Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–9
© 2013 Informa UK Ltd. DOI: 10.3109/14756366.2013.782298

informa healthcare

ORIGINAL ARTICLE

Indirect inactivation of tyrosinase in its action on 4-*tert*-butylphenol

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Abstract

Under anaerobic conditions, the *o*-diphenol 4-*tert*-butylcatechol (TBC) irreversibly inactivates *met* and *deoxy*tyrosinase enzymatic forms of tyrosinase. However, the monophenol 4-*tert*-butylphenol (TBF) protects the enzyme from this inactivation. Under aerobic conditions, the enzyme suffers suicide inactivation when it acts on TBC. We suggest that TBF does not directly cause the suicide inactivation of the enzyme in the hydroxylase activity, but that the *o*-diphenol, which is necessary for the system to reach the steady state, is responsible for the process. Therefore, monophenols do not induce the suicide inactivation of tyrosinase in its hydroxylase activity, and there is a great difference between the monophenols that give rise to unstable *o*-quinones such as L-tyrosine, which rapidly accumulate L-dopa in the medium and those like TBF, after oxidation, give rise to a very stable *o*-quinone.

Keywords

Irreversible inhibition, suicide inactivation, TBC, TBF, tyrosinase

History

Received 14 December 2012
Revised 21 January 2013
Accepted 28 February 2013
Published online 8 April 2013

Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase widely distributed throughout the phylogenetic scale. Tyrosinase catalyses two types of reactions on phenolic substrates, in which molecular oxygen intervenes: (a) the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and (b) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), which, in turn, are polymerized to brown, red or black pigments[1]. Tyrosinase has two copper atoms in its active site, which may be in three coordination states or forms: (i) Cu^{2+}–Cu^{2+}, *met*-tyrosinase, (ii) Cu^{2+}–O_2^{2-}–Cu^{2+} with a peroxide group in its active site, *oxy*-tyrosinase or (iii) Cu^{1+}–Cu^{1+}, *deoxy*-tyrosinase[2,3].

Melanogenesis is the principal enzymatic pathway responsible for browning in fruits and vegetables[4], and it also causes pigmentation of the hair, eyes and skin in mammals and other animals[1,5]. This pathway can be divided into a proximal and distal phase as follows:

(a) The proximal phase begins with the hydroxylation of a monophenol (L-tyrosine) to its corresponding *o*-diphenol (L-dopa) and the oxidation of this *o*-diphenol to its corresponding *o*-quinone (*o*-dopaquinone). These two reactions correspond to those described as monophenolase and diphenolase activities of tyrosinase, respectively[6]. Then, through non-enzymatic reactions, the *o*-dopaquinone evolves towards dopachrome.
(b) The distal phase begins with the chemical decarboxylation of dopachrome towards 5,6-dihydroxyindole (DHI) or with an enzymatic tautomerization of the dopachrome towards 5,6-dihydroxyindole-2-carboxylic acid (DHICA)[1,6]. These compounds then polymerize giving rise to melanins.

Due to the wide specificity of tyrosinase, several types of substrate can be considered according to the stability of the corresponding quinone.

S_A: **Substrates yielding stable *o*-quinone.** The clearest example is 4-*tert*-butylcatechol (TBC), whose *o*-quinone (*o*-TBQ) is very stable. In this case, the *o*-quinone can be measured and V_0 can be calculated accurately[7]. The diphenolase activity can be determined, but not the monophenolase activity[8].

S_B: **Substrates yielding a stable coupling product.** Substrates which produce a very unstable *o*-quinone, but that evolve to a stable product through a first-order reaction. An example is 3,4-dihydroxymandelic acid (DOMA), whose *o*-quinone evolves to 3,4-dihydroxybenzaldehyde (DOBA)[9]. Other examples of type S_B substrate are L-dopa, dopamine, L-α-methylnoradrenaline, isoproterenol, epinine and L-dopa methyl ester, whose *o*-quinone evolves to aminochrome[10].

S_C: **Substrates yielding a stable nucleophile-adduct.** The oxidation of this type of substrate gives rise to an unstable *o*-quinone which suffers from the attack of a potent nucleophilic reagent (N) and produces a chromophoric adduct (NQ) with a clear stoichiometry. Among the nucleophilic reagents used are L-proline (Pro)[10], MBTH[11–13] and L-cysteine (Cys)[10]. The accumulation of the chromophoric product permits us to characterize the monophenolase and diphenolase activities (L-tyrosine,

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tyramine, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, L-dopa, dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid)[10].

S_D: Substrates measured by means of ascorbic acid. The substrates give rise to a very unstable *o*-quinone that evolves with no clear stoichiometry, even after MBTH attack. These substrates can only be characterized through a reduction by ascorbic acid (AH_2), either directly or spectrophotometrically following the disappearance of ascorbic acid or using chronometric methods that measure the time necessary for the ascorbic acid to be consumed due to the reaction with *o*-quinone[7]. In this case, measurement by reference to the reduction of the *o*-quinone by ascorbic acid makes it possible to characterize the diphenolase but not the monophenolase activity[7,8].

A large number of studies have been made of the action of tyrosinase on different types of monophenols such as highly unstable *o*-quinones[10,14] and slightly unstable *o*-quinones[8,10,15].

As indicated above, tyrosinase is the main enzyme involved in melanization, but is also responsible for the loss of quality of fruit and vegetables during post-harvest handling and processing[4]. In addition, tyrosinase may cause disorders in pigmentation[16]. In insects, the enzyme is associated with three processes: cuticle schlerotization, defensive encapsulation and melanization of foreign organisms, and wound healing[17]. Therefore, inhibitors of this enzyme, which can be isolated from natural sources or be synthesized chemically, have been sought due to the many possible applications[18–20]. Moreover, the enzyme may be inactivated when it acts on a series of substrates, which act as suicide substrates; see the work by Muñoz-Muñoz[21] for a revision. The study of reversible and irreversible inhibitors and of suicide substrates is an interesting option for controlling the activity of this enzyme.

Both 4-*tert*-butylphenol (TBF) and 4-*tert*-butylcatechol (TBC) have been described as depigmenting agents since they act as tyrosinase inhibitors[22]. The same compounds have been described as apoptosis inducers in human melanocytes even without the tyrosinase activity of human melanocytes[23,24]. These phenolic compounds can act as alternative tyrosinase substrates, following a pathway other than the melanin biosynthesis pathway. At the same time, the *o*-diphenol TBC can act as a suicide substrate for this enzyme[21,25].

Tyrosinase undergoes an inactivation process when it reacts with its *o*-diphenolic substrates, a phenomenon that has long been known in the case of enzymes from a variety of natural sources, including fungi, plant and animals[21]. The study of enzymatic inactivation by suicide substrates or mechanism-based inhibitors is of growing importance because of possible pharmacological applications[26,27]. In addition, suicide substrates and mechanism-based enzyme inactivators may be useful for studying enzymatic mechanisms and designing new drugs[27]. Given that tyrosinase participates in different physiological processes, such as fruit and vegetable browning and pigmentation in animals[1], the suicide inactivation process of this enzyme is of even more interest.

Our group has made several kinetic studies of the suicide inactivation of tyrosinase using mushroom tyrosinase[21,25], frog epidermis tyrosinase[28] and peroxidases from different sources[29,30]. We have also studied the suicide inactivation of an enzyme that can be measured from coupled reactions[31] and have published an experimental study, which used several experimental designs to kinetically study suicide inactivation[32].

In the mechanism proposed by our research group, the bifurcation of the catalytic and suicide inactivation pathways is considered to occur through the E_{ox} form[21,25,33]. Other groups have also proposed that the suicide inactivation occurs through the same form, E_{ox}[34,35].

The main aim of kinetic studies with suicide substrates is their kinetic characterization through the parameters: λ_{max} (maximum apparent inactivation constant), r (a partition ratio between the catalytic and the inactivation pathways), k_{cat} (catalytic constant) and K_m^S (Michaelis constant for the substrate)[21,25,29–31].

The kinetic mechanism for tyrosinase acting on monophenols and *o*-diphenols has been the subject of several studies, and various structural mechanisms have been proposed[36–39], e.g. substrates which give highly unstable *o*-quinones, such as L-tyrosine, L-dopa or dopamine[28,33,40]. These studies showed that *o*-diphenol accumulation in the medium through chemical reactions is responsible for suicide inactivation when the enzyme acts on monophenols.

Here, we study the action of tyrosinase in aerobic and anaerobic conditions on monophenols and *o*-diphenols which, after oxidation, give rise to very stable *o*-quinones, as in the case of TBF and TBC. In the case of TBC, the enzyme is inactivated both in aerobic (suicide inactivation) and anaerobic (irreversible inactivation) conditions. However, TBF protects the enzyme in both conditions because the *o*-quinone is stable and cannot regenerate *o*-diphenol in the medium to inactivate the tyrosinase. But when catalytic concentrations of TBC are added to the reaction medium to follow the monophenolase activity, suicide inactivation occurs due to the action of the enzyme on the added *o*-diphenol. We try to demonstrate experimentally and confirm by computer simulations that tyrosinase does not suffer suicide inactivation when it acts on monophenols, but does so when acting on the *o*-diphenol that accumulates in the medium and which is necessary for the steady state to be reached. The stability of *o*-quinones, then, plays a fundamental role in melanogenesis during the formation of melanins and the suicide inactivation of the enzyme.

Notation

For clarity and for the sake of brevity, we will use the following notations in the text.

Species and concentrations

E, Tyrosinase; $[E]$, instantaneous concentration of E; $[E]_0$, initial concentration of E; E_a, active enzyme; $[E_a]$, instantaneous concentration of E_a; E_m, *met*-tyrosinase $[E_m]$, instantaneous concentration of *met*-tyrosinase; $[E_m]_0$, initial concentration of *met*-tyrosinase; E_d^R, *Deoxy*-tyrosinase relaxed form; $[E_d^R]$, instantaneous concentration of *deoxy*-tyrosinase relaxed form; $[E_d^R]_0$, initial concentration of *deoxy*-tyrosinase relaxed form; E_d^T, *deoxy*-tyrosinase tense form after the transition of E_d to E_d^T; $[E_d^T]$, instantaneous concentration of E_d^T; $[E_d^T]_0$, initial concentration of E_d^T; E_i, inactive enzyme; $[E_i]$, instantaneous concentration of E_i; TBC, 4-*tert*-butylcatechol; [TBC], instantaneous concentration of TBC; $[TBC]_0$, initial concentration of TBC; D, *o*-diphenol (TBC); [D], instantaneous concentration of D; $[D]_0$, initial concentration of D; Q, *o*-quinone product of the enzymatic reaction; [Q], instantaneous concentration of Q; $[Q]_\infty$, *o*-quinone concentration produced at the end of the reaction, $t \to \infty$; TBF, 4-*tert*-butylphenol; M, monophenol (TBF); [M], instantaneous concentration of monophenol; $[M]_0$, initial concentration of monophenol; O_2, molecular oxygen; $[O_2]_0$, initial concentration of oxygen; $[O_2]$, instantaneous concentration of oxygen; $[O_2]_f$, oxygen-value at $t \to \infty$; $[O_2]_\infty$, oxygen consumed at $t \to \infty$, i.e. $[O_2]_\infty = [O_2]_0 - [O_2]_f$.

Kinetic parameters

$V_0^{D(Q)}$, initial rate of tyrosinase acting on D, measuring Q; $V_0^{D(Q)}$, initial rate of tyrosinase acting on D, measuring O_2; $\lambda_{E_{ox}}^{M}$, apparent constant of suicide inactivation of tyrosinase in the

presence of monophenol; $\lambda^{M}_{E_{ox}(max)}$, maximum value of $\lambda^{M}_{E_{ox}}$ for saturating substrate; $\lambda^{D(M)}_{E_d^R}$, apparent constant of irreversible inhibition of E_d^R by D in the presence of the monophenol M; $\lambda^{D(M)}_{E_d^T}$, apparent constant of irreversible inhibition of E_d^T by D in the presence of the monophenol M; r^{Q}_{D}, partition ratio for the diphenolase activity measuring Q; $r^{O_2}_{D}$, partition ratio for the diphenolase activity measuring O_2; $r^{O_2}_{M}$, number of total turnovers (in both cycles, hydroxylase and oxidase) made by one molecule of enzyme acting on M before its suicide inactivation, obtained by measuring the oxygen consumed; $r^{O_2}_{M(O)}$, number of turnovers in the oxidase cycle made by one molecule of enzyme acting on M before its suicide inactivation, measuring oxygen consumed; $r^{O_2}_{M(H)}$, number of turnovers in the hydroxylase cycle made by one molecule of enzyme acting on M before its suicide inactivation, obtained by measuring the oxygen consumed; K^{M}_{m}, Michaelis constant of tyrosinase for M; k^{M}_{cat}, catalytic constant of the monophenolase activity for M; $K^{D(M)}_{E_d^T}$, dissociation constant of the complex E_d^TD in the presence of the monophenol; $K^{M}_{E_d^T}$, dissociation constant of the complex E_d^TM; $K^{D(M)}_{E_d^R}$, dissociation constant of the complex E_d^RD in the presence of the monophenol; $K^{M}_{E_d^R}$, dissociation constant of the complex E_d^RM.

Material and methods

Reagents

Mushroom tyrosinase (*o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1) (8300 units/mg) was supplied by Sigma (Madrid, Spain). The enzyme was purified as described by Rodriguez-Lopez et al.[36]. Protein concentration was determined by the Lowry method[41]. The substrates TBC and TBF were purchased from Sigma (Madrid, Spain). All other chemicals were of analytical grade. Stock solutions of the diphenolic substrates were prepared in 0.15 mM of phosphoric acid to prevent auto-oxidation. Milli-Q system (Millipore Corp., Billerica, MA) ultrapure water was used throughout the experiment.

Spectrophotometric assays

Diphenolase activity

These assays were carried out with a Perkin-Elmer Lambda-35 spectrophotometer (Perkin-Elmer Corp., Waltham, MA), on line interfaced to a PC-computer, where the kinetic data were recorded, stored and later analyzed. The product of the enzyme reaction 4-*tert*-*o*-benzoquinone is stable at long assay times, for which reason, the reaction was followed by measuring the appearance of Q at 410 nm[7,10], with $\varepsilon = 1200\,M^{-1}\,cm^{-1}$. The inactivation kinetics was studied in 30 mM sodium phosphate buffer (pH 7.0).

Monophenolase activity

These assays were carried out with the same apparatus as in the diphenolase activity assays. The reaction was followed by measuring the Q accumulated in the reaction medium at $\lambda = 410$ nm ($\varepsilon = 1200\,M^{-1}\,cm^{-1}$)[7,10]. The inactivation kinetics was studied in 30 mM sodium phosphate buffer (pH 7.0), adding the amount of *o*-diphenol (TBC) necessary for the system to reach the steady state when $t \to 0$, that is an initial steady state.

Oxymetric assays

Measurements of dissolved oxygen concentration were made with a Hansatech (Kings Lynn, Cambs, UK) oxygraph unit controlled by a PC. The oxygraph used a Clark-type silver/platinum electrode with a 12.5 μm Teflon membrane. The sample was continuously stirred during the experiment and maintained at 25 °C. The zero oxygen level for the calibration and experiments was obtained by bubbling oxygen-free nitrogen through the sample for 10 min[42]. TBF was studied by means of this method, adding the amount of TBC necessary to reach the steady state at $t \to 0$.

Kinetic data analysis of monophenolase and diphenolase activities

The experimental recordings of oxygen consumption in the action of tyrosinase on monophenol follow Equation (1):

$$[O_2] = [O_2]_f + [O_2]_\infty e^{-\lambda^{M}_{E_{ox}} t} \quad (1)$$

whose parameters can be obtained by a non-linear regression[43]. Thus, the experimental recordings obtained follow Equation (1), from which the corresponding inactivation parameters, $[O_2]_f$ (oxygen remaining at the end of the reaction), $[O_2]_\infty$ (oxygen consumed by the end of the reaction) and $\lambda^{M}_{E_{ox}}$ (apparent inactivation constant), can be determined.

The experimental recordings of Q accumulation in the action of tyrosinase on monophenol fit Equation (2):

$$[Q] = [Q]_\infty (1 - e^{-\lambda^{D\text{ or }M}_{E_{ox}} t}) \quad (2)$$

whose parameters can be obtained by a non-linear regression[43]. The experimental recordings obtained fit Equation (2), from which the corresponding inactivation parameters, $[Q]_\infty$ and $\lambda^{M}_{E_{ox}}$, can be determined.

In the case of the diphenolase activity, the experimental data of time-based assays for Q accumulation in the action of tyrosinase on *o*-diphenol fit Equation (2). From this equation, $[Q]_\infty$ and $\lambda^{D}_{E_{ox}}$ can be determined.

Simulation assays

The simulation shows how the concentrations of the ligand and enzymatic species involved in the reaction mechanism here proposed for tyrosinase evolve. The respective systems of differential equations have been solved numerically for particular sets of values of the rate constants and of initial concentrations of the species of the reaction mechanism. The numerical integration is based on the Runge–Kuta–Fehlberg algorithm, implemented on a PC-compatible computer program (WES)[44].

Simulated data of time-based assays for the accumulation of Q were fitted to Equation (2) (see Supplementary material).

Generation of E_{ox}, E_d^R, E_d^T and E_m

The enzymatic forms were generated and their inactivation was studied following Muñoz-Muñoz et al.[40].

Evaluation of enzymatic species E_m, E_d^R and E_{ox} in an enzymatic preparation of tyrosinase

The evaluation of these enzymatic species in an enzymatic preparation of tyrosinase was carried out as described by Muñoz-Muñoz et al.[40].

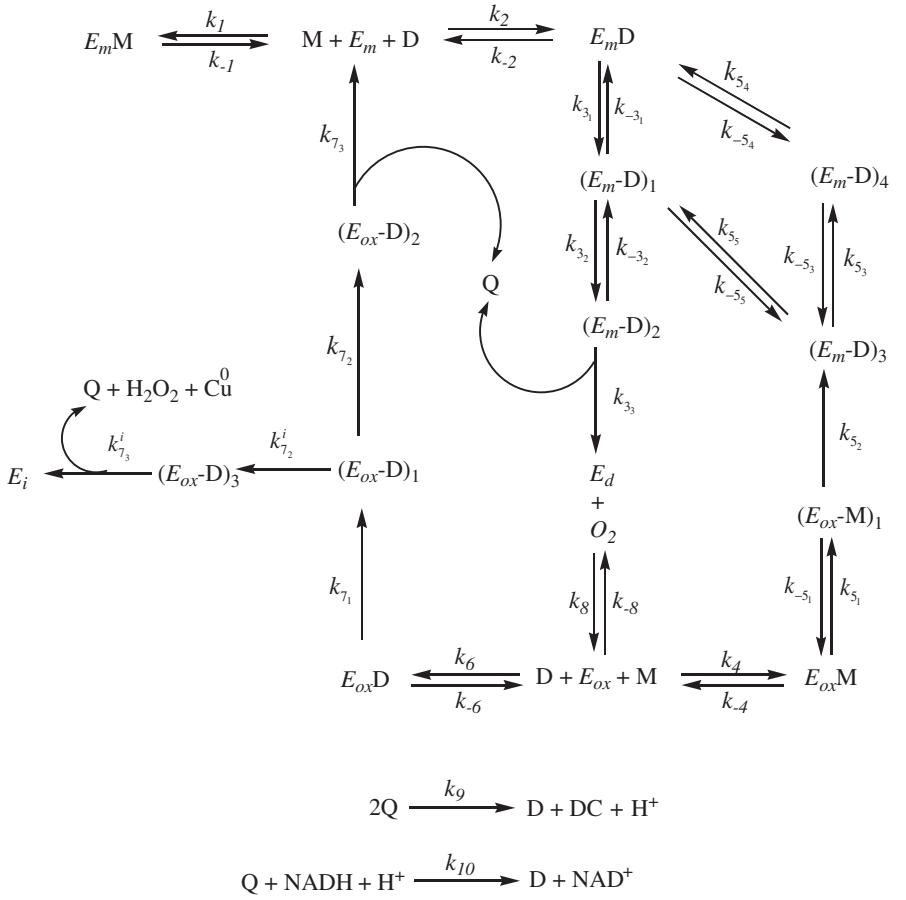
^{13}C-NMR assays

^{13}C-NMR spectra of TBC and TBF were obtained as described by Muñoz-Muñoz et al.[40].

Results and discussion

In this work, we study the reaction of different enzymatic forms of tyrosinase in its catalytic cycle with TBF/TBC both aerobic (E_{ox})

Scheme 1. Kinetic mechanism proposed to explain the monophenolase together with diphenolase and suicide inactivation pathways of tyrosinase acting on TBF/TBC (see Scheme 1SM for further details).



and anaerobic (E_m, E_d^R and E_d^T) conditions (Scheme 1 in its kinetic form and Scheme 1SM in its structural form).

Calculation of the *o*-diphenol concentration necessary for the system to reach its steady state

First, to eliminate the lag period characteristics of the monophenolase activity when TYR acts on L-tyrosine, the value of the dimensionless parameter *C*, which represents the ratio between $[D]_{ss}$ and $[M]_{ss}$ necessary for the system to reach the steady state, was calculated[10]. If the substrate varies very little in the assay, then $[M]_{ss} \approx [M]_0$ and *C* can be expressed as $C = [D]_{ss}/[M]_0$ [see the work by Garcia-Molina et al.[10]]. If the concentration of *o*-diphenol added exceeds the value predicted by *C*, a burst of *o*-quinone accumulation results. However, when the *o*-quinone does not generate *o*-diphenol in the medium, as is the case of TBF, *C* was calculated in an opposite sense. That is, first, a burst was generated by adding a concentration of *o*-diphenol above the steady state and, then, the *o*-diphenol concentration added was gradually reduced until the burst was eliminated (Figure 1)[8,10].

Figure 1 shows the *C* obtained by adding different concentrations of TBC while maintaining the initial concentration of TBF constant. As can be seen from Figure 1 curve (a), a burst appears in the accumulation of *o*-quinone as a consequence of the excessive concentration of TBC in the reaction medium. As this concentration diminishes [Figure 1, curves (b–g)], the burst is eliminated and the steady state is reached at $t \rightarrow 0$ [curve (g)]. The value obtained of *C* for the pair TBC/TBF is 0.05.

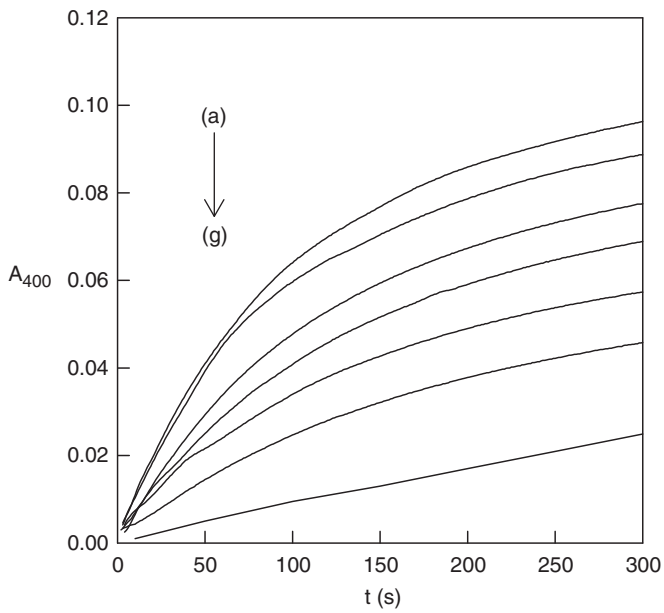


Figure 1. Spectrophotometric recordings to calculate the constant $[TBC]_{ss}/[TBF]_{ss}$ in the steady state, *C*. The experimental conditions are sodium phosphate buffer (pH 7.0) 30 mM, $[E]_0 = 36$ nM, $[TBF]_0 = 0.3$ mM and the initial $[TBC]_0$ (μM) concentrations are: (a) 35, (b) 30, (c) 28, (d) 25, (e) 20, (f) 18 and (g) 15.

Inactivation of tyrosinase in aerobic conditions: suicide inactivation

The kinetic mechanism proposed in Scheme 1 depicts the action of tyrosinase on monophenols and *o*-diphenols. It also depicts the suicide inactivation of the enzyme. The structural mechanism is depicted in Scheme 1SM (see Supplementary material).

In the mechanism proposed in Scheme 1, it can be seen how the enzyme is inactivated when its acts on the *o*-diphenol and, as

the number of turnovers that inactivates one mole of enzyme is a constant for a given enzyme, an analytical expression can be established that relates the number of turnovers in the diphenolase cycle (Scheme 1 in the absence of M) with the number of inactivating turnovers in the mechanism of Scheme 1.

The diphenolase activity (action of tyrosinase on TBC) can be monitored spectrophotometrically by measuring the formation of *o*-quinone, since, in each turnover (Scheme 1 in the absence of M) two molecules of *o*-quinone are formed at $t \rightarrow \infty$, the quantity of o-quinone is $[Q]_\infty$, and the number of turnovers is calculated by measuring the formation of *o*-quinone (Q) is:

$$r_D^Q = \frac{[Q]_\infty/2 - 2[E]_0/2}{[E]_0} = \frac{[Q]_\infty/2}{[E]_0} - 1 \approx \frac{[Q]_\infty/2}{[E]_0} \quad (3)$$

In the mechanism of Scheme 1, the number of turnovers made by the enzyme acting on M and measured by the consumption of oxygen is:

$$r_M^{O_2} = \frac{[O_2]_\infty}{[E]_0} \quad (4)$$

These turnovers are of two types, one for the hydroxylase cycle ($r_{M(H)}^{O_2}$) and the other for the oxidase cycle ($r_{M(O)}^{O_2}$). The abovementioned turnovers can produce inactivation, and, as two turnovers are produced in the hydroxylase cycle for every one in the oxidase cycle, the turnovers realized in the oxidase cycle as a function of the oxygen consumed will be[45]:

$$r_{M(O)}^{O_2} = \frac{[O_2]_\infty/3}{[E]_0} \quad (5)$$

The relation between Equations (3) and (5) is:

$$\frac{r_D^Q}{r_{M(O)}^{O_2}} = \frac{\dfrac{[Q]_\infty/2 - 2[E]_0/2}{[E]_0}}{\dfrac{[O_2]_\infty/3}{[E]_0}} = \frac{\dfrac{[Q]_\infty/2}{[E]_0} - 1}{\dfrac{[O_2]_\infty/3}{[E]_0}} \approx \frac{\dfrac{[Q]_\infty/2}{[E]_0}}{\dfrac{[O_2]_\infty/3}{[E]_0}} = 1 \quad (6)$$

If the activity on monophenols is followed by measuring the *o*-quinone formation, which, in this case, is stable, bearing in mind the stoichiometry $3O_2:4Q$ (in the oxidase cycle $1O_2:2Q$ and in the hydroxylase cycle $2O_2:2Q$), Equation (6) can be expressed as:

$$\frac{r_D^Q}{r_{M(O)}^Q} = \frac{\dfrac{[Q]_\infty/2 - 2[E]_0/2}{[E]_0}}{\dfrac{[Q]_\infty/2 - 2[E]_0/2}{[E]_0}} = \frac{\dfrac{[Q]_\infty/2}{[E]_0} - 1}{\dfrac{[Q]_\infty/2}{[E]_0} - 1} \approx \frac{\dfrac{[Q]_\infty/2}{[E]_0}}{\dfrac{[Q]_\infty/2}{[E]_0}} = 1 \quad (7)$$

Experimental demonstration of the validity of the analytical expressions shown in Equations (6) and (7) would confirm the hypothesis that tyrosinase is not inactivated in the hydroxylation of monophenols, as has been demonstrated in the case of L-tyrosine and L-dopa, compounds that generate very unstable *o*-quinones (*o*-dopaquinone) that lead to the accumulation of L-dopa in the medium[40].

In the present case, the monophenol used (TBF) originates a very stable *o*-quinone and the only way to study the process is to add a quantity of *o*-diphenol (TBC) to the medium to prevent the burst while ensuring that the enzyme remains in the steady state (Figure 1). Once $[D]_{ss}$ has been established, the ratio $[D]_{ss}/[M]_{ss} = C$ can be established and maintained in all the experiments.

Experimental approach

Since the substrate originates a stable quinone, a kinetic study of the suicide inactivation that occurs when tyrosinase acts on TBF can be carried out by measuring the consumption of oxygen or spectrophotometrically measuring the formation of *o*-quinone. Figure 2 depicts the oxygen consumption measured during the tyrosinase action on TBF, while the figure legend details the conditions. Analysis of the recordings depicted in Figure 2, according to Equation (1), enables $[O_2]_\infty$ and $\lambda_{E_{ox}}^M$ to be obtained. The inset in Figure 2 shows these parameters as a function of the initial enzyme concentration, and from the slope of $[O_2]_\infty$ versus $[E]_0$ the value of $r_M^{O_2}$ can be obtained – one-third of these turnovers take place in the oxidase cycle. The inset also shows how the apparent inactivation constant ($\lambda_{E_{ox}}^M$) does not depend on the initial enzyme concentration.

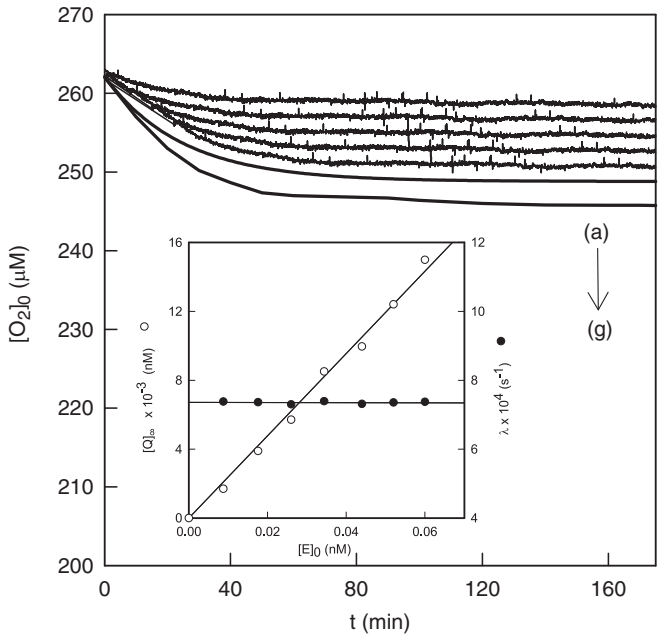


Figure 2. Oxymetric recordings of the suicide inactivation of tyrosinase in its action on TBF, varying the initial enzyme concentration. The experimental conditions are sodium phosphate buffer (pH 7.0) 30 mM, $[TBF]_0 = 0.5$ mM, $[TBC]_0 = 25$ μM and the initial enzyme (nM) concentrations are: (a) 0.008, (b) 0.017, (c) 0.026, (d) 0.034, (e) 0.044, (f) 0.052 and (g) 0.06. Inset. Representation of $[O_2]_\infty$ (○) and $\lambda_{E_{ox}}^M$ (●) versus $[E]_0$.

Figure 3 shows the spectrophotometric recordings of the accumulation of *o*-quinone in the action of tyrosinase on TBF. Analysis of the data by Equation (2) gives the values of $[Q]_\infty$ and $\lambda_{E_{ox}}^M$. The inset in Figure 3(a) shows the values of $[Q]_\infty$ versus $[E]_0$ which, from the slope of the straight line, give the value of r_M^Q. Taking Scheme 1 into account, half of $[Q]_\infty$ comes from the oxidase pathway and so the value of $r_{M(O)}^Q$ is $r_M^Q/2$. Note how $\lambda_{E_{ox}}^M$ is independent of the enzyme concentration[46,47]. The suicide inactivation of tyrosinase on TBC is described Muñoz-Muñoz et al.[25] and the values of the kinetic analysis are detailed in Table 1. The inset of Figure 3(b) shows the $[Q]_\infty$ versus $[E]_0$ and from the slope the value of r_D^Q can be obtained (Table 1).

The experimental results shown in Figures 2–3 and their kinetic analysis according to Equations (1) and (2), give the values of $r_{M(O)}^{O_2}$ and $r_{M(O)}^Q$, and the number of turnovers made by one mole of enzyme acting on TBF/TBC, measuring the consumption of oxygen or formation of *o*-quinone, respectively. From the data obtained for the inactivation of the enzyme acting on TBC[25], we obtain the value of $r_D^{NADH} = [NADH]_0/(2[E]_0)$, which is equivalent to $r_D^Q = [Q]_\infty/(2[E]_0)$. From these results and using Equations (6) and (7), we see that $r_D^Q/r_{M(O)}^{O_2} = 1.07 \pm 0.08$ and $r_D^Q/r_{M(O)}^Q = 1.01 \pm 0.10$.

These results are resumed in Table 2. Note that in the case of the pair TBF/TBC, the great stability of the *o*-quinones jeans that record the formation of *o*-quinone can be obtained both with the monophenol and *o*-diphenol, which cannot be done with the pair L-tyrosine/L-dopa due to the instability of the dopachrome[40].

Numerical integration of the mechanisms proposed

The numerical integration of the mechanisms proposed in Scheme 1 in the presence and the absence ($[M]_0 = 0$) of monophenol confirms the predictions indicated in Equation (7).

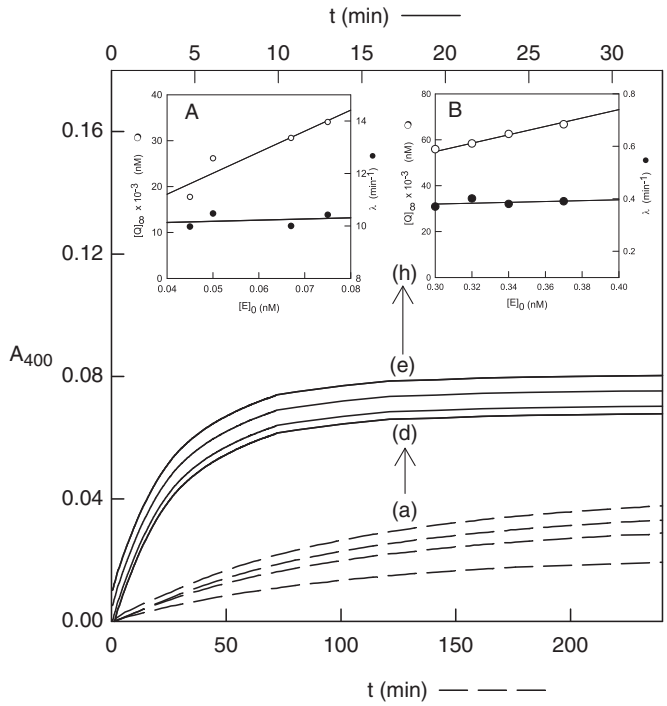


Figure 3. Spectrophotometric recordings of the suicide inactivation of tyrosinase in its action on TBF, varying the initial enzyme concentration. The experimental conditions are sodium phosphate buffer (pH 7.0) 30 mM, $[TBF]_0 = 0.1$ mM, $[TBC]_0 = 5$ μM and the initial enzyme (nM) concentrations are: (a) 0.045, (b) 0.05, (c) 0.067 and (d) 0.075. The curves (e)–(h) represent the action of the enzyme on TBC. The experimental conditions are the same as curves (a)–(d), but $[TBF]_0 = 0$ and $[TBC]_0 = 9$ mM. The initial enzyme concentrations (nM) are: 0.30 (e), 0.32 (f), 0.34 (g) and 0.37 (h). Inset A. Representation of $[Q]_\infty$ (○) and $\lambda^M_{E_{ox}}$ (●) versus $[E]_0$. Inset B. Representation of $[Q]_\infty$ (○) and $\lambda^D_{E_{ox}}$ (●) versus $[E]_0$.

Hence, in the ''Supplementary material'', we show the set of differential equations that express the monophenolase and diphenolase mechanism of tyrosinase.

Figure 1SM shows the recordings obtained for the formation of *o*-quinone (Q) in the diphenolase activity of the enzyme by varying the initial concentration. Analysis of the curves by means of Equation (2) gives the values of $[Q]_\infty$ and $\lambda^D_{E_{ox}(max)}$, while their dependence on $[E]_0$ is shown in Figure 1SM (inset). From this dependence of $[Q]_\infty$ versus $[E]_0$ we obtain the value $r^Q_D = 89040$.

Figure 2SM shows the recordings obtained for the formation of *o*-quinone (Q) in the monophenolase and diphenolase activity according to Scheme 1. Analysis of the curves by means of Equation (2) gives the values of $[Q]_\infty$ and $\lambda^M_{E_{ox}(max)}$, while their dependence on $[E]_0$ is shown in Figure 2SM (inset). From the values of $[Q]_\infty$ we obtain the value of $r^Q_{M(O)} = 89040$. As expected, these values comply with Equation (7) (Table 2).

Inactivation of E^R_d, E^T_d and E_m under anaerobic conditions by TBC and its protection by TBF

Figure 4 shows the residual activity of tyrosinase when E^R_d is preincubated with TBC. Substituting these data into Equation (8)

$$[E^R_d] = [E^R_d]_0 e^{-\lambda^D_{E^R_d} t} \quad (8)$$

gives the value of $\lambda^D_{E^R_d}$, and the analysis of these data according to Equation (9) (Figure 4, Inset ●), gives the values of $k^*_{i_D}$ and $K^D_{E^R_d}$ (Table 3).

$$\lambda^D_{E^R_d} = \frac{k^*_{i_D}[D]_0}{K^D_{E^R_d} + [D]_0} \quad (9)$$

In the presence of TBC and the monophenol (TBF) (Figure 4, Inset ○), the residual activity data fit Equation (10) and the values of $\lambda^{D(M)}_{E^R_d}$ fit Equation (11), giving $k^*_{i_{D(M)}}$ and $K^{D(M)}_{E^R_d}$. Analysis of these data by means of Equation (12) gives the values of $K^M_{E^R_d}$ (Table 3).

$$[E^R_d] = [E^R_d]_0 e^{-\lambda^{D(M)}_{E^R_d} t} \quad (10)$$

Figure 4 (inset) shows the values of $\lambda^D_{E^R_d}$ (●) and $\lambda^{D(M)}_{E^R_d}$ (○) versus $[TBC]_0$, which demonstrate how the monophenol protects the enzyme against inactivation by *o*-diphenol. Similar results are shown in Figure 5 and its inset for E^T_d. The results obtained are shown in Table 3. The protection presented by the monophenol against the irreversible inactivation caused by the *o*-diphenol is depicted in Schemes 2(A), 2(B) and 3. Possible molecular mechanisms of these inactivations are shown in the ''Supplementary material''. Analysis of the residual activity

Table 1. Kinetic constants which characterize the suicide inactivation of tyrosinase by TBC and values of the chemical shifts of this compound obtained by ^{13}C-NMR for the C-2 and C-1 carbons at pH 7.0*.

o-Diphenol	$\lambda^D_{E_{ox}}(max) \times 10^3$ (s^{-1})	$r = k^D_{cat}/\lambda^D_{E_{ox}}(max)$	k^D_{cat} (s^{-1})	V_{max} (μM/s)	K^D_m (mM)	$K^{O_2}_m$ (μM)	δ_2 (p.p.m.)	δ_1 (p.p.m.)
TBC	7.28 ± 0.28	$88\,788 \pm 1864$	640.1 ± 28.10	0.12 ± 0.01	1.45 ± 0.12	27.82 ± 2.52	147.75	146.24

*Data taken from the work by Muñoz-Muñoz et al.[25].

Table 2. Experimental confirmation of the relations described in Equations (6) and (7), according to which the theoretical value is 1.

Results	r^Q_D	$r^{O_2}_{M(O)}$	$r^Q_{M(O)}$	Equation (6) $r^Q_D/r^{O_2}_{M(O)} = \frac{[Q]_\infty/(2[E]_0)}{[O_2]_\infty/(3[E]_0)} = 1$	Equation (7) $r^Q_D/r^Q_{M(O)} = \frac{[Q]_\infty/(2[E]_0)}{[Q]_\infty/(2[E]_0)} = 1$
$[TBF]_0$ (0.5 mM)	–	$82\,891 \pm 8102$	$87\,099 \pm 7566$	–	–
$[TBC]_0$ (25 μM)	$88\,788 \pm 1864$	–	–	–	–
Applying Equations (6) and (7)				1.07 ± 0.08	1.01 ± 0.10

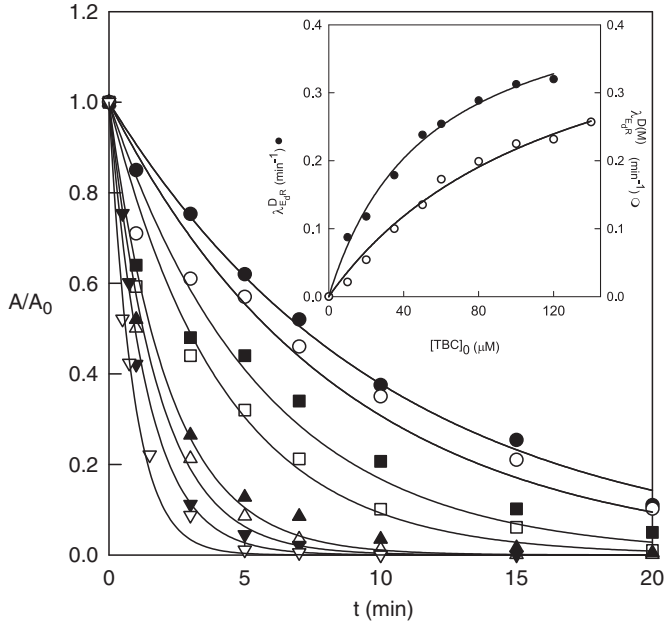


Figure 4. Residual activity, A/A_0, corresponding to the irreversible inactivation of E_d^R by TBC and its protection by TBF. The form E_d^R was obtained as described in the section ''Material and methods'' and immediately incubated with *o*-diphenol (TBC) in the presence of monophenol (TBF) or in its absence, taking aliquots at different times to measure the residual activity with $[TBC]_0 = 9\,mM$ ($\lambda = 400\,nm$). The experimental conditions were: 30 mM sodium phosphate buffer (pH 6.0), 25 °C, $[E]_0 = 0.1\,\mu M$ (see ''Material and methods'' section). The $[TBC]_0$ concentrations were (μM): (a) 10 •, (b) 20 ○, (c) 35 ■, (d) 50 □, (e) 60 ▲, (f) 80 △, (g) 100 ▼ and (h) 120 ▽. The experimental results follow Equation (8) and the apparent inactivation constants follow Equation (9). Inset. Representation of the values of $\lambda^D_{E_d^R}$ versus $[TBC]_0$ in the absence (•) of TBF. In the presence of TBF (○) ($[TBF]_0 = 40\,\mu M$), the experimental results follow Equation (10) to calculate $\lambda^{D(M)}_{E_d^R}$. These results were substituted into Equation (11) to obtain $K^{D(M)}_{E_d^R}$ and its analysis by Equation (12) gives $K^M_{E_d^R}$.

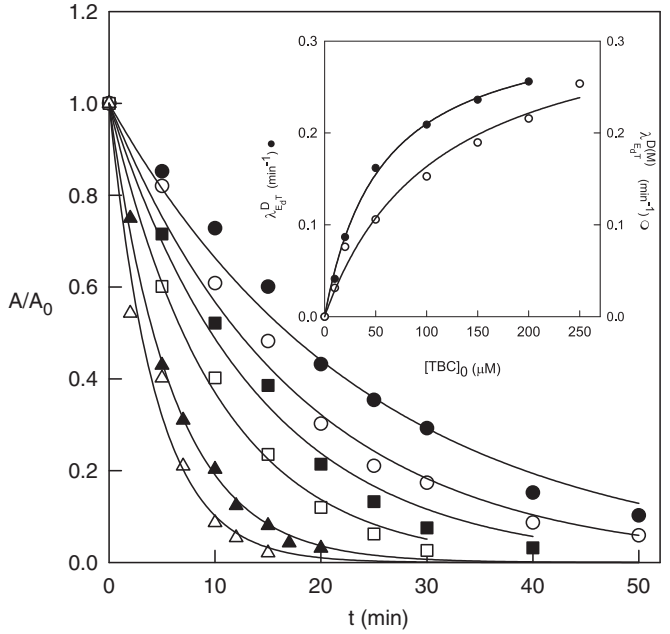
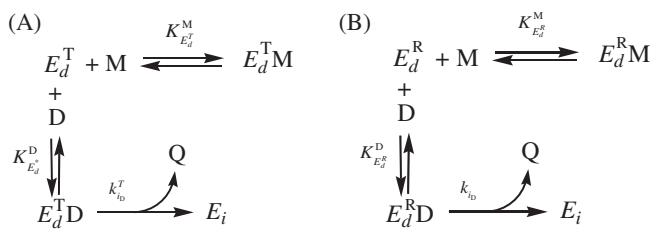


Figure 5. Residual activity, A/A_0, corresponding to the irreversible inactivation of E_d^T by TBC and its protection by TBF. The form E_d^T was obtained as described in the section ''Material and methods'' and immediately incubated with *o*-diphenol (TBC) in the presence of monophenol (TBF) or in its absence, taking aliquots at different times to measure the residual activity with 9 mM TBC ($\lambda = 400\,nm$). The experimental conditions were: 30 mM sodium phosphate buffer (pH 6.0), 25 °C, $[E]_0 = 0.1\,\mu M$ (see ''Material and methods'' section). The $[TBC]_0$ concentrations were (μM): (a) 10 •, (b) 20 ○, (c) 50 ■, (d) 100 □, (e) 150 ▲ and (f) 200 △. The experimental results follow Equation (8) and the apparent inactivation constants follow Equation (9). Inset. Representation of the values of $\lambda^D_{E_d^T}$ versus $[TBC]_0$ in the absence (•) of TBF. In the presence of TBF (○) ($[TBF]_0 = 40\,\mu M$), the experimental results were substituted into Equation (10) to calculate $\lambda^{D(M)}_{E_d^T}$. These results were again substituted into Equation (11) to obtain $K^{D(M)}_{E_d^T}$ and its analysis by Equation (12) gives $K^M_{E_d^T}$.

Table 3. Kinetic constants which characterize the inactivation of E_d^R, E_m and E_d^T by TBC and their protection by TBF under anaerobic conditions. The constants were obtained using the equations indicated.

Enzymatic form	Substrate	$K_E^{D(a)}$ (μM) [Equation (9)]	$k^*_{i_D} \times 10^3$ (min^{-1}) [Equation (9)]	$k^*_{i_D} \times 10^3$ (min^{-1}) [Equation (11)]	$K_E^{M\dagger}$ (μM) [Equation (12)]
E_d^T	TBC	55.46 ± 4.68	0.32 ± 0.01	–	–
	TBF	–	–	0.32 ± 0.03	110.27 ± 17.13
E_d^R	TBC	52.91 ± 5.90	0.47 ± 0.04	–	–
	TBF	–	–	0.47 ± 0.06	125.61 ± 17.51
E_m	TBC	52.91 ± 5.90	0.47 ± 0.04	–	–
	TBF	–	–	0.47 ± 0.05	125.61 ± 17.51

†In K_E^D and K_E^M, E corresponds to the enzymatic forms E_d^T or E_d^R. When the reaction is started with E_m, the data obtained correspond to E_d^R, because of the rapid transformation of E_m into E_d^R (Scheme 3).



Scheme 2. (A) Effect of monophenols on E_d^T, inactivation by *o*-diphenol. (B) Effect of monophenols on E_d^R, inactivation by *o*-diphenol.

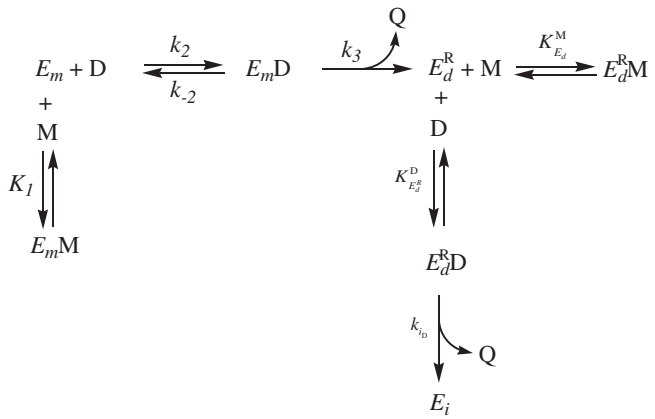
data (for E_d^R and E_d^T) in the presence of monophenol (TBF) fits Equation (10) but the apparent inactivation constant is now given by Equation (11):

$$\lambda^{D(M)}_{E_d} = \frac{k^*_{i_{D(M)}}[D]_0}{K^{D(M)}_{E_d} + [D]_0} \quad (11)$$

where $K^{D(M)}_{E_d}$ is expressed by Equation (12), which is used to calculate $K^M_{E_d}$.

$$K^{D(M)}_{E_d} = K^D_{E_d}\left(1 + \frac{[M]_0}{K^M_{E_d}}\right) \quad (12)$$

This analysis was also carried out for the E_m form (Table 3).



Scheme 3. Effect of monophenols on E_m, inactivation by *o*-diphenol.

Conclusions

In anaerobic conditions, monophenols protect the enzyme (E_d^R, E_d^T and E_m forms) against inactivation by *o*-diphenol. In aerobic conditions, the monophenols do not inactivate the enzyme (E_{ox} form) in the hydroxylase cycle directly, but, as this activity does not occur without the oxidase cycle acting on *o*-diphenol, it may be claimed that the monophenols inactivate tyrosinase indirectly. This implies that monophenols such as TBF, which, after oxidation, produce stable *o*-quinones that do not give rise to *o*-diphenol in the medium, do not bring about the suicide inactivation of the enzyme, but neither would the enzyme act on them due to the lack of *o*-diphenol in the medium.

Declaration of interest

The authors report no declarations of interest. This paper was partially supported by grants from: Ministerio de Educación y Ciencia (Madrid, Spain), project BIO2009-12956; Fundación Séneca (CARM, Murcia, Spain), projects 08856/PI/08 and 08595/PI/08, and Consejería de Educación (CARM, Murcia, Spain), BIO-BMC 06/01-0004. JLMM hold fellowships from Fundación Caja Murcia (Murcia, Spain).

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