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Tyrosinase inactivation in its action on dopa

J.L. Muñoz-Muñoz [a], J.R. Acosta-Motos [a], F. Garcia-Molina [a], R. Varon [b], P.A. Garcia-Ruíz [c], J. Tudela [a], F. Garcia-Cánovas [a,*], J.N. Rodríguez-López [a]

[a] GENZ: Grupo de Investigación Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biologia, Universidad de Murcia, E-30100, Espinardo, Murcia, Spain
[b] Departamento de Química-Física. Escuela de Ingenieros Industriales de Albacete. Universidad de Castilla la Mancha. Avda. España s/n. Campus Universitario, E-02071, Albacete, Spain
[c] QCBA: Grupo de Química de Carbohidratos y Biotecnología de Alimentos, Departamento de Química Orgánica. Facultad de Química, Universidad de Murcia, E-30100, Espinardo, Murcia, Spain

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ABSTRACT

Under aerobic or anaerobic conditions, tyrosinase undergoes a process of irreversible inactivation induced by its physiological substrate L-dopa. Under aerobic conditions, this inactivation occurs through a process of suicide inactivation involving the form *oxy*-tyrosinase. Under anaerobic conditions, both the *met*- and *deoxy*-tyrosinase forms undergo irreversible inactivation. Suicide inactivation in aerobic conditions is slower than the irreversible inactivation under anaerobic conditions. The enzyme has less affinity for the isomer D-dopa than for L-dopa but the velocity of inactivation is the same. We propose mechanisms to explain these processes.

1. Introduction

Tyrosinase or polyphenol oxidase (EC 1.14.18.1, *o*-diphenol:O_2 oxidoreductase) is a copper protein that uses molecular oxygen to catalyse the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [1,2]. Tyrosinase from a variety of sources undergoes an inactivation process when it reacts with its substrate under aerobic conditions [3–5]. The study of enzymatic inactivation by suicide substrates or mechanism-based inhibitors has grown in importance because of possible pharmacological applications [6,7]. Furthermore, these compounds may be useful for studying enzymatic mechanisms and for designing new pharmacological drugs [8].

The suicide inactivation of enzymes has been studied in several works since Waley in 1980 [9]. Our own group has made several kinetic studies of the suicide inactivation mechanism from a theoretical point of view [10,11] and using mushroom tyrosinase [12,13], frog skin tyrosinase [14,15] and peroxidase from different sources [16,17]. We have also studied the suicide inactivation of an enzyme that can be measured from coupled reactions [18] and published a review on the experimental designs for a kinetic study of suicide inactivation [19].

Several monophenols and *o*-diphenols are chiral tyrosinase substrates (L- and D-enantiomers). Some papers have reported the stereoselective action of several tyrosinases on these enantiomers, although the results have been unequal and even contradictory [20–22]. The use of a suitable measuring method to characterize monophenolase and diphenolase activities, using the chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH) [23,24], demonstrated that tyrosinase shows stereospecificity in its affinity towards its substrate and has a higher Michaelis constant for the isomers D (D-tyrosine and D-dopa) than for the L (L-tyrosine and L-dopa). However, it demonstrates the same velocity of catalysis for both isomers (L- and D-dopa and L- and D-tyrosine), confirming that the velocity is related with the charge density of the oxygen atoms of the hydroxyl groups of the benzene ring, which, in turn, is related with the chemical shifts δ_3 and δ_4. The same conclusion was reached using the immobilised enzyme [25].

The suicide inactivation of tyrosinase from different sources has been studied and many attempts have been made to find a mechanism that explains the phenomenon [26,27]. Recently, our group published a possible explication for this process [28]. In our mechanism (Scheme ISM), the E_{ox} form binds one molecule of substrate (*o*-diphenol) (Step 6). In this case, the base (B), possibly a histidine, is already protonated and cannot help the substrate bind to the copper. The proton of the hydroxyl group of C-4 must be transferred to the peroxide group, which acts as a base [29,30]. This would be the slow step of the turnover, controlled by k_{7_1} (Step 7) [30]. The fact that the peroxide acts as a base and is involved in the proton transfer from the hydroxyl group of C-4 and C-3 is important. The protonated peroxide draws the copper atoms in the E_{ox} form closer together, since the distance in the unprotonated form, 3.6 Å, is too great for the molecule of substrate to bind diaxially to them. However,

* Corresponding author. Tel.: +34 868 884764; fax: +34 868 883963.
E-mail address: canovasf@um.es (F. Garcia-Cánovas).

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this would be possible in the protonated E_{ox} form. With the substrate axially bound to one copper atom, there are two possibilities in Step 8: (a) the oxygen of the hydroxyl group of C-3 may attack the other copper atom, which is drawn closer following protonation of the peroxide group, transferring the proton to the histidine, binding to the substrate diaxially and co-planar with the copper atoms. In this way, the concerted redox reaction in Step 9 can occur, releasing *o*-quinone and E_m. (b) The hydroxyl group of C-3 may donate a proton to the protonated peroxide, remaining as $C–O^-$ in Step 10. The co-planarity of the substrate ring, the oxygen of C-4 and the copper atom favor the simultaneous oxidation of the substrate to *o*-quinone and the reduction of one Cu^{2+} atom to copper (0) through the ring, while copper (0) and hydrogen peroxide are released, and the enzyme is inactivated as a consequence (Step 11) (Scheme ISM).

For its part, under anaerobic conditions it has been demonstrated that a reductant like ascorbic acid inactivates the enzyme in its *met*-tyrosinase and *deoxy*-tyrosinase forms [31]. The capacity to inactivate the enzyme under both aerobic and anaerobic conditions may have an industrial application for protecting juices and other preparations from enzymatic browning: hence the interest in its study and characterisation.

The aims of this study were: to analyse the inactivation of tyrosinase by its physiological substrate L-dopa under aerobic conditions (suicide inactivation of *oxy*-tyrosinase form) and under anaerobic conditions (irreversible inactivation of *met*-tyrosinase and *deoxy*-tyrosinase) and to study the effect of the stereospecificity, using the isomers L- and D-dopa.

1.1. Notation

For clarity and for the sake of brevity, the following additional notations will be used in the text.

1.1.1. Species and concentrations

$[E]_0$	initial concentration of tyrosinase
E_a	active enzyme
$[E_a]$	instantaneous concentration of E_a
$[E_m]$	instantaneous concentration of *met*-tyrosinase
$[E_m]_0$	initial concentration of *met*-tyrosinase
$[E_d]$	instantaneous concentration of *deoxy*-tyrosinase
$[E_d]_0$	initial concentration of *deoxy*-tyrosinase
E_d^*	*deoxy*-tyrosinase after of E_d to E_d^* transition
$[E_d^*]$	instantaneous concentration of E_d^*
$[E_d^*]_0$	initial concentration of E_d^*
E_i	inactive enzyme
$[E_i]$	instantaneous concentration of E_i
D	L- or D-*o*-diphenol
$[D]_0$	initial concentration of D
Q	*o*-quinone product of the enzymatic reaction
[Q]	instantaneous concentration of Q
$[Q]_\infty$	*o*-quinone concentration at $t \to \infty$
NADH	nicotinamin adenin dinucleotide
$[NADH]_0$	initial concentration of NADH
[NADH]	instantaneous concentration of NADH
$[NADH]_f$	NADH-value at $t \to \infty$
$[NADH]_\infty$	NADH consumed at the end of the reaction, $t \to \infty$, i.e. $[NADH]_\infty = [NADH]_0 - [NADH]_f$

1.1.2. Kinetic parameters

$V_0^{D(NADH)}$	initial rate of tyrosinase acting on D in the presence of NADH.
$V_0^{D(Q)}$	initial rate of tyrosinase acting on D, $V_0^{D(Q)} = V_0^{D(NADH)}$.
$\lambda_{E_{ox}}^{D(L\text{ or }D)}$	apparent constant of suicide inactivation of tyrosinase in the presence of L- or D-dopa.
$\lambda_{E_{ox(max)}}^{D(L\text{ or }D)}$	maximum value of $\lambda_{E_{ox}}^{D(L\text{ or }D)}$ for saturating substrate.
$\lambda_{E_d}^{D(L\text{ or }D)}$	apparent constant of irreversible inhibition of E_d by L- or D-dopa.
$\lambda_{E_d^*}^{D(L\text{ or }D)}$	apparent constant of irreversible inhibition of E_d^* by L- or D-dopa.
$k_{i_{D(L\text{ or }D)}}$	maximum value of $\lambda_{E_d}^{D(L\text{ or }D)}$ for saturating substrate.
$k_{i_{D(L\text{ or }D)}}^*$	maximum value of $\lambda_{E_d^*}^{D(L\text{ or }D)}$ for saturating substrate.
k_T	rate constant of the transition E_d to E_d^*.
r_D	partition ratio for the diphenolase activity, $r_D = k_{7_2}/k_{7_2}^i = k_{cat}^D/\lambda_{E_{ox}(max)}^D$.
$K_m^{O_2}$	Michaelis constant of oxygen.
$K_m^{D(L\text{ or }D)}$	Michaelis constant of tyrosinase for L- or D-dopa.
$k_{cat}^{D(L\text{ or }D)}$	catalytic constant of the diphenolase activity for L- or D-dopa.
$K_{E_d^*}^{D(L\text{ or }D)}$	dissociation constant of the complex E_d^*D, where D is L- or D-dopa.
$K_{E_d}^{D(L\text{ or }D)}$	dissociation constant of the complex E_dD, where D is L- or D-dopa.

2. Experimental

2.1. Materials

Mushroom tyrosinase or polyphenol oxidase (*o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1) (8300 U/mg) and β-NADH were supplied by Sigma (Madrid, Spain). The enzyme was purified as previously described in [32]. Protein concentration was determined by the Lowry method [33]. The substrates used L- and D-dopa 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 phosphoric acid to prevent autooxidation. Milli-Q system (Millipore corp.) ultrapure water was used throughout.

2.2. Methods

2.2.1. Spectrophotometric assays

These assays were carried out with a Perkin-Elmer Lambda-35 spectrophotometer, on line interfaced to a PC-computer, where the kinetic data were recorded, stored and later analysed. The product of the enzyme reaction *o*-dopaquinone is unstable and evolves towards dopachrome, which is not stable at long assay times [34,35]. For this reason, the reaction was followed by measuring the disappearance of NADH at 340 nm, with $\varepsilon = 6230\ M^{-1}\ cm^{-1}$, which reduces *o*-dopaquinone to L-dopa [36]. The inactivation kinetics was studied in 30 mM sodium phosphate buffer (pH 6.0), since *o*-dopaquinone evolves rapidly to dopachrome at pH 7.0, while at lower pH the reaction slows down and *o*-dopaquinone is reduced by NADH [34].

2.2.2. Kinetic data analysis

2.2.2.1. Diphenolase activity. The experimental data of time-based assays for the disappearance of NADH (Fig. 1) follow the Eq. (1):

$$[NADH] = [NADH]_f + [NADH]_\infty e^{-\lambda_{E_{ox}}^D t} \quad (1)$$

$[NADH]_f$,$[NADH]_\infty = [Q]_\infty$ and $\lambda_{E_{ox}}^D$ can be obtained by non-linear regression [37]. There is always a slow spontaneous oxidation of *o*-diphenol and NADH (Fig. 1), which should be computer corrected in further NADH *vs.* time plots (in general this treatment is applied to all the kinetic recordings). The experimental recordings obtained in the following steps, therefore, fit Eq. (1), from which the corresponding inactivation parameters, $[NADH]_\infty = [Q]_\infty$ and $\lambda_{E_{ox}}^D$, can be determined.

2.2.3. Generation of E_{ox} and E_d

To kinetically characterize the inactivation of the E_d form of tyrosinase, E_d was generated from the native enzyme, adding

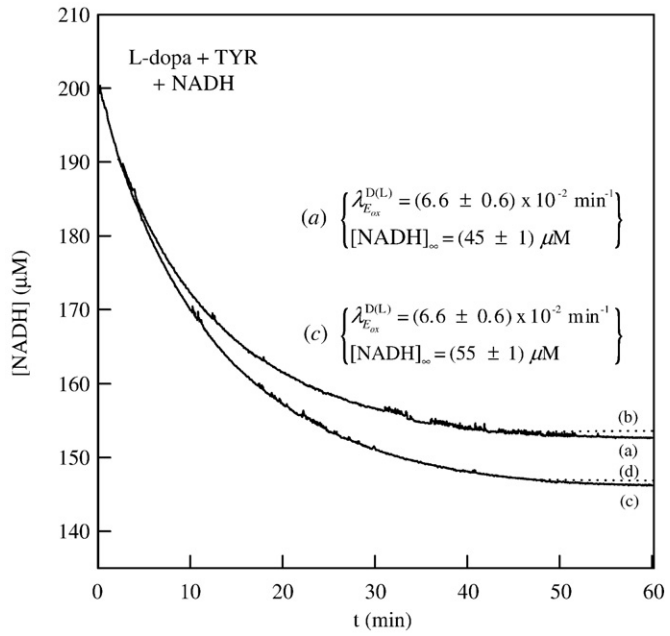


Fig. 1. Experimental recordings of the disappearance of NADH in the suicide inactivation of tyrosinase by L-dopa and curves corrected after applying the kinetic data analysis. Conditions were 30 mM sodium phosphate buffer (pH 6.0), 0.2 mM NADH, 0.26 mM O_2, 1 mM L-dopa and enzyme concentrations were: (a) 0.7 nM and (c) 0.85 nM. Curves (b) and (d) are the corrected recordings from (a) and (c), respectively, subtracting the L-dopa and NADH spontaneous oxidation.

micromolar concentrations (2 μM) of H_2O_2, so that, the E_m form passed to E_{ox}. Then, nitrogen was bubbled through the solution transforming all the E_{ox} to E_d ($E_{ox} \rightleftarrows E_d + O_2$) [38,39].

2.2.4. Generation of E_m

The inactivation of E_m was characterized by first generating it from the native enzyme in two ways: (a) adding catalase 2 μM, so that $E_{ox} \rightleftarrows E_m + H_2O_2$; the catalase acts on the H_2O_2 displacing the above equilibrium, $2H_2O_2 + \text{catalase} \rightarrow O_2 + 2H_2O$, so that all E_{ox} is transformed into E_m at the end of the reaction. (b) Adding 2 μM of H_2O_2, so that all the enzyme passes to E_{ox} and then adding 2 μM catalase [38,39].

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

It is known that in an enzymatic preparation of tyrosinase from any source is found in three forms, E_m, E_d and E_{ox} [39]. Several authors have proposed spectrophotometric methods for evaluating these forms [39–41]. Here, we propose a kinetic method based on the fact that the inactivation of these forms by 2-mercaptoethanol [42] occurs over a wide time range: inactivation constants of 0.014 s^{-1}, 4×10^{-5} s^{-1} and 1×10^{-5} s^{-1} for E_{ox}, E_m and E_d, respectively. Under aerobic conditions at oxygen concentrations of 0.26 mM, practically the only forms existing are E_{ox} and E_m [32]. Note that the difference between k_i^{ox} and k_i^m is three orders of magnitude, a difference that can be used to evaluate these enzymatic forms. The experimental method was described in [31].

2.2.6. ^{13}C-NMR assays

^{13}C-NMR spectra of several substrates were obtained in a Varian Unity spectrometer at 300 MHz. The spectra were obtained at pH = 6.0 by using ^2H_2O as solvent for the substrates. Chemical displacement (δ) values were measured relative to those for tetramethylsilane ($\delta = 0$). The maximum line breadth accepted in the ^{13}C-NMR spectra was 0.06 Hz. Therefore, the maximum accepted error for each peak of the spectrum was ±0.03 p.p.m.

3. Results

The active site of tyrosinase is found in three forms in the catalytic cycle: *met*-tyrosinase ($Cu^{2+}Cu^{2+}$), *deoxy*-tyrosinase ($Cu^{+}Cu^{+}$) and *oxy*-tyrosinase ($Cu^{2+}Cu^{2+}O_2^{2-}$) [39]. These enzymatic forms may undergo a substrate-induced irreversible inactivation. Under aerobic conditions, the enzyme undergoes a process of suicide inactivation, localised in *oxy*-tyrosinase, while under anaerobic conditions the *met*-tyrosinase and *deoxy*-tyrosinase forms undergo irreversible inactivation. These processes are studied below with *o*-diphenols (L- and D-dopa), under both aerobic and anaerobic conditions.

3.1. Tyrosinase action on o-diphenols

3.1.1. Inactivation of tyrosinase in its action on o-diphenols under aerobic conditions: suicide inactivation

The kinetic mechanism proposed to explain the suicide inactivation of tyrosinase acting on *o*-diphenols is based on the structural mechanism proposed in Scheme ISM and can be outlined as follows in Scheme I.

When $[D]_0$, $[O_2]_0 >> [E]_0$ and $[Q]_\infty << [D]_0$, $[O_2]_0$, it is possible a derivation of the analytical expression which establishes the accumulation of the product (*o*-quinone) with time, as is detailed in reference [28].

The variation of [Q] with time is given by Eq. (2) [28].

$$[Q] = [Q]_\infty \left(1 - e^{-\lambda^D_{E_{ox}} t}\right) \quad (2)$$

When $t \rightarrow \infty$, $[Q] = [Q]_\infty$ and, according to [28]:

$$[Q]_\infty = \frac{2k_{7_2}}{k^i_{7_2}} [E]_0 \quad (3)$$

The velocity of the suicide inactivation is regulated by the apparent inactivation constant, $\lambda^D_{E_{ox}}$, whose expression is given by Eq. (4). Taking into account the saturation of tyrosinase by oxygen ($[O_2]_0 \rightarrow \infty$), thus according to [28]:

$$\lambda^D_{E_{ox}} = \frac{\lambda^D_{E_{ox}(max)} [D]_0}{K^D_m + [D]_0} \quad (4)$$

The partition ratio, r_D, between the catalytic and suicide inactivation pathways is:

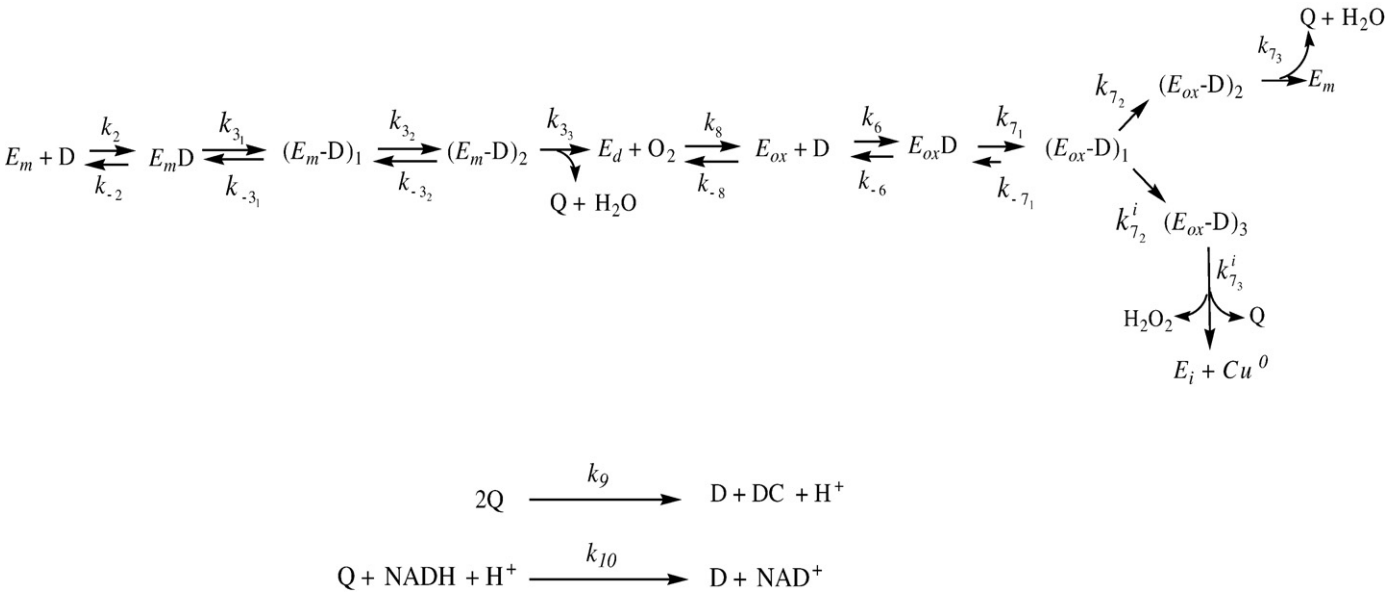
$$r_D = \frac{k_{7_2}}{k^i_{7_2}} = \frac{k^D_{cat}}{\lambda^D_{E_{ox}(max)}} = \frac{[Q]_\infty}{2[E]_0} = \frac{[NADH]_\infty}{2[E]_0} \quad (5)$$

where k_{7_2} is the substrate binding constant to the copper atom through the C-3 hydroxyl (axial) and $k^i_{7_2}$ is the rate constant of transfer of a H^+ to the protonated peroxide (see Scheme ISM).

Therefore, from Eq. (1–5), we can obtain the kinetic constants that characterize the kinetic behaviour of a suicide substrate: r_D, $\lambda^D_{E_{ox}(max)}$, K^D_m and k^D_{cat}. Note that if dopachrome or oxygen consumption is being measured, Eqs. (1–5) are totally applicable and it is only necessary to take into account the stoichiometric relations: 2Q:1 DC:1O_2 and that Q and DC are originated in the medium by the action of the enzyme, while the oxygen concentration falls.

3.1.1.1. Experimental study of suicide inactivation. The study of the suicide inactivation of tyrosinase was developed in three steps and the *o*-diphenols used in this work were L-dopa and D-dopa.

Step 1. Fig. 1 shows the spectrophotometric recordings of the disappearance of NADH, curves (a) and (c), and their corrections by fitting to Eq. (1), after subtracting the NADH and *o*-diphenol spontaneous oxidation, Fig. 1, curves (b) and (d). These corrected recordings are analysed as described in the Kinetic Data Analysis section. By means



Scheme I. Kinetic mechanism proposed to explain the suicide inactivation of tyrosinase acting on dopa enantiomers (see text and Scheme ISM for further details).

of these preliminary studies, the concentration of enzyme is optimised so that $[Q]_\infty << [O_2]_0, [D]_0$.

Step 2. Variation in enzyme concentration. The $[E]_0$-value is varied while the substrate concentration is kept constant. The results obtained are shown in Fig. 2A and B. The concentration of the substrate in these experiments does not change because the *o*-quinone is reduced by NADH. However, the concentrations of O_2 and NADH do change. In the case of tyrosinase, $K_m^{O_2}$ is very small [43], which permits an oxygen consumption of more than 10%. The NADH must be in excess. Note how the values of $[NADH]_\infty$ are directly proportional to $[E]_0$, while $\lambda_{E_{ox}}^{D(L\text{ or }D)}$ does not vary with $[E]_0$ (Fig. 2A Inset and B Inset).

Fig. 2A Inset and B Inset show the dependence of the values obtained for the product at the end of the reaction, $[NADH]_\infty$ *vs.* $[E]_0$, from whose slopes and, according to Eq. (5), r_D can be determined (Table 1).

Step 3. Variation in substrate concentration. The recordings for the disappearance of NADH during the action of tyrosinase on L-dopa and D-dopa obtained by varying the concentration of substrate are shown in Fig. 3A and B, respectively. Fitting by non-linear regression to Eq. (1) gives the apparent inactivation constant $\lambda_{E_{ox}}^{D(L\text{ or }D)}$. The hyperbolic dependence of $\lambda_{E_{ox}}^{D(L\text{ or }D)}$ *vs.* $[D]_0$ is shown in Fig. 3A Inset and B Inset. Fitting these data by non-linear regression according to Eq. (4) gives $\lambda_{E_{ox}(max)}^{D(L\text{ or }D)}$ and $K_m^{D(L\text{ or }D)}$ for each of the isomers (Table 1). These values of $\lambda_{E_{ox}(max)}^{D(L\text{ or }D)}$ and r_D (Table 1) permit us to obtain $k_{cat}^{D(LorD)}$ (Table 1).

3.1.2. Tyrosinase inactivation under anaerobic conditions: irreversible inactivation

The three enzymatic forms of the turnover of tyrosinase are in equilibrium, as shown by the following scheme (Scheme II).

In the presence of oxygen (0.26 mM), the principal forms are E_m and E_{ox}, whose concentration in an enzymatic preparation was determined by measuring the inactivation of the native enzyme by irreversible inhibition with 2-mercaptoethanol, as described in [31].

3.1.2.1. Inactivation of deoxy-tyrosinase by o-diphenols. The form *deoxy*-tyrosinase was generated as described in Materials and Methods sections [31]. Maintaining anaerobic conditions, E_d evolves slowly towards another enzymatic form that we denominate E_d^* [44], as described in the Scheme III.

Through analogy with that observed in hemocyanin, it has been proposed that the copper atoms in the enzymatic form E_d^* are more separated than in E_d [44,45].

The forms E_d and E_d^* show different affinities for *o*-diphenols and hence different activities ($K_{m,E_d}^{D(L)} = 0.52$ mM and $K_{m,E_d^*}^{D(L)} = 0.99$ mM in the case of L-dopa, and $K_{m,E_d}^{D(D)} = 3.95$ mM and $K_{m,E_d^*}^{D(D)} = 5.36$ mM in the case of D-dopa), so that the transition of E_d and E_d^* can be followed by measuring the initial rate with L-dopa, as shown in Fig. 4. Analysis of the data of Fig. 4 according to Eq. (6)

$$\frac{A}{A_0} = \frac{[E_a]}{[E_d]_0} = \alpha + \beta e^{-k_T t} \tag{6}$$

gives the apparent constant of the transition in the conditions described in Fig. 4 ($k_T = (3.9 \pm 0.4) \times 10^{-2}$ min^{-1}) and where A is the instantaneous enzymatic activity.

3.1.2.1.1. E_d^* inactivation by L-dopa and D-dopa. From the form E_{ox}, the form E_d was generated by passing a nitrogen stream. After 90 min, the form E_d is transformed into E_d^*, which is stable under anaerobic conditions. In this section, we shall study the reaction of E_d^* with L-dopa and D-dopa.

At t = 0, a given concentration of L-dopa was added as indicated in Fig. 5A. Aliquots were taken and, when the activity was followed with time, an irreversible inactivation, which can be described kinetically by Scheme IV and whose structural interpretation is depicted in Scheme IISM, was observed.

The monoexponential behaviour observed in Fig. 5A (gradual loss of activity) can be fitted to Eq. (7):

$$\left[E_d^*\right] = \left[E_d^*\right]_0 e^{-\lambda_{E_d^*}^{D(L)} t} \tag{7}$$

where D(L) corresponds to L-dopa. The values of $\lambda_{E_d^*}^{D(L)}$ vary with the concentration of substrate used in the assays, according to Eq. (8):

$$\lambda_{E_d^*}^{D(L)} = \frac{k_{i_{D(L)}}^* [D]_0}{K_{E_d^*}^{D(L)} + [D]_0}. \tag{8}$$

The data analysis by non-linear regression shown in Fig. 5A Inset according to Eq. (8) gives the values of $k_{i_{D(L)}}^*$ and $K_{E_d^*}^{D(L)}$ shown in Table 2. When the same experiments were carried out with D-dopa, Fig. 5B and B Inset, the data shown in Table 2 were obtained.

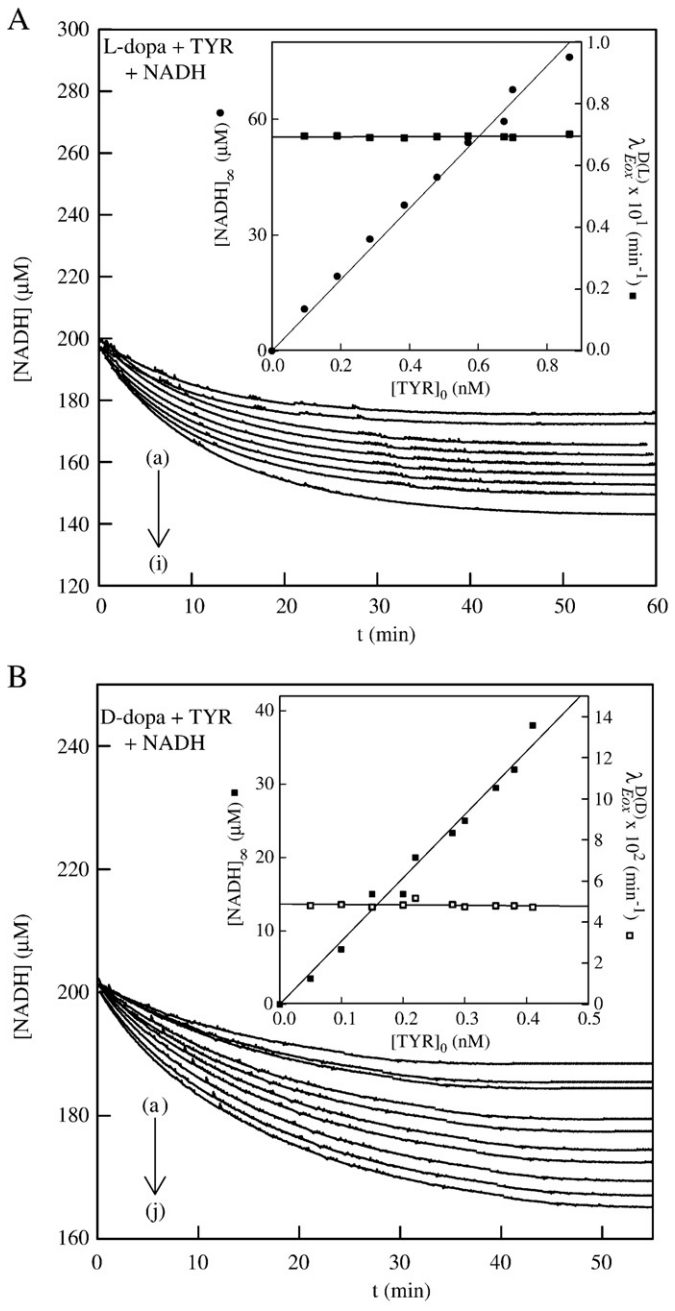


Fig. 2. Corrected recordings of the disappearance of NADH in the suicide inactivation of tyrosinase by *o*-diphenol for different enzyme concentrations. (A) L-dopa, (B) D-dopa. Conditions were: 30 mM sodium phosphate buffer (pH 6.0), 0.2 mM NADH, 0.26 mM O_2. (A). 1 mM L-dopa and $[E]_0$ (nM): (a) 0.09, (b) 0.19, (c) 0.28, (d) 0.38, (e) 0.48, (f) 0.57, (g) 0.67, (h) 0.70 and (i) 0.85. Inset. Representation of the values of $[NADH]_\infty$ (●) and $\lambda^{D(L)}_{E_{ox}}$ (■) *vs.* enzyme concentration for L-dopa. (B). 4 mM D-dopa and $[E]_0$ (nM): (a) 0.05, (b) 0.10, (c) 0.15, (d) 0.20, (e) 0.22, (f) 0.28, (g) 0.30, (h) 0.35, (i) 0.38 and (j) 0.41. Inset. Representation of the values of $[NADH]_\infty$ (●) and $\lambda^{D(D)}_{E_{ox}}$ (■) *vs.* enzyme concentration for D-dopa.

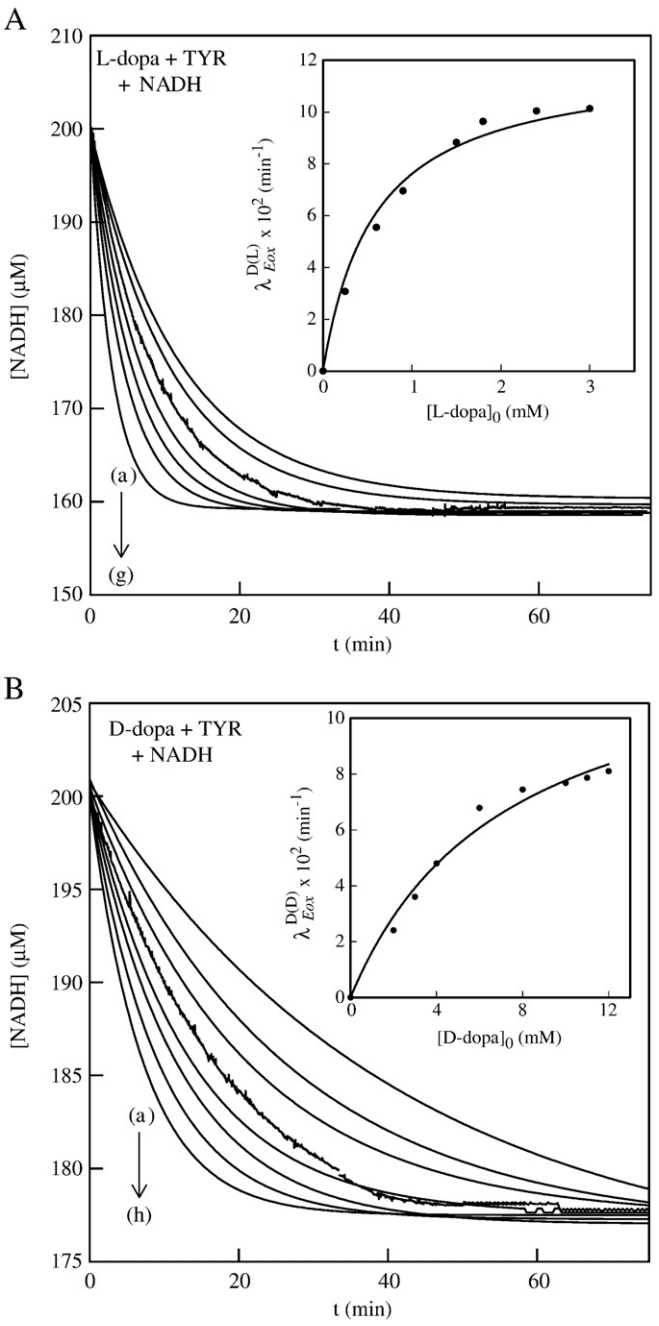
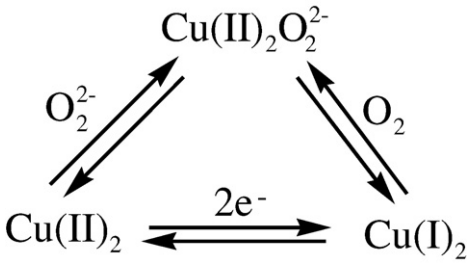


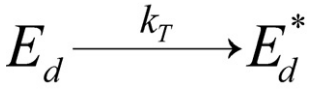
Fig. 3. Corrected recordings of the disappearance of NADH in the suicide inactivation of tyrosinase by different substrate concentrations. (A) L-dopa, (B) D-dopa. Conditions were 30 mM sodium phosphate buffer (pH 6.0), 0.26 mM O_2 and the initial concentrations of NADH and tyrosinase were: 0.2 mM and 0.6 nM for L-dopa and 0.2 mM and 0.2 nM for D-dopa, respectively. (A) L-dopa, the substrate concentrations were (mM): (a) 0.25, (b) 0.6, (c) 0.9, (d) 1.5, (e) 1.8, (f) 2.4 and (g) 3. Inset. Values of $\lambda^{D(L)}_{E_{ox}}$ for different L-dopa concentrations. (B) D-dopa, the substrate concentrations were (mM): (a) 2, (b) 3, (c) 4, (d) 6, (e) 8, (f) 10, (g) 11 and (h) 12. Inset. Values of $\lambda^{D(D)}_{E_{ox}}$ for different D-dopa concentrations.

Table 1
Kinetic constants which characterize the suicide inactivation of tyrosinase by the dopa isomers and values of the chemical shifts of these compounds obtained by ^{13}C-NMR for the C-3 and C-4 carbons at pH 6.0.

o-Diphenol	$\lambda^{D}_{E_{ox(max)}} \times 10^2$ (min^{-1})	$r = k^{D}_{cat}/\lambda^{D}_{E_{ox(max)}}$	k^{D}_{cat} (min^{-1})	K^{D}_{m} (mM)	$K^{O_2}_{m}$ (μM)	δ_3 (p.p.m.)	δ_4 (p.p.m.)
L-dopa	13.3 ± 0.6	46133 ± 2306	6156 ± 1152	0.55 ± 0.08	4.46 ± 0.89	146.92	146.06
D-dopa	12.9 ± 0.9	44895 ± 2573	5910 ± 1014	3.98 ± 0.57	4.28 ± 0.86	146.92	146.06



Scheme II. Equilibrium between E_{ox}, E_m and E_d forms of tyrosinase.



Scheme III. Kinetic mechanism of the transition of E_d to E_d^*.

3.1.2.1.2. E_d inactivation by L-dopa and D-dopa. The form E_d generated from E_{ox} under the nitrogen stream was made to react rapidly with different concentrations of L-dopa. The results (Fig. 6A) can be explained kinetically by Scheme V and, structurally, by a Scheme similar to Scheme IISM, although the distance between the coppers in E_d may be smaller, as occurs in [45]. Once again, monoexponential behaviour was observed, which can be fitted to Eq. (1SM), which is similar to Eq. (7), but for E_d , while the inactivation constant values better fit an equation similar to Eq. (8), but for E_d [Eq. (2SM)].

A fit, by non-linear regression, of the values of $\lambda_{E_d}^{D(L)}$ to Eq. (2SM), Fig. 6A Inset, gives $k_{i_{D(L)}}$ and $K_{E_d}^{D(L)}$ (Table 2). When the same experiments are carried out with D-dopa, Fig. 6B and B Inset, the values shown in Table 2 are obtained for $k_{i_{D(D)}}$ and $K_{E_d}^{D(D)}$.

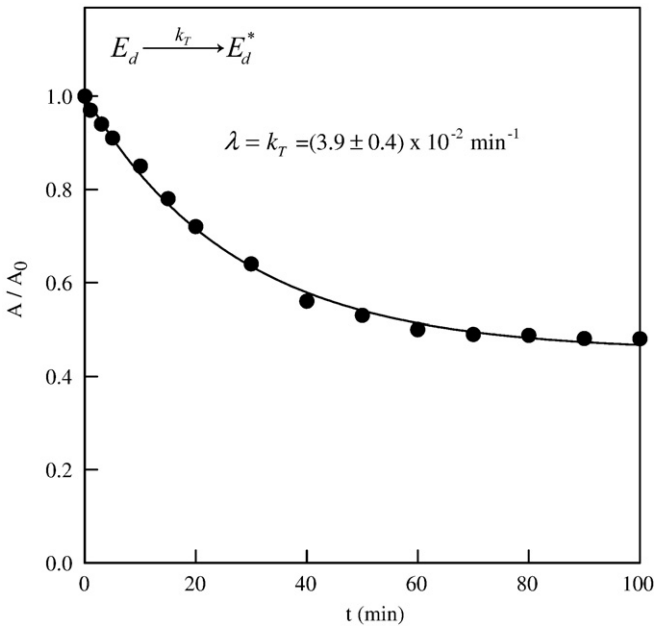


Fig. 4. Kinetic study of the transition of E_d to E_d^*. The enzyme form E_d was generated by passing a stream of nitrogen through a preparation of E_{ox}. $[E]_0 = 0.1$ μM (see Materials and Methods sections) and $[H_2O_2]_0 = 2$ μM. Aliquots were taken at different times and enzymatic activity was detected with 1 mM L-dopa ($\lambda = 475$ nm). The values obtained were fitted by non-linear regression to Eq. (6).

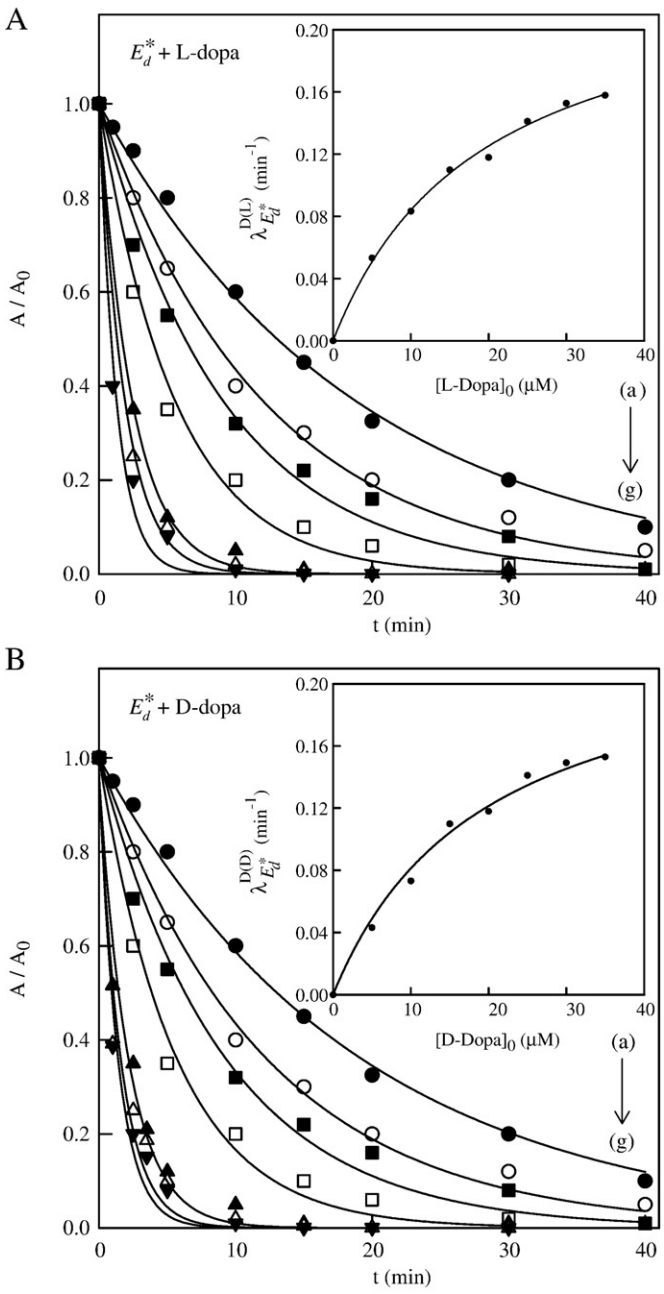
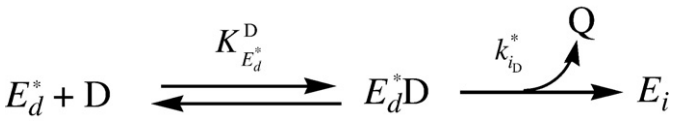


Fig. 5. Inactivation of the E_d^* form of tyrosinase by *o*-diphenols. The form E_d^* was obtained by allowing the form E_d obtained as described in Fig. 4 to evolve under anaerobic conditions for 90 min to obtain E_d^*. The *o*-diphenol was added at t = 0 and aliquots were taken at different times to measure the residual activity with 2.5 mM L-dopa ($\lambda = 475$ nm). The values obtained were fitted to Eq. (1SM) and the apparent inactivation constant was obtained at each substrate concentration. (A) L-dopa. The experimental conditions were: 30 mM sodium phosphate buffer (pH 6.0), 25 °C, $[E]_0 = 0.1$ μM (see Materials and Methods sections), $[H_2O_2]_0 = 2$ μM and L-dopa (μM): (a) 5 (●), (b) 10 (○), (c) 15 (■), (d) 20 (□), (e) 25 (▲), (f) 30 (△) and (g) 35 (▼). Inset. Representation of $\lambda_{E_d^*}^{D(L)}$ *vs.* $[L\text{-dopa}]_0$. (B) D-dopa. The experimental conditions were the same as in (A) but D-dopa (μM): (a) 5 (●), (b) 10 (○), (c) 15 (■), (d) 20 (□), (e) 25 (▲), (f) 30 (△) and (g) 35 (▼). Inset. Representation of $\lambda_{E_d^*}^{D(L)}$ *vs.* $[D\text{-dopa}]_0$.

3.1.2.1.3. E_m inactivation by L-dopa and D-dopa. E_m was generated from the native enzyme, as described in Materials and Methods sections [31]. Under anaerobic conditions, this form is stable, but in the presence of *o*-diphenols, it is inactivated, as described in kinetic form in Scheme VI and structurally in Scheme IIISM.

E_m was incubated with L-dopa and aliquots were taken at different times and revealed with L-dopa, the results being similar to those



Scheme IV. Kinetic mechanism of E_d^* inactivation by *o*-diphenols.

obtained with the E_d. According to the mechanism described in Scheme VI, the enzyme E_m rapidly passes to E_d and is inactivated (Scheme VI and Scheme IIISM). The values of the constants are shown in Table 2.

4. Discussion

This study reveals that the physiological substrate of tyrosinase, L-dopa, and its iosomer, D-dopa, act as tyrosinase inactivators both in aerobic and anaerobic conditions. Under aerobic conditions, L- or D-dopa act as a suicide substrate of the enzyme basically through the enzymatic species E_{ox} and, under anaerobic conditions, they act as irreversible inactivators of the enzyme in the enzymatic species E_m, E_d and E_d^*.

4.1. Aerobic conditions: suicide inactivation

Kinetic characterisation of an enzyme acting on a suicide substrate is determined from *r* (partition ratio), λ (apparent constant of suicide inactivation) and K_m (Michaelis constant). In the action of tyrosinase on L- or D-dopa these parameters are determined according to Eqs. (4) and (5).

From an analysis of the data shown in Fig. 2A Inset and B Inset and, according to Eq. (5), r_D can be determined, giving practically the same value for the two isomers (Table 1). From Eq. (5) and taking into account that $k_{cat}^{D(D)} \approx k_{cat}^{D(L)}$ [23], it is deduced that the inactivation constant is approximately equal in both isomers (Table 1).

When the effect of substrate concentration is studied by analysing the results shown in Fig. 3A and B, the corresponding apparent inactivation constants ($\lambda_{E_{ox}}^{D(L\text{ or }D)}$) are obtained, and their analysis according to Eq. (4) (Fig. 3A Inset and B Inset) enables us to obtain $\lambda_{E_{ox(max)}}^{D}$ and K_m^D (Table 1). Note that the values of $\lambda_{E_{ox(max)}}^{D}$ are practically the same for L and D. However, $K_m^{D(L)} < K_m^{D(D)}$, thus, the enzyme shows stereospecificity in its substrate binding. These results agree with the values of k_{cat}^D obtained from a study of the steady state, $k_{cat}^{D(D)} \cong k_{cat}^{D(L)}$ [23], since these values and those of $\lambda_{E_{ox(max)}}^{D}$ are related with the strength of the nucleophilic attack of the oxygens of the hydroxyls of C-3 and C-4, which, in turn, depends on δ_3 and δ_4 (Table 1).

The inactivation mechanism described here agree with the experimental result obtained by other authors [27], regarding to the loss of a 50% of copper during the oxidation of catechol, and with others [26] regarding to the impossibility of inactive enzyme reactivation after Cu^{2+} addition, perhaps indicating the need for a "caddie" protein [29].

Table 2
Kinetic constants which characterize the inactivation of E_d, E_m and E_d^* by the dopa isomers.

Enzymatic form	Substrate	$K_E^{D(a)}$ (μM)	$k_{i_D} \times 10^3$ (min^{-1})	$k_{i_D}^* \times 10^3$ (min^{-1})
E_d^*	L-dopa	19.24 ± 2.12	–	0.25 ± 0.01
	D-dopa	28.88 ± 2.93	–	0.26 ± 0.02
E_d	L-dopa	10.77 ± 1.12	0.37 ± 0.01	–
	D-dopa	25.34 ± 2.24	0.37 ± 0.03	–
E_m	L-dopa	10.77 ± 1.12	0.37 ± 0.01	–
	D-dopa	25.34 ± 2.24	0.37 ± 0.03	–

(a) In K_E^D, *E* corresponds to the enzymatic forms E_d^* or E_d. When the reaction is begun with E_m, the data obtained correspond to E_d, because of the rapid transformation of E_m into E_d (Scheme VI).

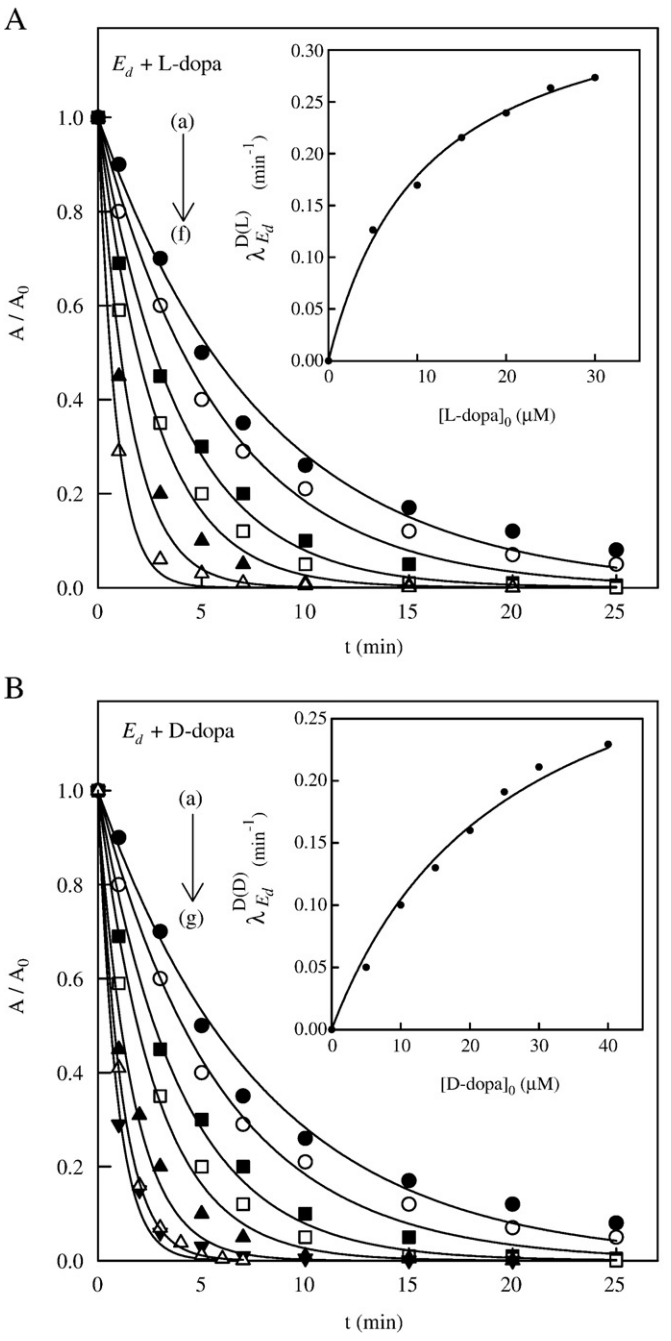
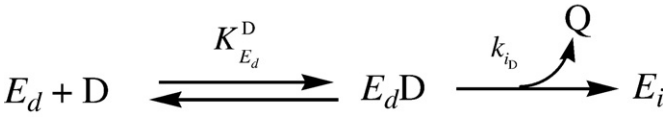
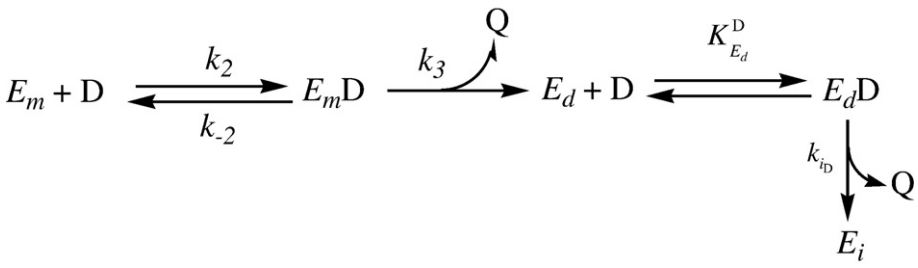


Fig. 6. Inactivation of the E_d form of tyrosinase by *o*-diphenols. The form E_d was obtained as is described in Fig. 4 and immediately incubated with *o*-diphenol, taking aliquots at different times to measure the residual activity with 2.5 mM L-dopa (λ = 475 nm). The values obtained were fitted to Eq. (2SM) and the apparent inactivation constant was obtained at each concentration of *o*-diphenol. (A) L-dopa. The experimental conditions were: 30 mM sodium phosphate buffer (pH 6.0), 25 °C, $[E]_0 = 0.1$ μM (see Materials and Methods sections), $[H_2O_2]_0 = 2$ μM and L-dopa (μM): (a) 5 (●), (b) 10 (○), (c) 15 (■), (d) 20 (□), (e) 25 (▲) and (f) 30 (△). Inset. Representation of $\lambda_{E_d}^{D(L)}$ *vs.* $[L\text{-dopa}]_0$. (B) D-dopa. The experimental conditions were the same as in (A) but D-dopa (μM): (a) 5 (●), (b) 10 (○), (c) 15 (■), (d) 20 (□), (e) 25 (▲), (f) 30 (△) and (g) 40 (▼). Inset. Representation of $\lambda_{E_d}^{D(L)}$ *vs.* $[D\text{-dopa}]_0$. In the case of E_m, the same procedure was followed as with E_d.



Scheme V. Kinetic mechanism of E_d inactivation by *o*-diphenols.



Scheme VI. Kinetic mechanism of E_m inactivation by *o*-diphenols.

On the other hand, the physiological implication of the suicide inactivation of tyrosinase by its physiological substrate (L-dopa) is not still clear, although it would result in a stop of action of the enzyme, since the products of its reaction are highly cytotoxic for the cell [46,47].

4.2. Inactivation of deoxy-tyrosinase by o-diphenols

The inactivation of E_d^*, E_d and E_m was studied in anaerobic conditions.

4.2.1. E_d^* inactivation by L-dopa and D-dopa

Analysis of the data of Fig. 5A and B according to Eq. (7) provides the apparent inactivation constant of E_d^* and its analysis according to Eq. (8) provides the values of k_i^* and $K_{E_d^*}$. The values of $k^*_{i_{D(L)}}$ and $k^*_{i_{D(D)}}$ are approximately equal (Table 2), which agrees with the values of δ_3 and δ_4 of the isomers (Table 1). However, the values of $K_{E_d^*}^{D(L)}$ and $K_{E_d^*}^{D(D)}$ are not the same (Table 2), ($K_{E_d^*}^{D(L)} < K_{E_d^*}^{D(D)}$), which indicates that this enzymatic form has more affinity for the L isomers.

4.2.2. E_d inactivation by L-dopa and D-dopa

When we study the inactivation of E_d by L- or D-dopa, analysis of the data in Fig. 6A and B according to Eq. (1SM) provides the apparent inactivation constant, and its analysis according to Eq. (2SM) gives the values k_i and K_{E_d}. The values of $k_{i_{D(L)}}$ and $k_{i_{D(D)}}$ are, approximately, equal (Table 2), since these values are directly related with the values of δ_3 and δ_4 of both isomers, which are equal (Table 1). However, $K_{E_d}^{D(L)} < K_{E_d}^{D(D)}$, which demonstrates the stereospecificity of the substrate binding. Furthermore, the values of $k_{i_{D(L)}}$ and $k_{i_{D(D)}}$ are greater than those of $k^*_{i_{D(L)}}$ and $k^*_{i_{D(D)}}$, respectively (Table 2), indicating that the structure of the enzyme in the form E_d^* transforms the substrate less efficiently than the form E_d, perhaps because the Cu^+ atoms in E_d^* are more separated [44]. As regards the affinity of these forms for the substrates, the E_d form shows greater affinity (lower $K_{E_d}^{D(L)}$ and $K_{E_d}^{D(D)}$) than E_d^* (Table 2).

4.3. E_m inactivation by L- or D-dopa

When the inactivation of E_m by L- or D-dopa is studied, the results are similar to those obtained in the study of the inactivation of E_d and E_d^* by the same *o*-diphenols. According to the kinetic mechanism described in Scheme VI and the structural mechanism described in Scheme IIISM, the enzyme E_m rapidly passes to E_d and is inactivated (Scheme VI and Scheme IIISM). The values of the constants are shown in Table 2.

4.4. Oxygen protects against the inactivation of tyrosinase

From the results obtained in this study and, especially, taking into account the inactivation processes, suicide inactivation (under aerobic conditions) occurs through E_{ox} and irreversible inactivation (under anaerobic conditions) occurs through E_m, E_d and E_d^*, both with *o*-diphenols. It is clear that the oxygen protects the enzyme from inactivation, since the value of $\lambda^D_{E_{ox(max)}}$ (maximum apparent inactivation constant) in suicide inactivation (Table 1) is lower than the inactivation constants of *Em*, *Ed* and E_d^* under anaerobic conditions (Table 2). This observation may have industrial applications since a nitrogen stream passed quickly through a solution of a fruit extract would prevent the enzyme from acting, the presence of *o*-diphenols provoking its inactivation. In this way, browning would be avoided. Besides, irreversible inactivation has the advantage over suicide inactivation because there is no turnover, so the product would be stoichiometric with the enzyme.

4.5. Conclusions

From a kinetic study of the inactivation of tyrosinase in the different forms involved in the catalytic cycle, E_{ox} (aerobic conditions), *Em*, *Ed* and E_d^* (anaerobic conditions), it can be deduced that the enzyme shows stereospecificity in the binding of isomers but not in catalysis or inactivation, since both processes are related with the nucleophilic power of the oxygens of the hydroxyls in C-3 and C-4 of the benzene ring. *o*-Diphenols inactivate the enzyme both under aerobic conditions (suicide inactivation through the form E_{ox}) and under anaerobic conditions (irreversible inactivation through the forms E_m, E_d and E_d^*).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2010.02.015.

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