaviour with the enzyme of *Streptomyces glaucescens* [8]. Data are also available for tyrosinases from *Neurospora crassa* [9], *Streptomyces antibioticus* [10], *Agaricus bisporus* [11–16], *Tuber melanosporum* [17], mammalian [18], melanoma B16 mouse tyrosinase [19] and Gerbil eye tyrosinase [20].

Furthermore, the stereospecificity of different tyrosinases has been studied in their action on several chiral

* Corresponding author. Present address: Biochemistry, University of Murcia, Apartado 4201, E30100 Murcia, Spain.
Tel.: +34 968364782; fax: +34 968364147.
*E-mail address:* canovasf@um.es (F. García-Cánovas).
*URL:* http://www.um.es/genz (F. García-Cánovas).

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monophenolic and *o*-diphenolic substrates [8,21,22]. A spectrophotometric method that uses the chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH) has provided quantitative data concerning the action of different animal and vegetal tyrosinases [22–32]. NMR studies of different substrates have revealed the chemical displacements, $\delta_3$ and $\delta_4$, which are related with the nucleophilic power of the oxygens of the phenolic OH in C-3 and C-4, values that have been used as predictors of their catalytic efficiency with tyrosinase. The results obtained for the enzymatic activity were compared with the NMR predictions [12–16,21,22].

Based on a detailed kinetic study and considering the kinetic information available [15], analytical expressions for the Michaelis constants of the enzyme towards monophenols ($K_m^M$) and *o*-diphenols ($K_m^D$) were obtained, along with the respective catalytic constants, $k_{cat}^M$ and $k_{cat}^D$. Information concerning the values of these kinetic constants for tyrosinases from different biological sources is available [8–22,26–33].

The object of the study described here is to determine the binding rate constants of monophenols and *o*-diphenols to the oxytyrosinase form of the tyrosinases from different sources, using the analytical expressions of the kinetic constants $K_m$ and $k_{cat}$. At the same time, the kinetic analysis carried out here provide a new kinetic constant, $K_m^{D(M)}$ (the Michaelis constant for a *o*-diphenol in the monophenolase activity). We correlate the NMR data obtained with the nucleophilic power of the oxygens of the phenolic OH's of the carbons C-3 and C-4, which, in turn, are related with the $\delta_3$ and $\delta_4$ values obtained by NMR. Lastly, we attempt to generalise the proposed kinetic reaction mechanism to tyrosinases from the whole phylogenetic scale.

## 2. Results and discussion

The melanin biosynthesis pathway begins with the hydroxylation of L-tyrosine by tyrosinase using molecular oxygen to give L-dopa. In a subsequent step, the L-dopa is oxidised with molecular oxygen to *o*-dopaquinone, which undergoes a series of chemical reactions to dopachrome [1,5] (Scheme 1).

### 2.1. Diphenolase activity

The reaction of tyrosinase on *o*-diphenols is known as diphenolase activity, the mechanism of which involves the oxidase cycle and non-enzymatic coupling reactions (Scheme 2, considering M = 0). Applying the steady-state approach, the expression for dopachrome formation rate is obtained [14]:

$$V_{ss}^{D,Cr} = \frac{\alpha[D]_0[O_2]_0[E]_0}{\beta_0 + \beta_1[D]_0 + \beta_2[O_2]_0 + \beta_3[D]_0[O_2]_0} \quad (1)$$

This rate is equal to the oxygen consumption rate $V_{ss}^{D,O_2}$ ($V_{ss}^{D,Cr} = V_{ss}^{D,O_2}$) with:

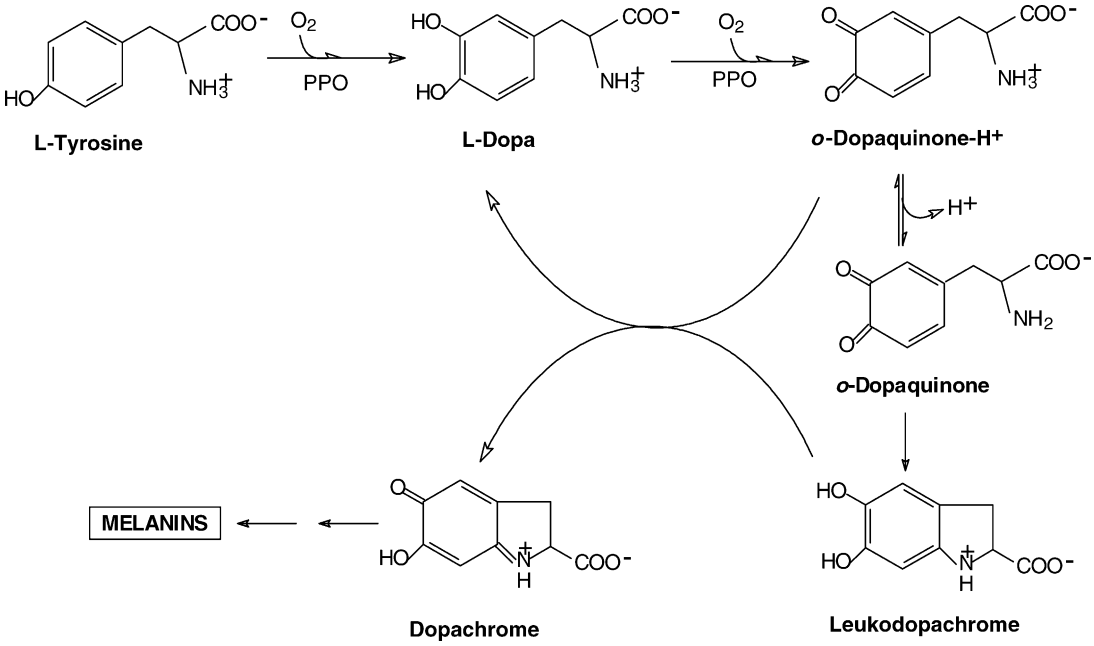
$$\alpha_1 = k_{+2}k_{+3}k_{+6}k_{+7}k_{+8} \quad (2)$$

$$\beta_0 = k_{+2}k_{+3}k_{-8}(k_{-6} + k_{+7}) \quad (3)$$

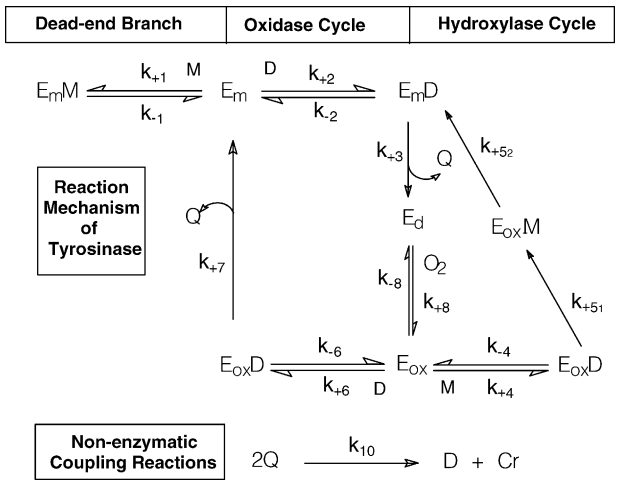
$$\beta_1 = k_{+2}k_{+3}k_{+6}k_{+7} \quad (4)$$

$$\beta_2 = k_{+8}[k_{+2}k_{+3}(k_{-6} + k_{+7})] + k_{+6}k_{+7}(k_{-2} + k_{+3}) \quad (5)$$

$$\beta_3 = k_{+2}k_{+6}k_{+8}(k_{+3} + k_{+7}) \quad (6)$$




Scheme 1. Melanogenesis pathway from L-tyrosine to dopachrome.




Scheme 2. Reaction mechanism of PPO acting on M and D with chemical reactions corresponding to Q evolution. $E_d$ is converted to $E_{ox}$ by binding $O_2$. $E_{ox}$ can react either with M or with D. In the first case, the $E_{ox}$M complex is transformed into $E_{ox}$–M, with a rate constant of $k_{+5_1}$, where M is bound to the copper of the active centre. Then, by means of the electrophilic attack controlled by $k_{+5_2}$, M evolves to D which 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 $E_d$, while $E_m$ may bind to M, generating the dead-end complex $E_m$M. Spontaneous endocyclisation of Q gives rise to Cr and D.

Hence, Eq. (1) can be written as

$$V_{ss}^{D,Cr} = \frac{V_{max}^{D,Cr}[D]_0[O_2]_0}{W + K_m^{O_2(D)}[D]_0 + K_m^D[O_2]_0 + [D]_0[O_2]_0} \quad (7)$$

where $V_{max}^{D,Cr}$ is the maximum rate of dopachrome formation from an *o*-diphenol; $K_m^D$ the Michaelis constant for *o*-diphenol and $K_m^{O_2(D)}$ the Michaelis constant for $O_2$ in presence of *o*-diphenol, with

$$V_{max}^{D,Cr} = k_{cat}^D[E]_0 \quad (8)$$

$$W = \frac{k_{+3}(k_{-6}+k_{+7})}{k_{+6}(k_{+3}+k_{+7})}K_s^{O_2} \quad (9)$$

$$K_m^{O_2(D)} = \frac{k_{+3}k_{+7}}{(k_{+3}+k_{+7})k_{+8}} \quad (10)$$

$$K_m^D = \frac{k_{+2}k_{+3}(k_{-6}+k_{+7}) + k_{+6}k_{+7}(k_{-2}+k_{+3})}{k_{+2}k_{+6}(k_{+3}+k_{+7})} \quad (11)$$

$$k_{cat}^D = \frac{k_{+3}k_{+7}}{k_{+3}+k_{+7}} \quad (12)$$

From the kinetic studies carried out to date using mushroom tyrosinase, it is known that:

(a) There is no rapid equilibrium in the binding with substrate D [11], which, kinetically, means that $k_{+3} \geq k_{-2}$ and $k_{+7} \geq k_{-6}$.
(b) The binding rate constant of substrate D to the $E_m$ form is faster than the binding rate constant to $E_{ox}$ form [14]. In other words, $k_{+2} \gg k_{+6}$.
(c) As deduced from studies of the isotopic effect, the step controlled by $k_{+7}$ is the limiting step [34,35].
(d) The binding of $O_2$ to $E_d$ form occurs by rapid equilibrium with a value of $K_s^{O_2} = 46.6 \pm 2.4\,\mu M$, being $k_{+8} = (2.3 \pm 0.4) \times 10^7\,M^{-1}\,s^{-1}$ [14].
(e) The Michaelis constant for $O_2$ in the diphenolase activity is very low, in order of μM. Kinetically, this means that the enzyme at an $O_2$ concentration of 0.26 mM (solubility of $O_2$ in air-saturated solutions) is saturated [36].

Taking into consideration (a)–(e), Eq. (7) can be simplified to:

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

with

$$k_{cat} = k_{+7} \quad (14)$$

$$K_m^D = \frac{k_{cat}^D}{k_{+6}} = \frac{k_{+7}}{k_{+6}} \quad (15)$$

$$K_m^{O_2(D)} = \frac{k_{cat}}{k_{+8}} = \frac{k_{+7}}{k_{+8}} \quad (16)$$

Hence, from kinetic studies of the steady-state $V_{max}^{D,Cr}$ and $K_m^D$ can be determined. Once $[E]_0$ is known, $k_{cat}^D$ can be obtained. Taking into consideration Eq. (15), it is possible to determine the binding rate constant of the substrate to $E_{ox}$, $k_{+6}$. When $K_m^{O_2(D)}$ and $k_{+7}$ is known, the binding rate constant of $O_2$ to $E_d$ form $k_{+8}$, can be determined [14].

### 2.2. Monophenolase activity

The reaction of tyrosinase activity on monophenols (M) is known as monophenolase activity, whose mechanism is described in Scheme 2 [12]. Using the steady-state approach, the expression for the dopachrome formation rate is

$$V_{ss}^{M,Cr} = \frac{b_0[D]_0[O_2]_0[E]_0}{c_0 + c_1[D]_0 + c_2[O_2]_0 + c_3[M]_0[O_2]_0 + c_4[D]_0[O_2]_0} \quad (17)$$

The $O_2$ consumption rate, ($V_{ss}^{M,O_2}$), is related with the dopachrome formation rate ($V_{ss}^{M,Cr}$) according to $V_{ss}^{M,O_2} = 1.5V_{ss}^{M,Cr}$ [7]. Accordingly:

$$b_0 = 2k_{+2}k_{+3}k_{+5_2}k_{+6}k_{+7}k_{+8}(k_{-4}+k_{+5_1}) \quad (18)$$

$$c_0 = k_{+2}k_{+3}k_{+5_2}k_{+8}(k_{-4}+k_{+5_1})(k_{-6}+k_{+7}) \quad (19)$$

$$c_1 = 3k_{+2}k_{+3}k_{+5_2}k_{+6}k_{+7}(k_{-4}+k_{+5_1}) \quad (20)$$

$$c_2 = k_{+5}, k_{+8}[k_{+6}k_{+7}(3k_{-2}+k_{+3}) + k_{+2}k_{+3}(k_{-6}+k_{+7})](k_{-4}+k_{+5_1}) \quad (21)$$

$$c_3 = k_{+5_2}k_{+6}k_{+7}k_{+8}(3k_{-2} + k_{+3})(k_{-4} + k_{+5_1})\left(\frac{1}{K_1}\right) + k_{+2}k_{+3}k_{+4}k_{+8}(k_{+5_1} + k_{+5_2})(k_{-6} + k_{+7}) \quad (22)$$

$$c_4 = k_{+2}k_{+5_2}k_{+6}k_{+8}(3k_{+7} + k_{+3})(k_{-4} + k_{+5_1}) \quad (23)$$

$$K_1 = \frac{k_{-1}}{k_{+1}} \quad (24)$$

$$R = \frac{[\mathrm{D}]_{ss}}{[\mathrm{M}]_{ss}} = \frac{k_{+4}k_{+5_1}(k_{-6} + k_{+7})}{2k_{+6}k_{+7}(k_{-4} + k_{+5_1})} \quad (25)$$

where $[\mathrm{D}]_{ss}$ and $[\mathrm{M}]_{ss}$ are the steady-state concentrations of D and M. Hence, Eq. (17) can be written as

$$V_{ss}^{M,Cr} = \frac{V_{max}^{M,Cr}[\mathrm{M}]_0[\mathrm{O_2}]_0}{W' + K_m^{O_2(M)}[\mathrm{M}]_0 + K_m^{M}[\mathrm{O_2}]_0 + [\mathrm{M}]_0[\mathrm{O_2}]_0} \quad (26)$$

where $V_{max}^{M,Cr}$ is the maximum rate of dopachrome formation from a monophenol; $K_m^M$ the Michaelis constant for monophenol and $K_m^{O_2(M)}$ the Michaelis constant for $O_2$ in presence of monophenol, with

$$V_{max}^{M,Cr} = \frac{b_0 R[\mathrm{E}]_0}{c_3 + c_4 R} = k_{cat}^{M}[\mathrm{E}]_0 \quad (27)$$

$$W' = \frac{c_0}{c_3 + c_4 R} \quad (28)$$

$$K_m^{O_2(M)} = \frac{c_1 R}{c_3 + c_4 R} \quad (29)$$

$$K_m^{M} = \frac{c_2}{c_3 + c_4 R} \quad (30)$$

or, taking into account Eq. (25), Eq. (17) can be written as

$$V_{ss}^{M,Cr} = \frac{V_{max}^{M,Cr}[\mathrm{D}]_0[\mathrm{O_2}]_0}{W'' + K_m^{O_2(M)}[\mathrm{D}]_0 + K_m^{D(M)}[\mathrm{O_2}]_0 + [\mathrm{D}]_0[\mathrm{O_2}]_0} \quad (31)$$

where $V_{max}^{M}$ is given by Eq. (27), $K_m^{O_2(M)}$ is given by Eq. (29) and where the following expressions apply

$$W'' = \frac{c_0 R}{c_3 + c_4 R} \quad (32)$$

and

$$K_m^{D(M)} = \frac{c_2 R}{c_3 + c_4 R} \quad (33)$$

The reaction mechanism of the monophenolase activity of tyrosinase (Scheme 2) includes the hydroxylase and oxidase cycles, the dead-end branch and the group of coupled enzymatic reactions until dopachrome (Cr) is formed. The affinity of tyrosinase for monophenols is inversely related with the respective Michaelis constant, $K_m^M$ (Eq. (30)). In the monophenolase activity, tyrosinase also shows an affinity for *o*-diphenols that is inversely related with the corresponding Michaelis constant, $K_m^{D(M)}$ (Eq. (33)). This new kinetic constant, described here for the first time in the literature, differs from the Michaelis constant of tyrosinase for *o*-diphenols in its diphenolase activity, $K_m^D$ (Eq. (15)). Later, we shall see the usefulness of both analytical expressions for establishing whether the same enzyme (tyrosinase) can show two different affinities for the same substrate (*o*-diphenol), in two distinct catalytic activities (monophenolase and diphenolase).

There is wide body of kinetic information based on transient phase data [14–16], steady-state studies [7,11–13,21,22,25–33] and isotopic effect experiments [34,35]. These experimental results (not assumptions) can be summarised as follows:

(a) The limiting step of the monophenolase activity corresponds to the nucleophilic attack of the phenolic OH on the copper in $E_{ox}$, so that the step controlled by $k_{+5_1}$ is the slowest [12,13,34,35].
(b) The electrophilic step of the peroxide on the benzene ring controlled by $k_{+5_2}$ must be equal or faster than the step controlled by $k_{+5_1}$ [12,13].
(c) As already mentioned, there is no rapid equilibrium in the diphenolase activity rate or in the monophenolase activity, since the rate equation is not of the form seen in Eq. (17). Therefore, $k_{+5_1} \geq k_{-4}$ [11].
(d) Since monophenols are poorer nucleophiles than *o*-diphenols (Table 4) [12], the value of their binding rate constant with $k_{+4}$ should be lower than the binding rate constant with *o*-diphenol, $k_{+6}$.

Taking into consideration the above experimental results (a)–(d), Eq. (26) can be simplified to:

$$V_{ss}^{M,Cr} = \frac{V_{max}^{M,Cr}[\mathrm{M}]_0}{K_m^{M} + [\mathrm{M}]_0} \quad (34)$$

and Eq. (31) can be simplified to

$$V_{ss}^{M,Cr} = \frac{V_{max}^{M,Cr}[\mathrm{D}]_0}{K_m^{D(M)} + [\mathrm{D}]_0} \quad (35)$$

with

$$k_{cat}^{M} = \frac{k_{+5_1}k_{+5_2}}{k_{+5_1} + k_{+5_2}} \quad (36)$$

$$K_m^{M} = \frac{k_{cat}^{M}}{k_{+4}} \quad (37)$$

$$K_m^{D(M)} = \frac{k_{cat}^{M}}{2k_{+6}} \quad (38)$$

and

$$K_m^{O_2(M)} = \frac{3k_{cat}^{M}}{2k_{+8}} \quad (39)$$

Therefore, knowing $K_m^M$ and $k_{cat}^M$ and using Eq. (37), it is possible to determine the binding rate constant of M to $E_{ox}$, $k_{+4}$.

### 2.3. Experimental design

The kinetic analysis led us to propose an experimental design which would permit a more rigorous characterisation of the kinetic reaction mechanism of tyrosinase. The experimental design consists of

(a) Diphenolase activity: to study $V_{ss}^{D,Cr}$ versus $[D]_0$ to give $V_{max}^{D,Cr}$ and $K_m^D$.
(b) Monophenolase activity: to study $V_{ss}^{M,Cr}$ versus $[M]_0$ to give $V_{max}^{M,Cr}$ and $K_m^M$.
(c) Knowing the concentration of the enzyme, $[E]_0$, and using Eqs. (8) and (27) to give the catalytic constants:

$$k_{cat}^{D} = \frac{V_{max}^{D,Cr}}{[E]_0} \tag{40}$$

and

$$k_{cat}^{M} = \frac{V_{max}^{M,Cr}}{[E]_0} \tag{41}$$

(d) From Eqs. (15) and (37), $k_{+6}$ and $k_{+4}$, respectively, can be obtained:

$$k_{+6} = \frac{k_{cat}^{D}}{K_m^{D}} \tag{42}$$

and

$$k_{+4} = \frac{k_{cat}^{M}}{K_m^{M}} \tag{43}$$

(e) With the information obtained, $K_m^{D(M)}$ can be calculated from Eq. (38).
(f) Knowing $k_{cat}^{D}$ for different *o*-diphenols, a study of different *o*-diphenols with respect of oxygen will give $K_m^{O_2(D)}$ and, according to Eq. (16), we can obtain $k_{+8}$, the binding rate constant of oxygen to the $E_d$ form [14].
(g) Lastly, the Michaelis constant of tyrosinase with respect to oxygen in the monophenolase activity can be determined analytically according to Eq. (39).

If the concentration of the enzyme is not known (it is not completely purified) the kinetic study should follow steps (a) and (b). With the information obtained ($V_{max}^{D,Cr}$, $K_m^D$,

Table 1
Kinetic constants characterising the reaction of tyrosinases from different biological sources on *o*-diphenols

| Tyrosinase from | *o*-Diphenol | $k_{cat}^{D}$ ($s^{-1}$) | $K_m^D$ (mM) | $k_{+6}$ ($M^{-1}\,s^{-1}$) | $K_m^{D(M)} = k_{cat}^{M}/2k_{+6}$ (μM) |
|---|---|---|---|---|---|
| *S. glaucescens* [8] | *t*BuCat | 3320.0 | 0.91 | $3.6 \times 10^6$ | |
| | DHPPA | 1830.0 | 1.05 | $1.7 \times 10^6$ | 39.4 |
| | 4MeCat | 2340.0 | 1.90 | $1.2 \times 10^6$ | |
| | CGA | 785.0 | 0.74 | $1.0 \times 10^6$ | |
| | LDopa | 1445.0 | 5.77 | $2.5 \times 10^5$ | 26.4 |
| | DHPAA | 142.0 | 1.16 | $1.2 \times 10^5$ | 203.0 |
| | DLDopa | 1542.0 | 12.55 | $1.2 \times 10^5$ | |
| | Cat | 395.0 | 4.50 | $8.7 \times 10^4$ | |
| | Dopamine | 875.0 | 11.90 | $7.3 \times 10^4$ | |
| | DDopa | 184.0 | 15.02 | $1.2 \times 10^4$ | |
| *N. crassa* [9] | Dopamine | 1350.0 | 0.28 | $4.8 \times 10^6$ | 17.0 |
| | LDopa | 1070.0 | 1.04 | $1.0 \times 10^6$ | 160.0 |
| | DDopa | 340.0 | 0.38 | $8.9 \times 10^5$ | 90.0 |
| | DHPAA | 850.0 | 4.40 | $1.9 \times 10^5$ | 658.0 |
| *A. bisporus* [12,13,22] | Cat | 877.6 | 0.30 | $2.9 \times 10^6$ | 2.2 |
| | Pirogallol | 1263.0 | 2.16 | $5.8 \times 10^5$ | |
| | Dopamine | 439.0 | 2.2 | $2.0 \times 10^5$ | 64.7 |
| | LDopa | 107.4 | 0.8 | $1.3 \times 10^5$ | 30.4 |
| | 4MeCat | 841.6 | 2.36 | $3.5 \times 10^5$ | |
| | DHPPA | 553.5 | 1.89 | $2.9 \times 10^5$ | 115.0 |
| | 4*t*BuCat | 641.8 | 2.80 | $2.2 \times 10^5$ | |
| | DHPAA | 631.6 | 5.10 | $1.2 \times 10^5$ | 184.6 |
| | DLDopa | 107.0 | 1.40 | $7.6 \times 10^4$ | 53.3 |
| | LDopaMeE | 35.5 | 0.57 | $6.2 \times 10^4$ | 27.4 |
| | DDopa | 107.4 | 4.50 | $2.3 \times 10^4$ | 174.0 |
| | LαMeDopa | 44.3 | 6.80 | $6.5 \times 10^3$ | 46.1 |
| | DLαMeDopa | 44.3 | 8.00 | $5.5 \times 10^3$ | 54.5 |
| | LIsoprot | 29.4 | 7.10 | $4.2 \times 10^3$ | |
| | DLIsoprot | 29.4 | 9.70 | $3.0 \times 10^3$ | |
| | DIsoprot | 29.8 | 18.10 | $1.6 \times 10^3$ | |

*t*BuCat: *tert*-butylcatechol; DHPPA: 3,4-dihydroxyphenyl propionic acid; 4MeCat, 4-methylcatechol; CGA: chlorogenic acid; cat: catechol; DHPAA: 3,4-dihydroxyphenyl acetic acid; LDopaMeE: L-dopamethylester; LαMeDopa: L-α-methyldopa; DLαMeDopa: DL-α-methyldopa; LIsoprot: L-isoproterenol; DLIsoprot: DL-isoproterenol. These data were obtained from references.

Table 2
Kinetic constants characterising the reaction of tyrosinases from different biological sources on monophenols

| Tyrosinase from | Monophenol | $k_{cat}^{M}$ ($s^{-1}$) | $K_m^M$ (mM) | $k_{+4}$($M^{-1}$ $s^{-1}$) | $k_{+6}/k_{+4}$ |
|---|---|---|---|---|---|
| *S. glaucescens* [8] | 4HPPA | 134.0 | 0.48 | $2.7 \times 10^5$ | 6.3 |
| | LTyrMeE | 235.0 | 1.66 | $1.4 \times 10^5$ | |
| | 4HPAA | 48.8 | 1.16 | $4.1 \times 10^4$ | 2.9 |
| | LTyr | 13.2 | 0.41 | $3.2 \times 10^4$ | 7.8 |
| *N. crassa* [9] | 4HPAA | 250.0 | 0.18 | $1.3 \times 10^6$ | 0.2 |
| | LTyr | 320.0 | 0.59 | $5.4 \times 10^5$ | 1.8 |
| | DTyr | 160.0 | 0.32 | $5.0 \times 10^5$ | 1.8 |
| | Tyramine | 160.0 | 0.67 | $2.3 \times 10^5$ | 20.8 |
| *S. antibioticus* [10] | 4FP | 10.5 | 2.90 | $3.6 \times 10^3$ | |
| | 3FP | 3.0 | 6.90 | $4.0 \times 10^2$ | |
| *A. bisporus* [12,13,22] | 4MeOP | 184.0 | 0.08 | $2.3 \times 10^6$ | |
| | 4EtOP | 132.0 | 0.17 | $7.7 \times 10^5$ | |
| | 4HPPA | 66.7 | 0.44 | $1.5 \times 10^5$ | 1.9 |
| | 4HBA | 88.2 | 1.10 | $8.0 \times 10^4$ | |
| | Tyramine | 25.9 | 0.51 | $5.0 \times 10^4$ | 4.0 |
| | LTyr | 7.9 | 0.21 | $3.7 \times 10^4$ | 3.5 |
| | 4HPAA | 44.3 | 1.91 | $2.3 \times 10^4$ | 5.2 |
| | P | 12.7 | 0.70 | $1.8 \times 10^4$ | |
| | DLTyr | 8.1 | 0.90 | $9.0 \times 10^3$ | 8.4 |
| | LTyrMeE | 3.4 | 0.38 | $8.9 \times 10^3$ | 7.0 |
| | 3MeOP | 46.8 | 5.40 | $8.6 \times 10^3$ | |
| | DTyr | 8.0 | 1.86 | $4.3 \times 10^3$ | 5.3 |
| | 3HBA | 12.1 | 10.10 | $1.2 \times 10^3$ | |
| | LαMeTyr | 0.6 | 1.20 | $5.0 \times 10^2$ | 13.0 |
| | DLαMeTyr | 0.6 | 1.45 | $4.1 \times 10^2$ | 13.4 |

4HPPA: 4-hydroxyphenyl propionic acid; 4HPAA: 4-hydroxyphenyl acetic acid; LTyrMeE: L-tyrosinemethyl ester; LTyr: L-tyrosine; DTyr: D-tyrosine; 4FP: 4-fluorophenol; 3-FP: 3-fluorophenol; 4MeOP: 4-methoxyphenol; 4EtOP: 4-ethoxyphenol; 4HBA: 4-hydroxybenzyl alcohol; P: phenol; DLTyr: DL-tyrosine; 3MeOP: 3-methoxyphenol; 3HBA: 3-hydroxybenzyl alcohol; LαMeTyr: L-α-methyltyrosine; DLαMeTyr: DL-α-methyltyrosine. These data were obtained from references.

$V_{max}^{M,Cr}$ and $K_m^M$), the following kinetic constants can be calculated:

(h) The $k_{+6}/k_{+4}$ ratio, according to:

$$\frac{k_{+6}}{k_{+4}} = \frac{V_{max}^{D,Cr} K_m^M}{V_{max}^{M,Cr} K_m^D} \quad (44)$$

(i) The $K_m^{D(M)}$, according to the equation:

$$K_m^{D(M)} = \frac{V_{max}^{M,Cr} K_m^D}{V_{max}^{D,Cr}} \quad (45)$$

(j) The value of $K_m^{O_2(M)}$ can be determined from the kinetic information for the action of tyrosinase on *o*-diphenol ($V_{max}^{D,Cr}$ and $K_m^{O_2(D)}$) and on its corresponding monophenol $V_{max}^{M,Cr}$. Taking into consideration Eqs. (14), (16), (36) and (39), the value of $K_m^{O_2(D)}$ can be determined according to:

$$K_m^{O_2(M)} = \frac{2}{3} \frac{V_{max}^{M,Cr}}{V_{max}^{D,Cr}} K_m^{O_2(D)} \quad (46)$$

Therefore, based on the kinetic analysis developed in this work, it is possible to obtain new kinetic information from the steady-state kinetic parameters described in the bibliography since, in principle, the tyrosinase mechanism functions throughout the phylogenetic scale [25–33].

To obtain the kinetic constants ($k_{cat}^D$, $K_m^D$, $k_{cat}^M$ and $K_m^M$) described in Tables 1 and 2 and ($V_{max}^D$, $K_m^D$, $V_{max}^M$ and $K_m^M$) described in Table 3, experimental measurements of the steady-state rate ($V_{ss}$) at different concentrations of D or M must be made. Such measurements are difficult to make because the products of the enzymatic reaction are *o*-quinones, which are unstable and which evolve non-enzymatically to polymers [1]. The difficulty increases when the substrate is M since, for the system to reach the steady state, a given quantity of D must have accumulated in the medium [7]. The scarcity of reliable quantitative data on the monophenolase activity of tyrosinase has been described by Solomon et al. [4]. The problem can be partially solved by optimising a spectrophotometric method based on the nucleophilic attack of MBTH on the *o*-quinones generated by the enzyme [23,24].

Furthermore, from an NMR study of the different monophenols and *o*-diphenols, it is possible to correlate the values obtained for the binding rate constants of the different substrates to the $E_{ox}$ form ($k_{+4}$ and $k_{+6}$, respectively) and the kinetic constants $k_{cat}$ and $K_m$. In general, the oxygens of the OH groups bound to carbons of low $\delta$ are good nucleophiles and vice versa (Table 4). This means that, in the first case, the binding rate constant and the catalytic constant are high. The contrary is true when the $\delta_4$ is high (Tables 1, 2 and 4). The kinetic analysis provides us with a new kinetic constant, $K_m^{D(M)}$, the Michaelis constant for *o*-diphenol in the monophenolase

Table 3
Relation between the kinetic binding rate constants of *o*-diphenols ($k_{+6}$) and monophenols ($k_{+4}$) in different monophenol/*o*-diphenol pairs binding to *oxy*tyrosinase for enzymes from different biological sources

| Tirosinase from | Monophenol/diphenol | $k_{+6}/k_{+4}$[a] |
|---|---|---|
| *Tuber melanosporum* [17] | LTyr/LDopa | 13.2 |
| Mouse-wild type [18] | LTyr/LDopa | 7.4 |
| Mouse-H389L [18] | LTyr/LDopa | 2.6 |
| Mouse-Q378H [18] | LTyr/LDopa | 0.1 |
| B16 Mouse Melanoma-H[b] [37] | LTyr/LDopa | 14.2 |
| B16 Mouse Melanoma-L[c] | LTyr/LDopa | 17.3 |
| Gerbil eye [20] | LTyr/LDopa | 8.0 |
| B16 mouse Melanoma [19] | 4-s-CAP/4-s-CAC | 12.6 |
| Apple cv. *verdedoncella* [27] | Tyramine/dopamine | 205.1 |
| | 4HPAA/DHPAA | 219.0 |
| | 4HPPA/DHPPA | 160.1 |
| Pear cv *blanquilla* [27] | Tyramine/dopamine | 265.5 |
| | 4HPAA/DHPAA | 54.6 |
| | 4HPPA/DHPPA | 20.8 |
| Strawberry cv *tioga* [32] | 4HPPA/DHPPA | 27.5 |
| Avocado cv *Haas* [31] | 4HPPA/DHPPA | 4.4 |

4-s-CAP: 4-cysteaminylphenol; 4-s-CAC: 4-cysteaminylcatechol.
[a] $k_{+6}/k_{+4} = V_{max}^{D,Cr} K_m^M / V_{max}^{M,Cr} K_m^D$.
[b] High electrophoretic mobility.
[c] Low electrophoretic mobility.

activity (Table 1). Table 1 shows the kinetic parameters determined for different tyrosinases, from which the values of $k_{+6}$, the binding rate constant of D to $E_{ox}$ form, can be obtained. An analogous study was carried out for monophenols (Table 2). Using the information of both tables, $K_m^{D(M)}$ (see Table 1) and $k_{+6}/k_{+4}$ can be calculated (see Table 2).

Table 3 shows the $k_{+6}/k_{+4}$ ratios, which can be obtained from the kinetic constants $V_{max}^{D,Cr}$, $K_m^D$ and $V_{max}^{M,Cr}$, $K_m^M$, for non-purified enzymes. Note that the $k_{+6}/k_{+4}$ ratio for the different monophenol/*o*-diphenol pairs studied and for enzymes from different sources (Table 3) is lowest in the case of mouse tyrosinase, in which there has been a mutation in the active centre involving H389L and Q378H, which correspond to aminoacids of the active site in the region of CuB [18]. Kinetic studies carried out with wild tyrosinase and the above-mentioned mutant tyrosinase species indicate that mutation changes the enzyme's affinity for *o*-diphenols ($K_m^D$ increases) but hardly affects the affinity for monophenols. Furthermore, the maximum rates are hardly modified [18]. These results have led to a mechanism being suggested in which monophenols bind to the CuA, while the *o*-diphenols begin the binding process to the CuB [18], the unchanging maximum rates being in accordance with studies on stereoisomers from different tyrosinases and indicating that the rate-limiting step is governed by the nucleophilia of the oxygen of the phenolic OH [12,13,22]. Table 4 shows the values of $\delta_3$ and $\delta_4$ obtained for the different monophenols and *o*-diphenols studied. These parameters are related with the charge density of the carbon atom which supports the OH and is an indicator of the nucleophilic power of the OH oxygen.

## 3. Conclusions

- The binding rate constants of *o*-diphenols to $E_{ox}$ ($k_{+6}$) are higher than those of the respective monophenols ($k_{+4}$). That is, $k_{+6} > k_{+4}$ (Tables 2 and 3). The exceptions, which are listed in Table 3, are mutants [18].
- In general, the value of $k_{+4}$ in monophenols (Table 2) decreases with increasing values of $\delta_4$ (Table 4). The presence of electron-donating (low $\delta_4$) or electron-withdrawing (high $\delta_4$) groups in C-1 (Table 4) influences the electronic richness of the benzene ring and, in turn, helps or hinders the binding of the substrate (Table 2).
- In *o*-diphenols, the effect is similar (Table 1), except that, since all the values of $\delta_3$ and $\delta_4$ are similar (Table 4). The steric hindrance resulting from the presence of a group R in C-1 and/or the presence of charges affects the kinetics of all tyrosinases (Table 1).
- The new kinetic constant determined, $K_m^{D(M)} = k_{cat}^M/2k_{+6}$, has a kinetic significance parallel to $K_m^M = k_{cat}^M/k_{+4}$ and $K_m^{O_2,M} = 3k_{cat}^M/2k_{+8}$. Thus, in the monophenolase activity tyrosinase becomes saturated with small quantities of *o*-diphenol [7], and so $K_m^{D(M)}$ is at the μM level (Table 1). In the diphenolase activity, however, tyrosinase shows lower affinity for *o*-diphenols, and so $K_m^D$ is at the mM level (Table 1). There is the same parallelism between $K_m^D$, Eq. (15), and $K_m^{D(M)}$ Eq. (38), as there is between $K_m^{O_2(D)}$,

Table 4
Values of $\delta_3$ and $\delta_4$ for carbons C-3 and C-4, respectively, of the benzene ring of monophenols and *o*-diphenols

| Monophenol | $\delta_3$ (ppm) | $\delta_4$ (ppm) | Diphenol | $\delta_3$ (ppm) | $\delta_4$ (ppm) |
|---|---|---|---|---|---|
| 4MeOP | 118.9 | 152.29 | Pirogallol | 148.07 | 135.03 |
| 4EtOP | 119.01 | 152.39 | Cat | 146.59 | 146.59 |
| 4HBA | 116.54 | 156.63 | 4-MeCat | 146.43 | 144.06 |
| 4HPPA | 118.02 | 156.13 | DHPAA | 146.43 | 144.96 |
| 3MeOP | 159.49 | 110.97 | DHPPA | 146.51 | 144.61 |
| 4HPAA | 118.05 | 156.49 | *t*BuCat | 146.24 | 144.09 |
| Tyramine | 118.49 | 157.28 | Dopamine | 146.79 | 145.58 |
| P | 118.13 | 158.15 | LDopa (D and DL) | 146.92 | 146.06 |
| 3HBA | 158.44 | 117.41 | LDopaMeE | 146.90 | 146.18 |
| LTyr (D and DL) | 118.08 | 158.86 | LαMeDopa(DL) | 146.74 | 146.19 |
| LTyrMeE | 118.53 | 157.75 | LIsoprot (D and DL) | 146.87 | 146.82 |
| LαMeTyr (DL) | 118.11 | 159.10 | CGA | 146.41 | 149.29 |

Eq. (16), and $K_m^{O_2(M)}$, Eq. (39), and so $K_m^{D(M)} \ll K_m^D$ and $K_m^{O_2(M)} \ll K_m^{O_2(D)}$, since these expressions have a kinetic significance and arise because the mechanism of tyrosinase on *o*-diphenols is linear, while that on monophenols consists of two cycles with common intermediates. Although it might be thought that two binding sites exist with different affinities for $O_2$ and D, it must be emphasised that this is not the case since the explication is kinetic and not related with the binding.

- The data shown in Tables 1–3 point to a similar behaviour for all tyrosinases from the whole phylogenetic scale and therefore confirm the validity of the kinetic reaction mechanism proposed (Scheme 2).

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