Suicide Inactivation of the Diphenolase and Monophenolase Activities of Tyrosinase

ARTICLE *in* INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY LIFE · JULY 2010

Impact Factor: 3.14 · DOI: 10.1002/iub.348 · Source: PubMed

IUBMB *Life*, 62(7): 539–547, July 2010

Critical Review

Suicide Inactivation of the Diphenolase and Monophenolase Activities of Tyrosinase

Jose Luis Muñoz-Muñoz[1], Francisco Garcia-Molina[1], Ramon Varon[2], Pedro A. Garcia-Ruíz[3], Jose Tudela[1], Francisco Garcia-Cánovas[1] and Jose Neptuno Rodríguez-López[1]

[1]*GENZ: Grupo de Investigación Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biologia, Universidad de Murcia, Espinardo, Murcia, Spain*

[2]*Departamento de Química-Física, Escuela de Ingenieros Industriales de Albacete, Universidad de Castilla la Mancha, Avda. España s/n, Campus Universitario, Albacete, Spain*

[3]*QCBA: Grupo de Química de Carbohidratos y Biotecnología de Alimentos, Departamento de Química Orgánica, Facultad de Química, Universidad de Murcia, Espinardo, Murcia, Spain*

Summary

The suicide inactivation mechanism of tyrosinase acting on its phenolic substrates has been studied. Kinetic analysis of the proposed mechanism during the transition phase provides explicit analytical expressions for the concentrations of *o*-quinone *versus* time. The electronic, steric, and hydrophobic effects of the phenolic substrates influence the enzymatic reaction, increasing the catalytic speed by three orders of magnitude and the inactivation by one order of magnitude. To explain this suicide inactivation, we propose a mechanism in which the enzymatic form oxy-tyrosinase is responsible for the inactivation. In this mechanism, the rate constant of the reaction would be directly related with the strength of the nucleophilic attack of the C-1 hydroxyl group, which depends on the chemical shift of the carbon C-1 (δ_1) obtained by ^{13}C-NMR. The suicide inactivation would occur if the C-2 hydroxyl group transferred the proton to the protonated peroxide, which would again act as a general base. In this case, the coplanarity between the copper atom, the oxygen of the C-1 and the ring would only permit the oxidation/reduction of one copper atom, giving rise to copper (0), hydrogen peroxide, and an *o*-quinone, which would be released, thus inactivating the enzyme. One possible application of this property could be the use of these suicide substrates as skin depigmenting agents.

IUBMB *Life*, 62(7): 539–547, 2010

Keywords mechanism-based inhibitors; suicide; inactivation; polyphenol oxidase; *o*-diphenols.

Received 2 March 2010; accepted 23 April 2010

Address correspondence to: Francisco Garcia-Cánovas, Facultad de Biologia, Campus de Espinardo, E-30100 Espinardo, Murcia, Spain. Tel: +34-968-364782. Fax: +34-968-363963. E-mail: canovasf@um.es

ISSN 1521-6543 print/ISSN 1521-6551 online
DOI: 10.1002/iub.00348

INTRODUCTION

Skin, hair, and eye pigmentation has been long and widely studied (*1*). The process is caused by melanins, heterogeneous polymers with a complex molecular structure, whose color ranges from yellow to black (*1*). Melanins are formed and accumulated in specialized organelles called melanosomes, which are to be found in cells known as melanocytes (*2*). Melanins are polymers that are formed in a series of enzymatic and non-enzymatic reactions from L-tyrosine and L-dopa. The melanin formation pathway (melanogenesis) includes chemical and enzymatic reactions (*1*).

Tyrosinase or polyphenol oxidase (EC 1.14.18.1) is widely distributed through the animal, vegetal, bacterial, and fungal kingdoms (*3, 4*). Tyrosinase catalyses two types of reactions on phenolic substrates, in which molecular oxygen intervenes: (*a*) the *ortho*-hydroxylation of the 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*).

Besides oxidizing their natural substrates (L-tyrosine and L-dopa), tyrosinase has a wide substrate specificity on monophenols (α-methyl-L-tyrosine, L-tyrosine methyl ester, etc.) and *o*-diphenols (dopamine, catechol, etc.) (*5*).

Tyrosinase undergoes an inactivation process when it reacts with their phenolic substrates, a phenomenon that has long been known in the case of enzymes from a variety of natural sources, including fungi, plant, and animals (*5–11*). The refining of spectrophotometric methods has allowed a kinetic study of the suicide inactivation of tyrosinase during its action on *o*-diphenols and triphenols (*7, 12, 13*). The study of enzymatic inactivation by suicide substrates or mechanism-based inhibitors is of

growing importance, because of possible pharmacological applications, for studying enzymatic mechanisms and designing new drugs (*14*). As 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.

The main aim of kinetic studies of 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), and K_m^S (Michaelis constant for the substrate) (*7–9*).

The kinetic mechanism of tyrosinase acting on monophenols and *o*-diphenols has been the object of several studies and has been reviewed (*15*). However, the suicide inactivation process of the enzyme is less well known, and so the purpose of this review is to summarize the kinetic studies on the suicide inactivation of tyrosinase acting on their different kinds of substrates, to propose reaction mechanism and look at possible physiological implications and applications.

KINETIC ANALYSIS OF THE SUICIDE INACTIVATION OF TYROSINASE

The kinetic mechanism proposed to explain the suicide inactivation of tyrosinase acting on *o*-diphenols is based on the structural mechanism proposed in Scheme 1 (*7*).

The derivation of the analytical expression, which establishes the accumulation of the product (Q) with time, when the initial substrate concentration $[S]_0$, is much higher than the initial concentration of enzyme $[E]_0$ and is described in detail in Supplementary material section of (*7*) (http://www.biochemj.org/bj/416/bj4160431add.htm).

The variation of $[Q]$ with time is given by Eq. (1) [see Supplementary material of (*7*)]:

$$[Q] = [Q]_\infty(1 - e^{-\lambda_{E_{ox}}t}) = \frac{V_0}{\lambda_{E_{ox}}}(1 - e^{-\lambda_{E_{ox}}t}) = \frac{V_{max}}{\lambda_{E_{ox}(max)}}(1 - e^{-\lambda_{E_{ox}}t}), \quad (1)$$

where $[Q]_\infty$ is the concentration of product accumulated at the end of the reaction, $\lambda_{E_{ox}}$ is the apparent inactivation constant of the E_{ox} form, $\lambda_{E_{ox}}(max)$ is the maximum value of the apparent inactivation constant, V_0 is the initial velocity, and V_{max} is the maximum velocity. When $t \to \infty$:

$$[Q]_\infty = \frac{2k_{cat}}{\lambda_{E_{ox}(max)}}[E]_0 = 2r[E_0], \quad (2)$$

where k_{cat} is the catalytic constant.

Bearing in mind that $K_m^{O_2}$ values are very low (*16*), and that the initial oxygen concentration is 0.26 mM, the enzyme is saturated with O_2, and from Eqs. (S8) and (S9) of (*7*), we obtain:

$$\lambda_{E_{ox}} = \frac{\lambda_{E_{ox}(max)}[S]_0}{K_m^S + [S]_0} \quad (3)$$

and

$$V_0 = \frac{2k_{cat}[S]_0[E]_0}{K_m^S + [S]_0} \quad (4)$$

When the disappearance of oxygen is measured:

$$[O_2] = [O_2]_f + [O_2]_\infty e^{-\lambda_{E_{ox}}t}, \quad (5)$$

where $[O_2]$ is the instantaneous concentration of oxygen, $[O_2]_f$ is the oxygen remaining at $t \to \infty$, and $[O_2]_\infty$ is the concentration of O_2 consumed at end of the reaction.

As *o*-quinones are unstable, reducing agents, such as nicotinamide adenine dinucleotide (NADH) or ascorbic acid (AH_2), can be used, and the corresponding equations for measuring the disappearance of NADH or AH_2 are as follows:

$$[NADH] = [NADH]_f + [NADH]_\infty e^{-\lambda_{E_{ox}}t}, \quad (6)$$

where [NADH] is the instantaneous concentration of NADH, $[NADH]_f$ is the NADH value at $t \to \infty$, and $[NADH]_\infty$ is the NADH consumed at the end of the reaction ($t \to \infty$). If the disappearance of AH_2 is measured, we obtain:

$$[AH_2] = [AH_2]_f + [AH_2]_\infty e^{-\lambda_{E_{ox}}t}, \quad (7)$$

where $[AH_2]$ is the instantaneous concentration of AH_2, $[AH_2]_f$ is the AH_2 value at $t \to \infty$, and $[AH_2]_\infty$ is the AH_2 consumed at the end of the reaction ($t \to \infty$).

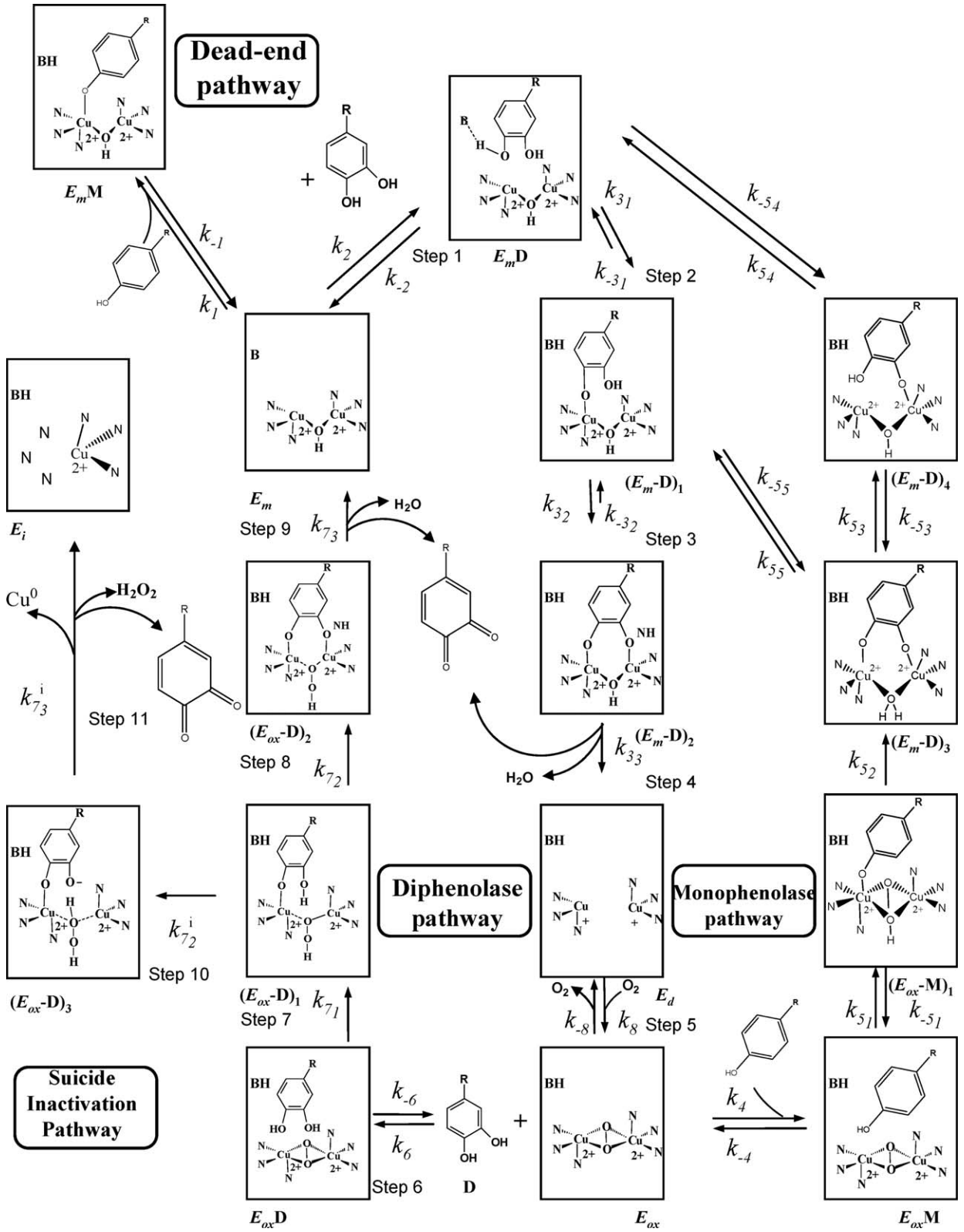
Experimental Design

The experimental study of the kinetics of suicide substrates or mechanism-based enzyme inhibitors is important for obtaining reliable kinetic parameters ($\lambda_{E_{ox}(max)}$, K_m^S, and r) that will permit any *in vitro* information gained to be extrapolated to *in vivo* situations (*17*).

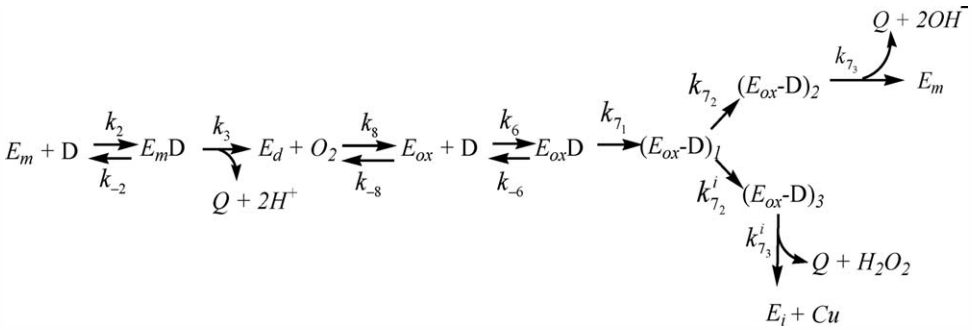
In a previous study, we outlined the methodology necessary for kinetically characterizing a suicide substrate following the reaction continuously (*9*). In this work, we apply the experimental design described in (*9*) to the suicide inactivation of tyrosinase as it acts on *o*-diphenols since, although the products obtained, *o*-quinones, are unstable, they can be reduced by NADH or AH_2 and the disappearance of these coupled reagents can be measured. Alternatively, the consumption of oxygen can be measured in an oxygraph because oxygen is also a substrate of the enzyme.

SUICIDE INACTIVATION OF TYROSINASE ACTING ON DIPHENOLIC SUBSTRATES

Numerous models on the tyrosinase reaction mechanism have appeared over the last 3 decades to explain the characteris-



Scheme 1. Structural mechanism proposed to explain the diphenolase, monophenolase, and suicide inactivation pathways of tyrosinase acting on monophenols and *o*-diphenols. E_m, met-tyrosinase; E_d, deoxy-tyrosinase; E_{ox}, oxy-tyrosinase; E_{ox}D, oxy-tyrosinase/*o*-diphenol complex; $(E_{ox}\text{-D})_1$, oxy-tyrosinase/*o*-diphenol complex axially bound to a Cu atom; $(E_{ox}\text{-D})_2$, oxy-tyrosinase/*o*-diphenol complex axially bound to the two Cu atoms; E_mD, met-tyrosinase/*o*-diphenol complex; $(E_m\text{-D})_1$, met-tyrosinase/*o*-diphenol complex axially bound to a Cu atom; $(E_m\text{-D})_2$, met-tyrosinase/*o*-diphenol complex axially bound to the two Cu atoms; $(E_m\text{-D})_3$, met-tyrosinase/*o*-diphenol complex axial-equatorially bound to the two Cu atoms; $(E_m\text{-D})_4$, met-tyrosinase/*o*-diphenol complex equatorially bound to a Cu atom; E_mM, met-tyrosinase/monophenol complex; E_{ox}M, oxy-tyrosinase/monophenol complex; $(E_{ox}\text{-M})_1$, oxy-tyrosinase/monophenol complex axially bound to a Cu atom. $(E_{ox}\text{-D})_3$, oxy-tyrosinase/*o*-diphenol complex axially bound to one Cu atom and the deprotonated hydroxyl group of C-3; E_i, inactive form of tyrosinase.



Scheme 2. Kinetic mechanism proposed to explain the suicide inactivation of tyrosinase acting on *o*-diphenols (see Scheme 1 for further details).

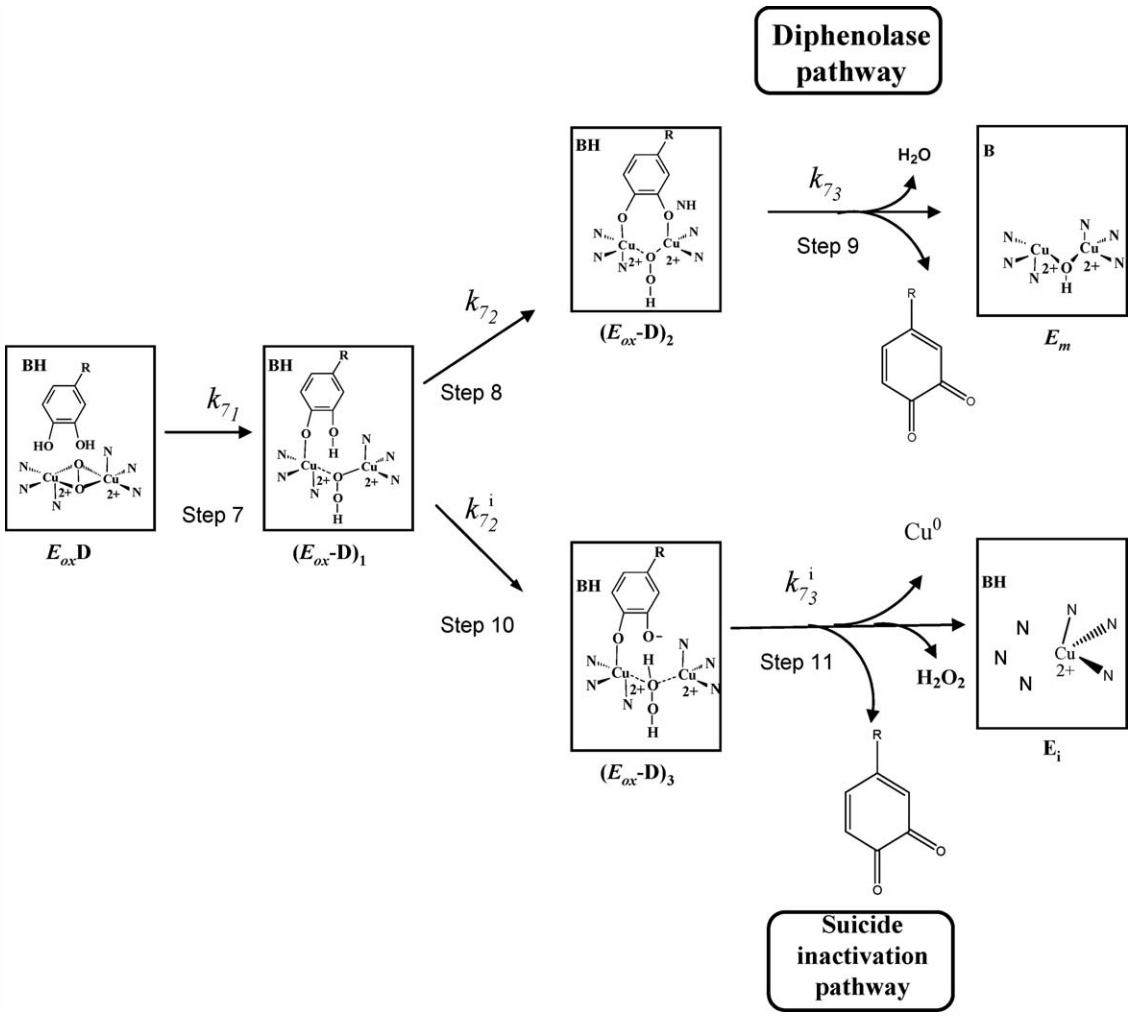
tics of monophenolase and diphenolase activities of tyrosinase, and, in particular, to clarify the presence of the lag period in its monophenolase activity (*15*). Our group has published a revision of the kinetic mechanism of tyrosinase activity (*15*) based on the structural aspects of the enzyme described by Dietler and Lerch (*4*) and Solomon et al. (*3*).

The catalytic cycle of tyrosinase acting on monophenols and *o*-diphenols is described in Scheme 1 (*15, 18*). The process of tyrosinase suicide inactivation has long been known, and we recently proposed a kinetic and structural mechanism to explain it (*7*).

When the enzyme acts on *o*-diphenols (Schemes 2 and 3), the suicide inactivation process is evident. The binding of the *o*-diphenol to the oxy form of the enzyme may begin with the transfer of an H^+ of the OH group in C-1 to the peroxide, while the phenolate attacks the copper (CuB) (*19*) (E_{ox}D). From this complex, two possibilities can exist: (*a*) the oxygen of the hydroxyl group of C-2 may attack the other copper atom (CuA), which is drawn closer following protonation of the peroxide, transferring the proton to the histidine (*19*), binding to the substrate diaxially and coplanar with the copper atoms. In this way, the concerted redox reaction (Step 9) can occur, releasing *o*-quinone and E_m. (*b*) The hydroxyl group of C-2 may cede a proton to the protonated peroxide, remaining as $C—O^-$ (Step 10). The coplanarity of the substrate ring, the oxygen of C-1 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). Note that the observation concerning the possibility of a bidentated union of the *o*-diphenol with one copper atom may also lead to suicide inactivation (*20*). Axial/equatorial-type bidentated structures have also been proposed for the binding of *o*-diphenols to copper (but only one copper) and have been seen by X-ray diffraction to be coplanar (*21*). However, the axial/equatorial binding to one copper atom in tyrosinase is not coplanar, and so the oxidation/reduction reaction cannot take place. For this reason, Steps 10 and 11 have been proposed in the mechanism of Schemes 2 and 3. The inactivation mechanism is consistent with the experimental observation that 50% of the copper is lost from the active site during catechol inactivation (*4*) and also with the experiments carried out (*22*) concerning the impossibility of reactivating the inactivated enzyme by adding Cu^{2+}, perhaps indicating the need for a "caddie" protein (*19*).

From the data shown in Table 1, the parallelism between the maximum apparent inactivation constant ($\lambda_{E_{ox}}$(max)) and the catalytic constant (k_{cat}) can be seen (Table 1), confirming the kinetic analysis as both are related by Eqs. (5)–(7). In this way, the best substrates, such as pyrogallol, catechol, 4-chlorocatechol, and 4-methylcatechol (with hydrogen or donating group in C-4), show higher catalytic (k_{cat}) and suicide inactivation ($\lambda_{E_{ox}(max)}$) constants, whereas the worst substrates, such as protocatechuic acid, protocatechuic aldehyde, and 4-nitrocatechol (with an electron-withdrawing group in C-4), have a low k_{cat} and $\lambda_{E_{ox}(max)}$. It should be noted that the electronic, steric, and hydrophobic effects on substrate–enzyme binding and on the inactivation and catalysis processes affect the catalysis (constants around 10^3) and suicide inactivation (constants around 10) processes. Note how pyrogallol inactivates the enzyme in the same way as the *o*-diphenols; furthermore, as it is the best substrate of the enzyme (greater k_{cat}), the $\lambda_{E_{ox}(max)}$ is also greater (Table 1).

The parameter $r = k_{7_2}/k^i_{7_2}$ (k_{7_2} and $k^i_{7_2}$ are the rate constants indicated in Scheme 2) indicates the probability that the hydroxyl group of C-2 will carry out a nucleophilic attack on the second copper (CuA), transferring the proton to the histidine and taking up a diaxial position (see Scheme 3). This assumption is derived from the flexible feature of the residue histidine previously proposed for *Streptomyces castaneoglobisporus* tyrosinase (*19*). Initially, it was proposed that the histidine bound axially to the E_{ox} form was His^{54}, although, subsequently, this axial position was attributed to His^{63} (*23, 24*). Another alternative, perhaps, is that it transfers the proton to the protonated peroxide, remaining in position C-1 and axially bound to the copper and to C-2 as $C—O^-$, a step favored by the charge density of the copper atom bound to the substrate. When this has a C-4 electron-donating group, the positive charge density in the



Scheme 3. Detail of the structural mechanism proposed to explain the suicide inactivation of tyrosinase during its action on *o*-diphenols (see Scheme 1 for further details).

copper will be lower, and the proton will be transferred to the histidine. In this way, the hydroxyl group in C-2 will be better able to carry out the nucleophilic attack on the copper, and the proton will be transferred to the histidine, binding to the substrate in a diaxial position. When the substrate has a withdrawing group in C-4, the positive charge density of the copper atom will be greater, influencing the hydroxyl group of C-2 and favoring deprotonation, the proton being transferred to the protonated peroxide (see Scheme 3). In addition, hydrophobic and esteric effects influence both process (catalysis and inactivation) (Table 1).

In the first case, the catalysis involves a concerted oxidation/reduction reaction, releasing *o*-quinone and E_m as the oxygens are coplanar with the two copper atoms. However, in the second case, the oxidation/reduction may take place on the one copper atom, releasing *o*-quinone, copper (0), hydrogen peroxide, and inactive enzyme.

Table 1 shows the values of k_{cat}, r, $\lambda_{E_{ox}(max)}$, and of the chemical shifts of the different suicide substrates obtained by ^{13}C-NMR for the C-1 and C-2 carbons. Note how the values of r decrease as the group in C-4 becomes more electron-withdrawing because of closer values of k_{7_2} and $k^i_{7_2}$ (the lower corresponds to 4-nitrocatechol). It is important to note the high relation, in some cases, between the ^{13}C-NMR data compared with the values of k_{cat} and $\lambda_{E_{ox}(max)}$ shown in Table 1. In the case of pyrogallol, the electron density of the oxygen atom of the hydroxyl group of C-1 is very high and has the highest k_{cat} and $\lambda_{E_{ox}(max)}$ values. However, the value of r is also at its maximum for all the substrates studied, which can be explained by the greater influence of the value of δ_1 on k_{cat} (10^3 range) than on $\lambda_{E_{ox}(max)}$ (10 range). Furthermore, as this compound is vicinal trihydroxylated, the probability of catalysis taking place is doubled as there are two possible *o*-diphenol orientations. According to Scheme 3, in the complex $(E_{ox}\text{-D})_r$, with the sub-

Table 1
Kinetic constants which characterize the suicide inactivation of tyrosinase by different substrates and values of the chemical shifts of the different suicide substrates of tyrosinase obtained by ^{13}C-NMR for the C-1 and C-2 carbons

o-Diphenol	$\lambda_{E_{ox}(max)} \times 10^3$ (s^{-1})	$r = k^S_{cat}/\lambda_{E_{ox}(max)}$	k^S_{cat} (s^{-1})	K^S_m (mM)	$K^{O_2}_m$ (μM)	δ^7_1 (ppm)	δ^7_2 (ppm)
Pyrogallol	12.01 ± 0.31	122,093 ± 2,740	1280.3 ± 47.1	1.96 ± 0.03	55.61 ± 2.81	135.19	148.22
Catechol	8.92 ± 0.27	99,994 ± 1,604	874.1 ± 30.2	0.16 ± 0.01	38.02 ± 2.61	146.59	146.59
4-Chlorocatechol	8.54 ± 0.25	99,356 ± 1,788	859.2 ± 28.3	0.60 ± 0.03	37.31 ± 1.62	145.04	147.35
4-Methylcatechol	8.21 ± 0.25	98,366 ± 1,377	842.1 ± 26.1	0.10 ± 0.01	36.62 ± 2.70	144.06	146.43
4-Ethylcatechol	7.95 ± 0.23	97,562 ± 1,951	802.3 ± 26.1	0.17 ± 0.01	34.83 ± 2.63	143.99	146.27
4-*tert*-Butylcatechol	7.28 ± 0.28	88,788 ± 1,864	640.1 ± 28.1	1.45 ± 0.12	27.82 ± 2.52	144.09	146.24
DHPPA	6.93 ± 0.27	87,646 ± 1,315	607.2 ± 25.1	0.70 ± 0.09	26.43 ± 2.13	144.96	146.43
DHPAA	6.89 ± 0.37	62,838 ± 1,187	433.1 ± 25.9	1.30 ± 0.10	18.82 ± 1.31	144.61	146.51
L-Dopa	2.21 ± 0.11	46,133 ± 2,306	102.6 ± 20.1	0.55 ± 0.08	4.46 ± 0.89	146.92	146.06
D-Dopa	2.15 ± 0.15	44,895 ± 2,573	98.5 ± 17.0	3.98 ± 0.57	4.28 ± 0.86	146.92	146.06
Gallic acid methyl ester	1.70 ± 0.09	47,444 ± 501	80.2 ± 3.9	0.12 ± 0.01	3.41 ± 0.11	141.42	147.34
Gallic acid	1.57 ± 0.07	18,666 ± 466	28.2 ± 0.9	0.18 ± 0.01	1.12 ± 0.11	140.64	147.29
Protocatechuic acid	0.85 ± 0.03	10,186 ± 224	8.1 ± 0.3	0.07 ± 0.01	0.34 ± 0.02	150.00	146.04
Protocatechuic aldehyde	0.61 ± 0.02	2,554 ± 40	1.5 ± 0.1	0.15 ± 0.02	0.06 ± 0.01	155.28	146.71
4-Nitrocatechol	0.45 ± 0.02	2,254 ± 41	0.9 ± 0.1	0.10 ± 0.01	0.04 ± 0.01	155.87	148.07

strate bound axially to the copper by C-1 (which has a lower positive charge density), the adjacent hydroxyl group has a greater possibility to bind to the second copper, transferring its proton to the histidine (*7*).

Catechol has no steric or hydrophobic effects that hinder the process, even though the values of δ_1 and δ_2 are not good; however, as the OH groups are equivalent, the probability of catalysis and inactivation increases. The value of r is high, since, as in the previous case, the effect on k_{cat} is much higher than on $\lambda_{E_{ox}(max)}$, favoring the catalytic pathway. The group of substrates, 4-chlorocatechol, 4-methylcatechol, and 4-ethylcatechol, with their very close δ_1 values, gives similar values of k_{cat}, $\lambda_{E_{ox}(max)}$, and r (although in the case of 4-ethylcatechol, steric effects exist). The effect of the donating group in C-4 is responsible for the powerful nucleophilic attack of the hydroxyl group in C-1. In the case of 4-*tert*-butylcatechol, although its δ_1 is similar to that of catechol, the size of the group in C-4 may cause steric hindrance, which decreases its k_{cat}, $\lambda_{E_{ox}(max)}$, and r.

As regards 3,4-dihydroxyphenylpropionic acid (DHPPA) and 3,4-dihydroxyphenylacetic acid (DHPAA), although their δ_1 are better than that of catechol, the effect of the negative charge of the carboxylic group decreases the k_{cat} and $\lambda_{E_{ox}(max)}$. Gallic acid and gallic acid methyl ester are trihydroxylated compounds with low δ_1 values, although the withdrawing effect of the carboxyl group means that their k_{cat} and $\lambda_{E_{ox}(max)}$ are low, as is their r. The withdrawing effect increases the possibility of the copper being bound to the substrate, bringing the second hydroxyl group in C-2 nearer, inducing deprotonation and proton transfer to the protonated peroxide, facilitating enzyme suicide. Lastly, poor tyrosinase substrates, such as protocatechuic acid, protocatechuic aldehyde, and 4-nitrocatechol, whose δ_1 are very high, order themselves in accordance with the values described in Table 1. Their very low values of k_{cat}, $\lambda_{E_{ox}(max)}$, and r can be explained by the same withdrawing effect of the group in C-4 (*7*).

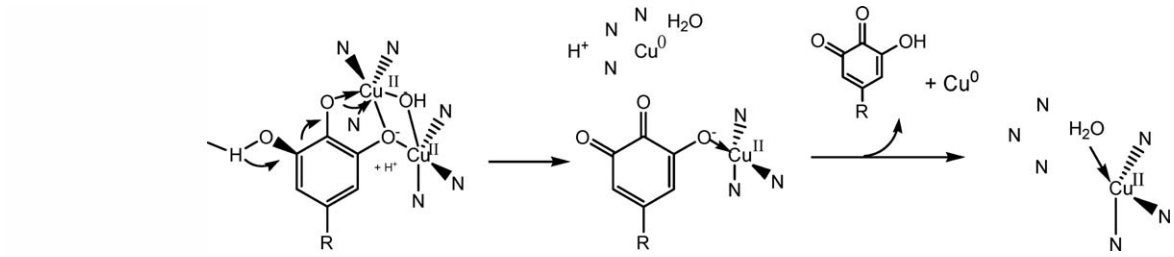
Similarly, several studies have compared the stereospecific effects of the isomers, L- and D-dopa, and calculated their respective suicide inactivation parameters (*8*). From Table 1, it can be seen that the suicide inactivation process shows no specificity as regards $\lambda_{E_{ox}(max)}$, but does so with respect to K^S_m. This agrees with studies published on the substrate stereospecificity of tyrosinase in its action on monophenols and *o*-diphenols (*12*), as both L- and D-dopa show the same δ_1 and δ_2 (Table 1).

SUICIDE INACTIVATION OF TYROSINASE ACTING ON MONOPHENOLS

As regards the monophenolase activity, the enzymatic forms E_m and E_{ox} can bind to the monophenol, giving rise to the complexes E_m-M (inactive) and E_{ox}-M (active), respectively. The kinetic mechanism for the monophenolase activity is described in (*15, 18*).

The suicide inactivation kinetic of tyrosinase in its action on monophenols such as L-tyrosine has not been studied in depth for two reasons: (*a*) the presence of a lag period in the monophenolase activity, and (*b*) experimentally, there is no possibility that the phenolic substrate will remain constant through the reaction time.

The suicide inactivation kinetic of tyrosinase has been studied in the presence of L-tyrosine (results not published) from an experimental point of view and through simulation of the set of differential equations that make up the mechanism of Scheme 1. The overall results of the study show that the enzyme is not inactivated when it acts on monophenols (L-tyrosine), a hydrox-



Scheme 4. Proposed model to explain the suicide inactivation of tyrosinase during its monophenolase activity when it acts on *o*-diphenols (*22*).

ylation reaction, but is inactivated as it acts on the L-dopa accumulated in the medium as a result of the chemical evolution of *o*-dopaquinone (Scheme 2). Thus, L-tyrosine does not directly inactivate the enzyme (suicide inactivation).

PROPOSED MECHANISMS TO EXPLAIN THE SUICIDE INACTIVATION OF TYROSINASE ACTING ON MONOPHENOLIC AND DIPHENOLIC SUBSTRATES

To date, three types of mechanism have been proposed to explain the suicide inactivation of tyrosinase:

(a) an attack by the *o*-quinone product on a sensitive nucleophilic group vicinal to the active site (*25*),
(b) a free radical attack on the active site by the reactive oxygen species generated during the catalytic oxidation (*10*), and, more recently,
(c) the processing of a catechol as though it was a monophenol ("cresolase"-type presentation) (*22, 26, 27*) (Scheme 4).

The first two hypotheses have been rejected because the inactivation takes place even in the presence of NADH or ascorbic acid whose presence prevents the *o*-quinone binding to the enzyme (*4*). Experiments that try to protect the enzyme from the action of free radicals were also unsuccessful (*4, 28*). Neither was it possible to mark the enzyme in the process of suicide inactivation in experiments using [U-C^{14}]phenol (*5*).

As regards the third hypothesis, which implies the hydroxylation of a catechol as though it was a monophenol ("cresolase"-type presentation), the authors (*22, 26, 27*) suggested that an intermediate product is formed that undergoes deprotonation and reductive elimination; this assumes that the orientation of the phenyl ring in the enzyme–substrate complex is approximately orthogonal to the plane defined by the copper and oxygen atoms (*23*).

Moreover, the inactivation mechanism proposed in (*22, 26, 27*) indicates that the chemical structure of the substrates is very important in the suicide inactivation process. Therefore, the substrate must have at least two hydroxyl groups in ortho in the benzene ring. Hence, when the substrate is a trihydroxybenzene (*e.g.*, pyrogallol), the proposed mechanism (Scheme 4) would indicate that this compound is not a suicide substrate (*22*), although our experimental results (*7*) suggest that it is the most powerful suicide substrate of mushroom tyrosinase. The oxygen consumption that is observed is due to autooxidation (*7*), not to the action of the enzyme. Indeed, the addition of superoxide dismutase and catalase to the medium inhibits this autooxidation process, underlining that pyrogallol is really a suicide substrate.

In a more recent work, Ramsden et al. (*26*), to support its suicide inactivation mechanism, used the compound 3,6-difluorocatechol. As this has positions 3 and 6 occupied, this compound would prevent the presentation of the *o*-diphenol as a monophenol and so, in the absence of hydroxylation, the enzyme is not inactivated. The experiments depicted in Fig. 1 of reference (*26*) compare, by means of oxymetric measurements, the inactivation of the enzyme acting on 3,6-difluorocatechol with the inactivation of 4-fluorocatechol (which has positions 3 and 6 free and could present itself to the enzyme as a monophenol). According to the model of these authors, 3,6-difluorocatechol would not produce suicide inactivation. If the experiments with 4-fluorocatechol and 3,6-difluorocatechol are fitted on the same time scale, it seems that 3,6-difluorocatechol does not inactivate the enzyme. We studied the suicide inactivation of the enzyme acting on 3-isopropyl-6-methylcatechol, which is available commercially and has a chemical structure which is similar to 3,6-difluorocatechol. According to the mechanism proposed in (*22*), 3-isopropyl-6-methylcatechol should not inactivate the enzyme, but we have showed that this substrate does indeed inactivate tyrosinase (results not published), although the inactivation constant ($\lambda^{D}_{E_{ox}}$) is 3×10^{-4} s^{-1} when the substrate concentration is 4 mM, indicating that the suicide inactivation provoked by the substrate is very slow (results not published). The experimental observation of this process would require a longer reaction time than that recorded (15 min) by other authors (*22, 26, 27*) to study inactivation by 3,6-difluorocatechol, Fig. 1 of reference (*26*). Note the smaller value of the suicide inactivation constant, which agrees with the chemical structure of the substrate, since, having groups in positions 3 and 6, the value of K^{D}_{m} is very high (4.9 mM). However, the suicide inactivation of tyrosinase by this compound (3-isopropyl-6-methylcatechol) has been studied (results not published).

From these data, it can be concluded that positions 3 and 6 of a diphenol do not necessarily have to be free for suicide inactivation to occur.

It is well known that some enzymes exist, named catechol oxidases, that do not have hydroxylase activity, and these would in principle be suitable for confirming or rejecting the mechanism proposed in (*22*) to explain the suicide inactivation of tyrosinase. The catechol oxidases studied to date in this respect are those of *Ipomoea batatas*, *Lycopus europaeus, Populus nigra*, and *Aspergyllus orizae* (*29–31*). In a recent work (*27*), the banana enzyme (*Musa cavendishii*) was used to confirm the mechanism proposed in (*22*), because it was claimed that it had no monophenolase activity. However, hydroxylase activity has been observed in this enzyme (*32*) along with its suicide inactivation acting on catechol (*11*). These results show that the mechanism proposed in (c) (*22*) does not explain the experimental results, but that the mechanism described in (*7*) would. Furthermore, this mechanism (*7*) would explain the suicide inactivation described in other activities of tyrosinase, such as ascorbate oxidase (*33*).

PHARMACOLOGICAL IMPORTANCE OF SUICIDE SUBSTRATES

The study of suicide inactivation and irreversible inhibition is important in the functional design of synthetic inactivators for therapeutic application. For example, irreversible inactivators (insecticides) have been designed against acetylcholinesterase and suicide substrates of β-lactamases (clavulanic acid) that help the action of antibiotics (amoxycilin) (*14*).

In mammals, the suicide inactivation of tyrosinase by L-dopa is significant when there is a considerable decrease in the concentration of L-tyrosine. This may be due to the decreased biosynthesis of L-tyrosine in situations of melanocyte dysfunction, such as in tissue lesions of the skin. In such cases, the melanosomes may have deteriorated, so that they release oxidizing melanogens, which are cytotoxic for the melanocyte (*34, 35*). Suicide inactivation of tyrosinase by L-dopa would protect the skin from the damage caused by the melanogens outside their physiological compartment.

As regards the application of the suicide substrates of tyrosinase, the depigmenting activity of some suicide substrates has been studied. More specifically, 8-hydroxydaidzein, which has been described as a powerful suicide substrate of tyrosinase (*36*), has demonstrated its depigmenting activity in both mouse melanoma cells and in human volunteers (*37*).

In conclusion, we have reviewed the mechanism and kinetic of the suicide inactivation of tyrosinase as it acts on different types of substrate. From the kinetic analysis, an experimental design is proposed that enables the calculation of the kinetic parameters and constants that characterize a suicide substrate of tyrosinase. This information can be used in an attempt to inhibit browning of fruits and vegetables and to evaluate the depigmenting activity of these compounds.

ACKNOWLEDGEMENTS

This article 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, Consejería de Educación (CARM, Murcia, Spain) BIO-BMC 06/01-0004, and the Consejería de Salud y Bienestar Social de la Junta de Comunidades de Castilla La Mancha, project FISCAM PI-2007/53. JLMM has a fellowship from the Ministerio de Educación y Ciencia (Madrid, Spain) Reference AP2005-4721. FGM has a fellowship from Fundación Caja Murcia (Murcia, Spain).

REFERENCES

1. Prota, G., d'Ischia, M., and Napolitano, A. (1998) The chemistry of melanins and related metabolites. In *The Pigmentary System, Physiology and Pathology*. (Nordlund, J. J.,Boissy, R. E.,Hearing, V. J.,King, R. A., and Ortonne, J. P., eds.). pp. 307–332, Oxford University Press, Oxford.
2. Yamaguchi, Y. and Hearing, V. J. (2009) Physiological factors that regulate skin pigmentation. *Biofactors* **35**, 193–199.
3. Solomon, E. I., Sundaram, U. M., and Machonkin, T. E. (1996) Multicopper oxidases and oxygenases. *Chem. Rev.* **96**, 2563–2606.
4. Dietler, C. and Lerch, K. (1982) Reaction inactivation of tyrosinase. In *Oxidases and Related Redox Systems*. (King, T. E.,Mason, H. S., and Morrison, M., eds.). pp. 305–317, Pergamon Press, New York.
5. Canovas, F. G., Tudela, J., Madrid, C. M., Varon, R., Carmona, F. G., and Lozano, J. A. (1987) Kinetic study on the suicide inactivation of tyrosinase induced by catechol. *Biochim. Biophys. Acta* **912**, 417–423.
6. Tudela, J., Garcia-Canovas, F., Varon, R., Jimenez, M., Garcia-Carmona, F., and Lozano, J. A. (1987) Kinetic characterization of dopamine as a suicide substrate of tyrosinase. *J. Enzyme Inhib.* **2**, 47–56.
7. Muñoz-Muñoz, J. L., Garcia-Molina, F., Garcia-Ruiz, P. A., Molina-Alarcon, M., Tudela, J., Garcia-Canovas, F., and Rodriguez-Lopez, J. N. (2008) Phenolic substrates and suicide inactivation of tyrosinase: kinetics and mechanism. *Biochem. J.* **416**, 413–440.
8. Muñoz-Muñoz, J. L., Acosta-Motos, J. R., Garcia-Molina, F., Varon, R., Garcia-Ruíz, P. A., Tudela, J., Garcia-Cánovas, F., and Rodríguez-López, J. N. Tyrosinase inactivation in its action on L-dopa. *Biochim. Biophys. Acta* **1804**, 1467–1475.
9. Garcia-Canovas, F., Tudela, J., Varon, R., and Vazquez, A. M. (1989) Experimental methods for kinetic study of suicide substrates. *J. Enzyme Inhib.* **3**, 81–90.
10. Seiji, M., Sasaki, M., and Tomita, Y. (1978) Nature of tyrosinase inactivation in melanosomes. *Tohoku J. Exp. Med.* **125**, 233–245.
11. Padron, M. P., Lozano, J. A., and Gonzalez, A. G. (1975) Properties of o-diphenol:O_2 oxidoreductase from *Musa cavendishii*. *Phytochemistry* **14**, 1959–1963.
12. Espin, J. C., Garcia-Ruiz, P. A., Tudela, J., and Garcia-Canovas, F. (2000) Study of stereospecificity in mushroom tyrosinase. *Biochem. J.* **331**, 547–551.
13. Garcia-Molina, F., Muñoz, J. L., Varon, R., Rodriguez-Lopez, J. N., Garcia-Canovas, F., and Tudela, J. (2007) A review on spectrophotometric methods for measuring the monophenolase and diphenolase activities of tyrosinase. *J. Agric. Food Chem.* **55**, 9739–9749.
14. Silverman, R. B. (1995) Mechanism-based enzyme inactivators. *Methods Enzymol.* **249**, 240–283.
15. Sanchez-Ferrer, A., Rodriguez-López, J. N., García-Cánovas, F., and García-Carmona, F. (1995) Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1247**, 1–11.
16. Fenoll, L. G., Rodriguez-Lopez, J. N., Garcia-Molina, F., Garcia-Canovas, F., and Tudela, J. (2002) Michaelis constants of mushroom tyrosin-

ase with respect to oxygen in the presence of monophenols and diphenols. *Int. J. Biochem. Cell Biol.* **34**, 332–336.
17. Ghanbari, F., Rowland-Yeo, K., Bloomer, J. C., Clarke, S. E., Lennard, M. S., Tucker, G. T., and Rostami-Hodjegan, A. (2006) A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition with implications for in vitro-in vivo extrapolation. *Curr. Drug Metab.* **7**, 315–334.
18. Rodriguez-Lopez, J. N., Fenoll, L. G., Peñalver, M. J., Garcia-Ruiz, P. A., Varon, R., Martinez-Ortiz, F., Garcia-Canovas, F., and Tudela, J. (2001) Tyrosinase action on monophenols: evidence for direct enzymatic release of o-diphenol. *Biochim. Biophys. Acta* **1548**, 238–256.
19. Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., and Sugiyama, M. (2006) Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *J. Biol. Chem.* **281**, 8981–8990.
20. Decker, H., Schweikardt, T., Nillius, Dd., Salzbrunn, U., Jaenicke, E., and Tuczek, F. (2007) Similar enzyme activation and catalysis in hemocyanins and tyrosinases. *Gene* **398**, 183–191.
21. Speier, G., Tisza, S., Tyeklar, Z., Lange, C. W., and Pierpont, C. G. (1994) Coligand-dependent shifts in charge-distribution for copper-complexes containing 3,5-di-tert-butylcatecholate and 3,5-di-tert-butylsemiquinonate ligands. *Inorg. Chem.* **33**, 2041–2045.
22. Land, E. J., Ramsden, C. A., and Riley, P. A. (2007) The mechanism of suicide-inactivation of tyrosinase: a substrate structure investigation. *Tohoku J. Exp. Med.* **212**, 341–348.
23. Decker, H., Schweikardt, T., and Tuczek, F. (2006) The first crystal structure of tyrosinase: all questions answered? *Angew. Chem. Int. Ed.* **45**, 4546–4550.
24. Deeth, R. J. and Diedrich, C. (2010) Structural and mechanistic insights into the oxy form of tyrosinase from molecular dynamics simulations. *J. Biol. Inorg. Chem.* **15**, 117–129.
25. Ingraham, L. L., Corse, J., and Makower, B. (1952) Enzymatic browning of fruits. III. Kinetics of the reaction inactivation of polyphenol oxidase. *J. Am. Chem. Soc.* **74**, 2623–2626.
26. Ramsden, C. A., Stratford, M. R. L., and Riley, P. A. (2009) The influence of catechol structure on the suicide-inactivation of tyrosinase. *Org. Biomol. Chem.* **7**, 3388–3390.
27. Land, E. J., Ramsden, C. A., Riley, P. A., and Stratford, M. R. (2008) Evidence consistent with the requirement of cresolase activity for suicide inactivation of tyrosinase. *Tohoku J. Exp. Med.* **216**, 231–238.
28. Lerch, K. (1983) Neurospora tyrosinase: structural, spectroscopic and catalytic properties. *Mol. Cell. Biochem.* **52**, 125–138.
29. Klabunde, T., Eicken, C., Sachettini, J. C., and Krebs, B. (1998) Crystal structure of a plant catechol oxidase containing a dicopper center. *Nat. Struct. Biol.* **5**, 1084–1090.
30. Rompel, A., Fisher, H., Meiwes, D., Buldt-Karentzopoulos, K., Dillinger, R., Tuczek, F., Witzel, H., and Krebs, B. (1999) Purification and spectroscopic studies on catechol oxidase from *Lycopus europaeus* and *Populus nigra*: evidence for a dinuclear copper center of type and spectroscopic similarities to tyrosinase and hemocyanin. *J. Biol. Inorg. Chem.* **4**, 56–63.
31. Gasparetti, C., Faccio, G., Arvas, M., Buchert, J., Saloheimo, M., and Kruus, K. (2010) Discovery of a new tyrosinase-like enzyme family lacking a C-terminally processed domain: production and characterization of an *Aspergillus oryzae* catechol oxidase. *Appl. Microbiol. Biotechnol.* **86**, 213–226.
32. Padron, M. P., Gonzalez, A. G., and Lozano, J. A. (1974) O-diphenol:oxygen-oxidoreductase from *Musa cavendishii*. *Rev. Esp. Fisiol.* **30**, 167–176.
33. Munoz-Munoz, J. L., Garcia-Molina, F., Garcia-Ruiz, P. A., Varon, R., Tudela, J., Garcia-Canovas, F., and Rodriguez-Lopez, J. N. (2009) Stereospecific inactivation of tyrosinase by L- and D-ascorbic acid. *Biochim. Biophys. Acta* **1794**, 244–253.
34. Urabe, K., Aroca, P., Tsukamoto, K., Mascagna, D., Palumbo, A., Prota, G., and Hearing, V. (1994) The inherent toxicity of melanin precursors: a revision. *Biochim. Biophys. Acta* **1221**, 272–278.
35. Munoz-Munoz, J. L., Garcia-Molina, F., Varon, R., Tudela, J., Garcia-Canovas, F., and Rodriguez-Lopez, J. N. (2009) Generation of hydrogen peroxide in the melanin biosynthesis pathway. *Biochim. Biophys. Acta* **1794**, 1017–1029.
36. Chang, T. S. (2007) Two potent suicide substrates of mushroom tyrosinase. *J. Agric. Food Chem.* **55**, 2010–2015.
37. Tai, S. S.-K., Lin, C.-G., Wu, M.-H., Chang, T. S. (2009) Evaluation of depigmenting activity by 8-hydroxydaidzein in mouse B16 melanoma cells and human volunteers. *Int. J. Mol. Sci.* **10**, 4257–4266.