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RESEARCH ARTICLE

Suicide inactivation of tyrosinase in its action on tetrahydropterines

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Abstract

Tetrahydrobiopterin (BH_4), methyl-tetrahydropterin (MBH_4) and dimethyl-tetrahydropterin ($DMBH_4$) are oxidized by tyrosinase in a process during which the suicide inactivation of tyrosinase may occur. From the kinetic study of this process, $\lambda^{S_R}_{E_{ox}(max)}$ (apparent maximum constant for the suicide inactivation), $K^{S_R}_m$ (Michaelis constant for the substrate) and *r* (number of turnovers that the enzyme makes before the inactivation) can be obtained. From the results obtained, it can be deduced that the velocity of the inactivation governed by ($\lambda^{S_R}_{E_{ox}(max)}$) and the potency of the same ($\lambda^{S_R}_{E_{ox}(max)}/K^{S_R}_m$) follow the order: $BH_4 > MBH_4 > DMBH_4$.

Keywords: Tyrosinase, suicide inactivation, tetrahydropterines

Introduction

Tyrosinase or polyphenol oxidase (TYR; EC 1.14.18.1) is a widely distributed enzyme through the animal, vegetal, bacterial and fungal kingdoms[1].

In a recent work[2], we studied the effect of tetrahydropterines at micromolar concentrations on the monophenolase and diphenolase activities of tyrosinase and demonstrated that they are oxidized by *o*-dopaquinone and that the systems reach the steady state when all the tetrahydropterines have been exhausted[2]. Subsequently, we demonstrated that the effect of tetrahydropterines on the catalytic activities of tyrosinase can occur at three levels: (i) through non-enzymatic inhibition, the tetrahydropterines acting as reductants of *o*-dopaquinone, (ii) by acting as competitive substrates of TYR and (iii) by acting as irreversible inhibitors of the enzymatic forms deoxy- and met-tyrosinase[3].

Tyrosinase undergoes an inactivation process when it reacts with its phenolic substrates, a phenomenon that has long been known[4]. 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 for designing new drugs[4,5,6]. As regards the possible applications of the suicide substrates of tyrosinase, some of the substrates, such as 7,8,4´-trihydroxyisoflavone and 8-hydroxydaidzein, have been studied for their depigmenting activity, the latter in both mouse melanoma cells and in human volunteers[7].

Several mechanisms have been proposed to explain the suicide inactivation of tyrosinase [4,8,9].

The objective of this work was the kinetic characterisation of the suicide inactivation of tyrosinase as it acts on tetrahydrobiopterin (BH_4), methyl-tetrahydropterin (MBH_4) and dimethyl-tetrahydropterin ($DMBH_4$), and we propose a structural mechanism to explain the results.

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Material and methods

Reagents

L-tyrosine, 4-*tert*-butylcatechol (TBC), (6-R)-L-erythro-5,6,7,8-tetrahydro-biopterin dihydrochloride (BH_4), 6-(R,S)-methyl 5,6,7,8-tetrahydropterin monohydrochloride (MBH_4) and 6,7-(R,S)-dimethyl 5,6,7,8-tetrahydropterin monohydrochloride ($DMBH_4$) were purchased from Sigma (Madrid, Spain; Scheme 1). Stock solutions of the phenolic substrate were prepared in 0.15 mM phosphoric acid to prevent autooxidation. $6BH_4$, MBH_4 and $DMBH_4$ were prepared in 0.15 mM phosphoric acid, eliminating the oxygen by passing a current of nitrogen.

Enzyme source

Mushroom tyrosinase (TYR, 3300 U/mg) was purchased from Sigma (Madrid, Spain) and purified according to ref. (10). The enzyme concentration was calculated taking the value of M_r as 120,000. Protein content was determined by Bradford's method[11] using bovine serum albumin as standard. Superoxide dismutase (SOD, 4140 U/mg) was purchased from Sigma (Madrid, Spain).

Monophenolase activity of TYR

The monophenolase activity of TYR was followed spectrophotometrically, measuring the accumulation of dopachrome at a wavelength of 475 nm ($\varepsilon = 3500\ M^{-1}\ cm^{-1}$)[2] during the oxidation of L-tyrosine, using a Perkin-Elmer Lambda-35 spectrophotometer connected to a PC (Perkin-Elmer, North Billerica, MA). The conditions of the assay are specified in the corresponding figure legends.

Diphenolase activity of TYR

The diphenolase activity of TYR was followed spectrophotometrically, measuring the accumulation of dopachrome at a wavelength of 475 nm ($\varepsilon = 3500\ M^{-1}\ cm^{-1}$)[12] during the oxidation of L-dopa using the mentioned apparatus. The conditions of the assay are specified in the corresponding figure legends.

Kinetics of the suicide inactivation

The kinetics of the suicide inactivation of TYR in its action on tetrahydropterines, S_R, (BH_4, MBH_4 and $DMBH_4$) can be followed by measuring the formation of S_{ox} (BH_2, MBH_2 and $DMBH_2$), in a spectrophotometer. The assays were carried out in triplicate for each concentration of S_R and enzyme. These spectrophotometric assays were carried out as described above. The experimental data for the formation of S_{ox} (in the other cases is the same) with time follow the equation,

$$[S_{ox}] = [S_{ox}]_\infty (1 - e^{-\lambda_{E_{ox}}^{S_R} t}) \quad (1)$$

where $[S_{ox}]$ is the instantaneous concentration of S_{ox}, $[S_{ox}]_\infty$ is the S_{ox} formed at the end of the reaction, $t \mapsto \infty$, and $\lambda_{E_{ox}}^{S_R}$ the apparent inactivation constant for E_{ox} in the suicide inactivation of TYR under aerobic conditions.

Results and discussion

Under aerobic conditions, tetrahydropterines act as alternative substrates to the physiological substrates of the enzyme (L-tyrosine and L-dopa)[3]. Under such conditions, the tetrahydropterines may act as inactivators of TYR as a result of the enzyme's suicide inactivation as it acts on the substrates[4–8]. This suicide inactivation process is studied below during the action of TYR on BH_4, MBH_4 and $DMBH_4$.

Tyrosinase inactivation in its action on tetrahydropterines under aerobic conditions: suicide inactivation

The kinetic mechanism proposed to explain the suicide inactivation of tyrosinase acting on tetrahydropterines is described in Scheme 2:

When $[S_R]_0$, $[O_2]_0 >> [E]_o$ and $<< [S_{ox}]_\infty$ $[S_R]_0$, $[O_2]_0$, derivation of the analytical expression establishes the accumulation of the product (S_{Ox}) with time, as is detailed in ref. (4).

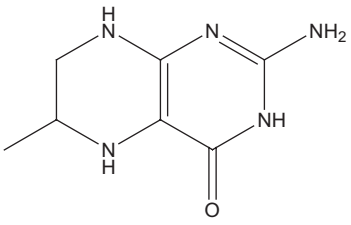
The variation of $[S_{ox}]$ with time is given by Eq. (2)[4].

$$[S_{ox}] = [S_{ox}]_\infty (1 - e^{-\lambda_{E_{ox}}^{SR} t}) \quad (2)$$

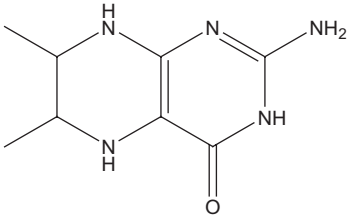
When $t \to \infty$, $[S_{ox}] = [S_{ox}]_\infty$ and according to (4),

$$[S_{ox}]_\infty = \frac{2k_{7_2}}{k_{7_2}^{i}}[E]_0 \quad (3)$$

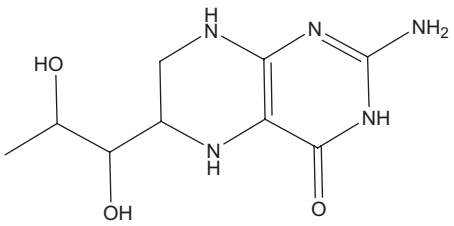
The velocity of the suicide inactivation is regulated by the apparent inactivation constant, $\lambda_{E_{ox}}^{S_R}$, whose expression is given by Eq. (4). Taking into account the saturation of tyrosinase by oxygen ($[O_2]_0 \to \infty$), thus according to (4),



(±)-6-Methyl-5,6,7,8-tetrahydropterine



6,7-Dimethyl-5,6,7,8-tetrahydropterine



(6R)-5,6,7,8-Tetrahydrobiopterin

Scheme 1. Chemical structures of the tetrahydropterines used in this work.

$$\lambda_{E_{ox}}^{S_R} = \frac{\lambda_{E_{ox}(max)}^{S_R}[S_R]_0}{K_m^{S_R} + [S_R]_0} \quad (4)$$

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

$$r = \frac{k_{12_2}}{k_{12_2}^i} = \frac{k_{cat}^{S_R}}{\lambda_{E_{ox}(max)}^{S_R}} = \frac{[S_{ox}]_\infty}{2[E]_0} \quad (5)$$

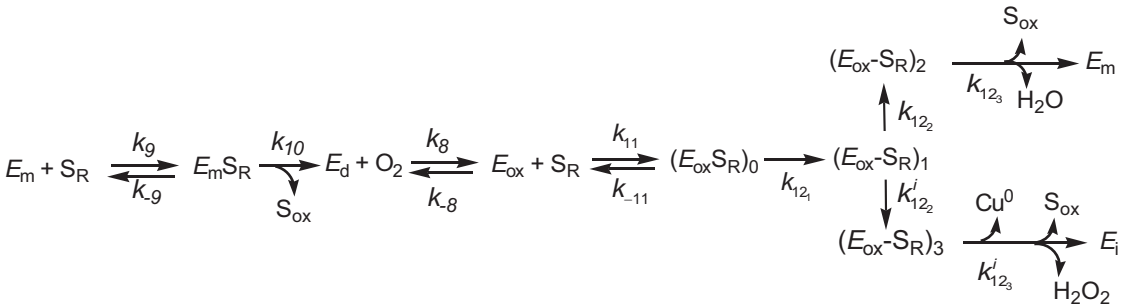
where k_{12_2} is the substrate binding constant to the copper atom through the C-3 hydroxyl (axial) and $k_{12_2}^i$ is the rate constant of transfer of a H^+ to the protonated peroxide (see Scheme 2 and supplementary Scheme 1SM).

Therefore, from Eq. (1–5), we can obtain the kinetic constants that characterise the kinetic behaviour of a suicide substrate: r, $\lambda_{E_{ox}(max)}^{S_R}$, $K_m^{S_R}$ and $K_{cat}^{S_R}$.

Experimental study of the suicide inactivation

The study of the suicide inactivation has been developed in three steps using BH_4, MBH_4 and $DMBH_4$ as substrates of TYR.

Step 1. The suicide inactivation kinetics is corrected when fitting to Eq. (1) so that the non-enzymatic oxidation of the substrates, BH_4, MBH_4 and $DMBH_4$ is eliminated (results not shown). These corrected recordings were analysed as described in the Kinetic Analysis section. By



Scheme 2. Kinetic mechanism to explain the pterin oxidase pathway and suicide inactivation pathway of tyrosinase in its action on tetrahydropterines.

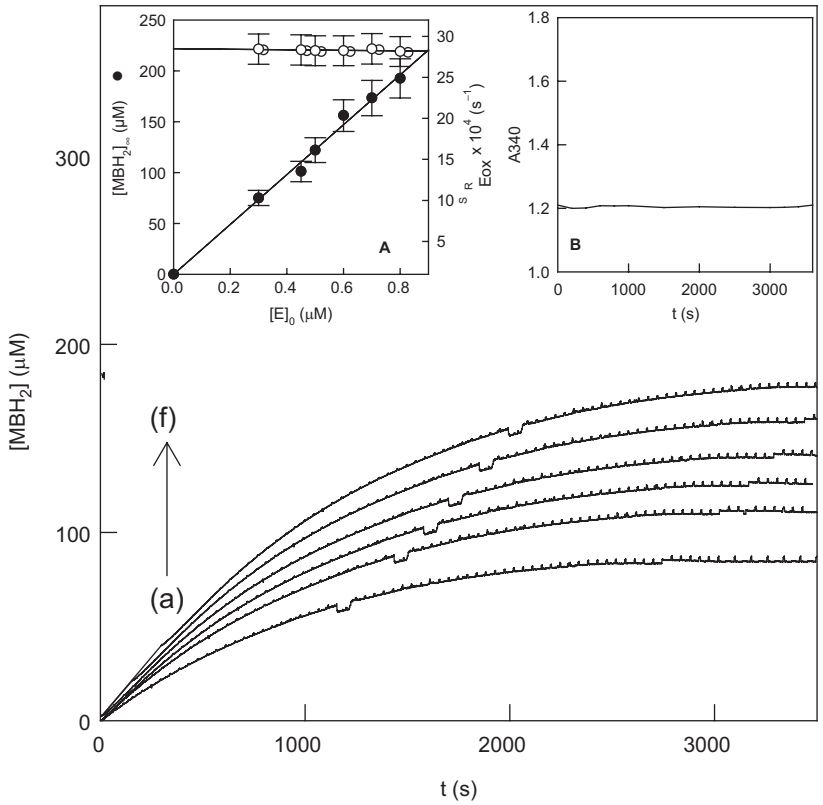


Figure 1. Corrected recordings of the appearance of MBH_2 in the suicide inactivation of tyrosinase (TYR) by MBH_4 for different enzyme concentrations. Conditions were 30 mM sodium phosphate buffer (pH 7.0), λ = 340 nm, 0.26 mM O_2, 0.5 mM $[MBH_4]_0$, 414 UI/m $[SOD]_0$, and $[E]_0$ (μM): (a) 0.3, (b) 0.45, (c) 0.5, (d) 0.6, (e) 0.7, and (f) 0.8. Inset A. Representation of the values of $[MBH_2]_\infty$ (•) and $\lambda_{E_{ox}}^{S_R}$ (O) vs. enzyme concentration. Inset B. Representation of absorbance at λ = 340 nm with time in the reaction of TYR on BH_2. The experimental conditions were 30 mM sodium phosphate buffer (pH = 7.0), 25°C, $[BH_2]_0$ = 0.2 mM and $[E]_0$ = 0.1 μM.

Table 1. Kinetic constants which characterize the suicide inactivation of tyrosinase by tetrahydropterines.

Substrate	$\lambda^{S_R}_{E_{ox}(max)} \times 10^3$ (s^{-1})	$r = k^{S_R}_{cat}/\lambda^{S_R}_{E_{ox}(max)}$	$k^{S_R}_{cat}$ (s^{-1})	$K^{S_R}_m$ * (mM)	$\lambda^{S_R}_{E_{ox}(max)}/K^{S_R}_m \times 10^3$ (s^{-1} mM^{-1})	$K^{O_2}_m$ (μM)
BH_4	0.035 ± 0.004	168 ± 19	5.98 ± 0.63	4.13 ± 0.87	8.51 ± 0.4	0.26 ± 0.03
MBH_4	0.026 ± 0.003	179 ± 22	4.29 ± 0.44	4.41 ± 0.91	5.47 ± 0.8	0.18 ± 0.02
$DMBH_4$	0.018 ± 0.003	199 ± 25	3.42 ± 0.32	5.01 ± 0.88	3.56 ± 0.7	0.14 ± 0.02

*Data taken from ref. (3).

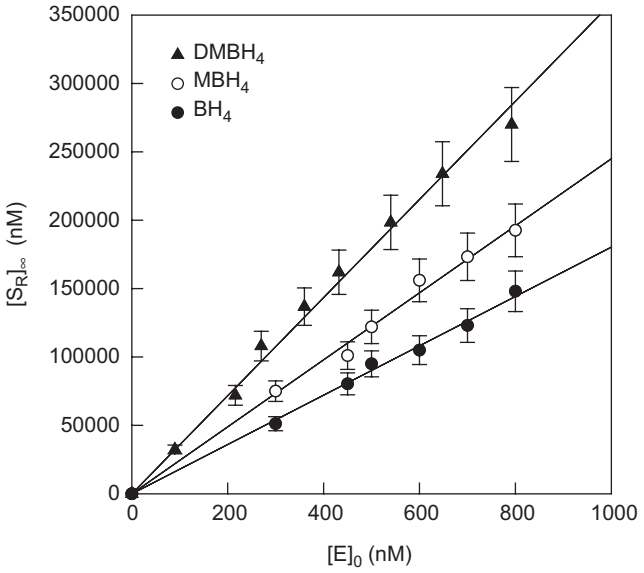


Figure 2. Representations of the values of $[S_{ox}]_\infty$ vs. enzyme concentration for the different substrates studied (BH4 •, MBH4 O and DMBH4 ▲). Experimental conditions were 30 mM sodium phosphate buffer (pH 7.0), 25°C and 414 UI/ml $[SOD]_0$. The substrates studied were recorded from the appearance of product. The initial concentration for every substrate was 0.8 mM. In every substrate, (—) linear regression fitting of the experimental data points.

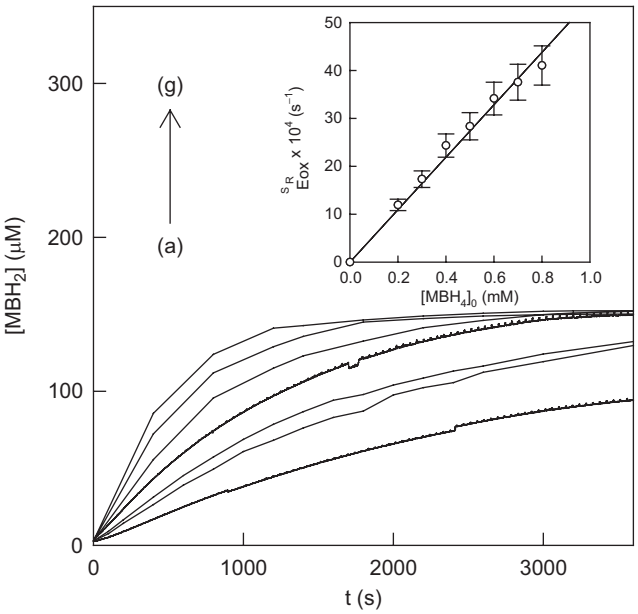


Figure 3. Corrected recordings of the appearance of MBH_2 in the suicide inactivation of TYR by different MBH_4 concentrations. Conditions were 30 mM sodium phosphate buffer (pH 7.0), 0.26 mM O_2, λ = 340 nm, 0.6 μM $[TYR]_0$, and 414 UI/ml $[SOD]_0$. The substrate concentrations were (mM): (a) 0.2, (b) 0.3, (c) 0.4, (d) 0.5, (e) 0.6, (f) 0.7, and (g) 0.8. Inset. Values of $\lambda^{S_R}_{E_{ox}}$ vs. $[MBH_2]_0$.

means of these preliminary studies, the concentration of enzyme was optimised so that $[S_{ox}]_\infty << [O_2]_0, [S_R]_0$.

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 Figure 1. The concentration of the substrate does not change significantly in these experiments so that $[S_{ox}]_\infty < 10\%$ $[S_R]_0$. However, the concentration of O_2 does change. In the case of tyrosinase, $K^{O_2}_m$ is very small (Table 1), which permits an oxygen consumption of more than 10%. Note how the values of $[MBH_2]_\infty$ are directly proportional to $[E]_0$, while $\lambda^{S_R}_{E_{ox}}$ does not vary with $[E]_0$ (Figure 1 Inset A). Figure 1 Inset B shows the stability of BH_2 with time, which demonstrates that the product of the reaction of TYR with BH_4 during the time that the suicide inactivation kinetics is being measured is stable.

Figure 2 shows the dependence of the values obtained for the product at the end of the reaction, $[S_{ox}]_\infty$ vs. $[E]_0$, from whose slopes, and according to Eq. (5), r can be determined for BH_4, MBH_4 and $DMBH_4$ (Table 1). Note that the value of r is greater in the case of $DMBH_4$ than in that of MBH_4 or BH_4, indicating that the enzyme needs to undergo a greater number of turnovers before inactivation when it acts on $DMBH_4$ than it does in the case of MBH_4 or BH_4.

Step 3. Variation in substrate concentration. The experimental recordings for the appearance of MBH_2 during the action of tyrosinase on MBH_4 obtained by varying the concentration of substrate are shown in Figure 3. Fitting by non-linear regression to Eq. (1) gives the apparent inactivation constant $\lambda^{S_R}_{E_{ox}}$. The dependence of $\lambda^{S_R}_{E_{ox}}$ vs. $[S_R]_0$ is shown in Figure 3 Inset. Due to the high degree of autooxidation of the tetrahydropterines and the long measuring times involved, it is not convenient to increase the substrate concentration by much, so that we shall work in the linear dependence zone, Figure 3 Inset. Fitting these data by linear regression according to Eq. (4) gives the relation $\lambda^{S_R}_{E_{ox}(max)}/K^{S_R}_m$ for each of the isomers (Figure 4 and Table 1). Bearing in mind the values of $K^{S_R}_m$ calculated from the initial velocity measurements obtained at short times[3], we can obtain the values of $\lambda^{S_R}_{E_{ox}(max)}$ and r (Table 1). Figure 4 represents the values of $\lambda^{S_R}_{E_{ox}}$ vs. $[S_R]_0$ for the substrates BH_4, MBH_4 and $DMBH_4$. Note how TYR is not saturated during its action on any tetrahydropterine (Figure 4). The value of the ratio $\lambda^{S_R}_{E_{ox}(max)}/K^{S_R}_m$ is $BH_4 > MBH_4 > DMBH_4$.

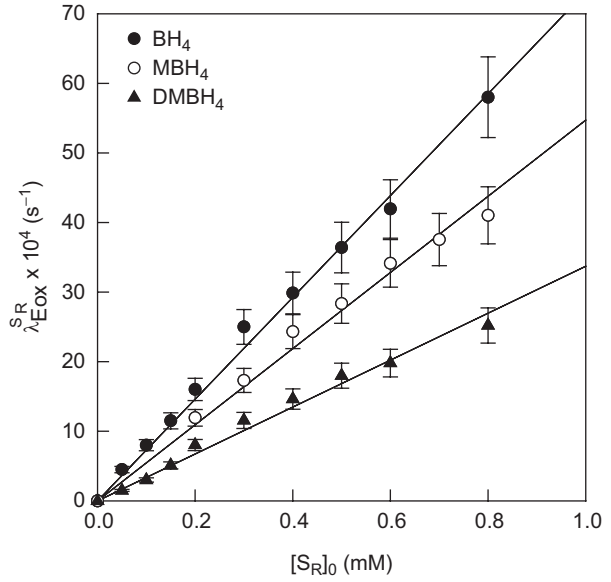


Figure 4. Representation of the values of the inactivation constant $\lambda^{S_R}_{E_{ox}}$ vs. $[S_R]_0$ for the different substrates studied. Conditions were 30 mM sodium phosphate buffer (pH 7.0), 25°C. Every substrates were recorded from the appearance of product (BH_2, MBH_2, and $DMBH_2$ for BH_4 •, MBH_4 O and $DMBH_4$ ▲, respectively). (—), Non-linear regression fitting to Eq. (4) of the data analysis. Enzyme concentration was, in each case, 0.6 μM. The initial concentration of superoxide dismutase (SOD) was 414 UI/ml.

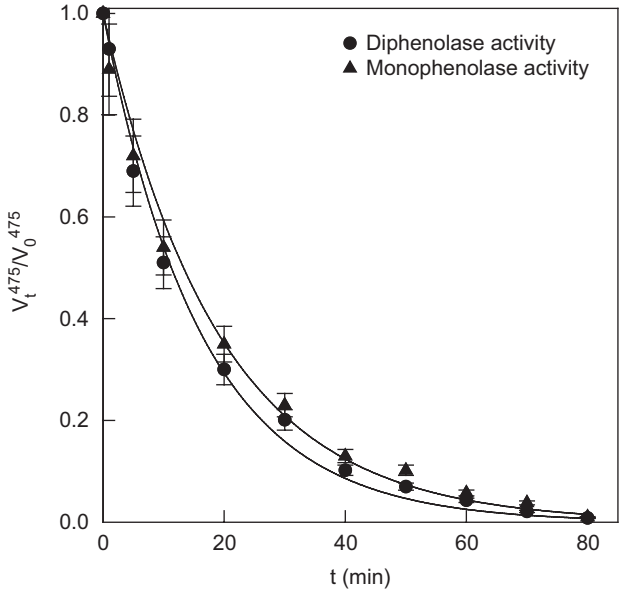


Figure 5. •Suicide inactivation kinetics of TYR in its action on $DMBH_4$. The process was followed by measuring the residual diphenolase activity of the enzyme with time. The experimental conditions were 30 mM sodium phosphate buffer (pH 7.0), $[O_2]_0 = 0.26$ mM, $[TYR]_0 = 0.61$ μM, $[SOD]_0 = 414$ UI/ml, and $[DMBH_4] = 0.8$ mM. Aliquots were taken at various times to measure the residual activity with 2.5 mM L-dopa (wavelength 475 nm). ▲Suicide inactivation kinetics of TYR in its action on $DMBH_2$. The process was followed by measurement of the residual monophenolase activity of the enzyme with time. The experimental conditions were 30 mM phosphate buffer (pH 7.0), $[O_2]_0 = 0.26$ mM, $[TYR]_0 = 0.61$ μM, $[SOD]_0 = 414$ UI/ml, and $[DMBH_4] = 0.8$ mM. Aliquots were taken at various times to measure the residual activity with 1 mM L-tyrosine and 46 μM L-DOPA (wavelength 475 nm). L-DOPA was added to eliminate the lag phase of the monophenolase activity of TYR.

In confirmation of the inactivation of tyrosinase as it acts on tetrahydropterines, Figure 5 shows the experimental values obtained for the residual diphenolase activity on TBC of the enzyme during the suicide inactivation of TYR with BH_4. This figure shows similar results obtained for monophenolase activity in its action on L-tyrosine. Note that the same value for the apparent inactivation constant, $\lambda^{S_R}_{E_{ox}}$, was obtained in both cases and the same as that in Figure 4 obtained with the same concentration of BH_4.

The inactivation mechanism, Scheme 2 and supplementary Scheme 1SM, is consistent with the experimental observation that 50% of the copper is lost from the active site during catechol inactivation[13], in the form of copper (0), and also with experiments carried out[8], concerning the impossibility of reactivating the inactivated enzyme by adding Cu^{2+}, which, in turn, suggests the need for a 'caddie' protein[14]. BH_4, MBH_4 and $DMBH_4$, therefore, are capable of irreversibly inactivating tyrosinase and may be useful as depigmenting agents or, at least, for designing the same.

Conclusions

In this article, the suicide inactivation process of tyrosinase acting on tetrahydropterines has been kinetically characterized. From the kinetic constants obtained, it can be deduced that the velocity of the inactivation governed by ($\lambda^{S_R}_{E_{ox}(max)}$) and its potency ($\lambda^{S_R}_{E_{ox}(max)}/K_m^{S_R}$) follow the order: $BH_4 > MBH_4 > DMBH_4$.

Acknowledgements

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