J. Enzyme Inhibition, 1987, Vol. 2, pp. 47–56
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KINETIC CHARACTERIZATION OF DOPAMINE AS A SUICIDE SUBSTRATE OF TYROSINASE

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(Received 5 January 1987)

A kinetic study of the inactivation of frog epidermis tyrosinase by a suicide substrate dopamine hydrochloride is described. The kinetic parameters and constants which characterize this reaction have been determined and the effects of pH and the stoichiometric inhibition by chloride have been considered.

KEY WORDS: Enzyme kinetics, suicide substrate, dopamine, tyrosinase.

INTRODUCTION

It has been established that the two major catabolic pathways of catecholamines are *O*-methylation and oxidative deamination.[1] Occurrence of a minor route in which the dihydric phenolic moieties are oxidized to the corresponding *o*-quinones is a further possibility. However, there is disagreement as to whether the products of oxidation are present or involved in physiological processes, as well as whether the reaction is part of a normal or an abnormal metabolic pathway.[2] The interest in studying this route increased on discovery of the antitumoral effect for malignant melanoma, *in vitro* as well as *in vivo* of catecholamines[3] and of dopamine in particular.[4] The enzyme tyrosinase has been detected in large amounts in malignant melanoma cells[5–7] and is the responsible for the antitumoral effects of the catecholamines by oxidising them into *o*-quinones which are very reactive with powerful nucleophile such as the thiol and amino groups of amino acids and proteins.[3,4,8–10]

In a previous paper we studied the oxidation of dopamine by tyrosinase, identifying the intermediates originated from the *o*-diphenolic substrate up to dopaminechrome.[11] This kinetic study was realized by using a spectrophotometer with a rapid response and capable of measuring on a seconds scale where the system apparently reaches the steady state.

However, measuring on a scale of several minutes, it is found that the initial steady state is breaking down and the activity is decreasing with time, the tyrosinase enzyme being inactivated whilst acting on the *o*-diphenolic substrates.[12] Thus, a transient phase is originated until the enzyme starts to lose activity in an irreversible manner.

The aim of the work described here was to determine by means of a kinetic study

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of the transient phase originated by the enzyme suicide inactivation, the parameters and kinetic constants which characterize the tyrosinase action on a suicide substrate such as dopamine. Also studied was the effect of pH and the inhibition by chloride on this reaction since this inhibitor is stoichiometric with the substrate, which is available in the hydrochloride form.

MATERIALS AND METHODS

Extraction and purification of frog epidermis tyrosinase has been previously described.[13] β-NADH was supplied by Sigma, St. Louis, Mo. (U.S.A.). [7-^{14}C]-dopamine was obtained from Amersham, Buckinghamshire (Great Britain). Dopamine HCl and other chemicals were of analytical grade and were purchased from E. Merck, Darmstadt (Fed. Rep. Ger.)

The suicide inactivation assays were conducted using a Perkin-Elmer Lambda-3 spectrophotometer, on line interfaced to a DS-3600 computer where the kinetic data were recorded, stored and later analyzed. The products of the enzyme reaction, the corresponding *o*-quinones cannot be experimentally detected during long assay periods due to their instability.[14,15] The assays were carried out in the presence of NADH as a reducing agent which, acting on *o*-quinones, regenerates *o*-diphenols and maintains their levels constant during the reaction. The course of the reaction was followed by measuring the disappearance of NADH at 340 nm, where $\varepsilon = 6230\,M^{-1}\,cm^{-1}$. This method yields a similar result to that obtained by using ascorbic acid,[14] although NADH allows better instrumental detection than ascorbic acid and also shows a lower spontaneous rate of oxidation by O_2 than ascorbic acid under the assay conditions used. The temperature used, 20 ± 0.1 °C, was regulated by means of a Hetofrig circulating bath equipped with a heater/cooler and controlled through a Cole-Parmer digital thermometer.

The effects of various reagents were studied. Their concentrations are given in the appropriate figures of the Results and Discussion section. In all assays, the following reagents were maintained constant: 0.26 mM O_2 (saturating), 0.4 mM NADH. Protein concentration was determined by the Hartree method.[17]

The radioactivity assays were carried out in a Intertechnic SL-30 liquid scintillation spectrometer using a scintillation fluid prepared from 100 g naphtalene, 4 g PPO, 0.2 g dimethyl-POPOP and 100 ml methanol, completed to 1:1 with dioxane. The retention of tyrosinase on a Sartorious filter was checked by measuring the enzyme activity of the filter and in the filtered solution. Tyrosinase (10 nmol) was incubated for 5 h. with [7-^{14}C] dopamine (2.68 nmol; specific activity 56 mCi/mmol) including 2.5 mM ascorbic acid and 0.1 M phosphate buffer, pH 6.0. The possible labelling of the enzyme was followed by measuring the radioactivity from the filter and from the filtered solution. The experimental data corresponding to the disappearance of NADH with time were fitted to the equation:

$$[NADH] = [NADH]_0 - P = [NADH]_0 - [c_1 + c_2 t + P_\infty(1 - e^{-\lambda t})] \quad (1)$$

where [NADH] is the concentration of this reagent remaining at each time t, $[NADH]_0$ is the initial concentration of reductant and [P] is the quantity of *o*-quinone formed in the reaction, which is equivalent to the concentration of the consumed NADH. Furthermore, c_1 represents the uncertainty on the zero time absorbance measurement, caused by enzyme addition at the start of the reaction, whereas c_2 corresponds to the

slow spontaneous oxidation of dopamine and NADH. The effects of the experimental factors c_1 and c_2 have been substracted by a computer in many NADH vs. time plots. P_∞ represents the quantity of accumulated product when $t \to \infty$, λ represents an exponential term constant. P_∞ is a function of the enzyme concentration and of the rate constants of the system, whereas λ is a function of the substrate concentration and of the rate constants.[18,19]

RESULTS AND DISCUSSION

In this section a series of steps have been carried out which allow determination of the kinetic parameters and constants that characterize the frog epidermis tyrosinase inactivation by the suicide substrate dopamine.

Inactivation curves

From the data fitting (Figure 1) to eqn. 1 by non-linear regression[20,21] and to equations with two, three and so on exponential terms, the corresponding values of the parameter χ^2, can be obtained and compared by the F test. This test is objective because it allows the equation providing the best fit to be determined.[22,23] The best fit has always corresponded to a uni-exponential instead of a multiexponential behaviour.

The kinetic parameters P_∞ and λ which characterize the suicide behaviour of the substrate are related to the rate constants and the reagent concentrations, E_0, S_0 and $[O_2]$ (as shown below and in Appendix).

Effect of E_0

This step consists firstly of choosing several E_0 values which give some P_∞ values so that the condition $P_\infty \ll S_0$ and $P_\infty \ll [O_2]$ is fulfilled. In this case, the dopamine

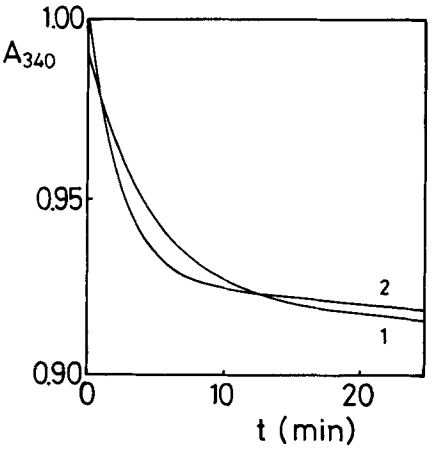


FIGURE 1 Experimental recordings of suicide inactivation of tyrosinase by dopamine. Conditions are as described in Materials and Methods and with: (1): 5.15 nM tyrosinase and 0.49 mM dopamine; (2) 5.42 nM tyrosinase and 4.91 mM dopamine, at pH 7.0.

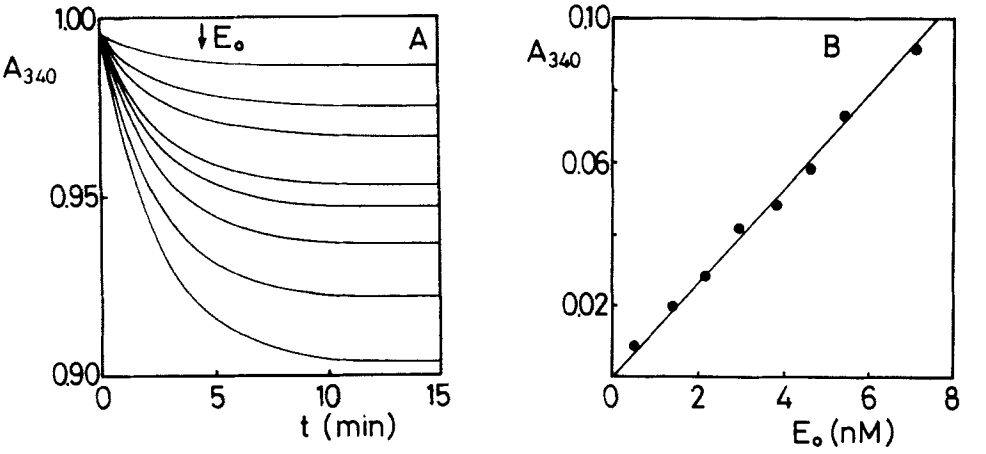


FIGURE 2 (A) Corrected recordings of suicide inactivation of tyrosinase by dopamine for different enzyme concentrations. Conditions are as described in Materials and Methods with 0.55–7.11 nM tyrosinase and 3 mM dopamine, at pH 7.0. (B) Corresponding values of P_∞ *vs.* E_0.

concentration is maintained constant by reduction of *o*-dopaminequinone with NADH. Therefore, the levels to the controlled are NADH and oxygen, the consumption of which were negligible in our assay conditions (see Figure 2A).

As shown in Figure 2A, where the recordings at different enzyme concentrations are presented (corrected with regard to the parameters c_1 and c_2), the fitting by non-linear regression according to eqn. 1 provides the parameters λ and P_∞, the first parameter being independent of the enzyme concentration and the second one directly proportional to it.

Taking into account that the definition of the partition ratio r is the number of turnovers given by one mol of enzyme before its inactivation, its value is P_∞/E_0^{24}. However, tyrosinase (see Scheme I in Appendix) gives two molecules of the product

TABLE I
Kinetic constants which characterize the suicide inactivation of tyrosinase by dopamine considering the effects of pH and of the inhibition by chloride

Constant	Value
r	1501 ± 49
λ_{max} (s^{-1})	(9.98 ± 0.33) × 10^{-3}
k_{cat} (s^{-1})	10.49 ± 0.34
K_S (mM)	0.65 ± 0.02
K'_S (mM)	7.23 ± 0.30
pK_{a1}	5.68 ± 0.05
pK_{a2}	4.63 ± 0.02
λ_{max}/K_S ($M^{-1}s^{-1}$)	15.35 ± 0.98
λ_{max}/K'_S ($M^{-1}s^{-1}$)	1.38 ± 0.10
$\lambda_{max}\cdot r/K_S$ ($M^{-1}s^{-1}$)	(16.14 ± 1.78) × 10^3
$\lambda_{max}\cdot r/K'_S$ ($M^{-1}s^{-1}$)	(1.45 ± 0.18) × 10^3
K_I (chloride) (mM)	0.11 ± 0.01

for each turnover and the partition ratio value is, in this case, $P_\infty/(2E_0)$. In Figure 2B the data obtained for P_∞ vs. enzyme concentration are plotted. A linear regression of these values allows the calculation of the slope (2r), which was much greater than one (see Table I). Since the partition ratio r is also defined as k_6/k_7 (see Scheme I in the Appendix), this value indicates that $k_6 \gg k_7$. Therefore, it is acceptable to consider that the catalytic route quickly reaches the steady state, and from thereon a transient phase is originated due to the irreversible inactivation of the enzyme, which is controlled by a slow step (k_7). For this reason, in the previous section the curves were fitted to only one significant exponential.

Effect of S_0

The application of the methods described in the former steps to assays carried out with different S_0 values, provides kinetic parameters (P_∞ and λ) for each value of S_0. The recordings are shown in Figure 3A. It is to be noted that the values of P_∞ are practically constant. Nevertheless the λ values vary in a hyperbolic manner with the substrate concentration, as is shown in Figure 3B. That is, the λ vs. S_0 values are fitted to an equation of the Michaelis type:

$$\lambda = aS_0/(b + S_0) \quad (2)$$

where a is the λ value when $S_0 \rightarrow \infty$ and b is equivalent to the necessary substrate concentration so that $\lambda = a/2$. The fitting by non-linear regression of data of λ *vs.* So according[20,21] to eqn. 3 allows the calculation of a and b (Figure 3B).

Effect of pH

This step consists of a series of studies at various pH values in a similar manner to those described in the above section. Thus, a parameter was obtained which was independent of pH, P_∞ and consequently r (see Table I). This indicates that the catalytic and inactivation steps (k_6 and k_7) do not depend on the protonation-

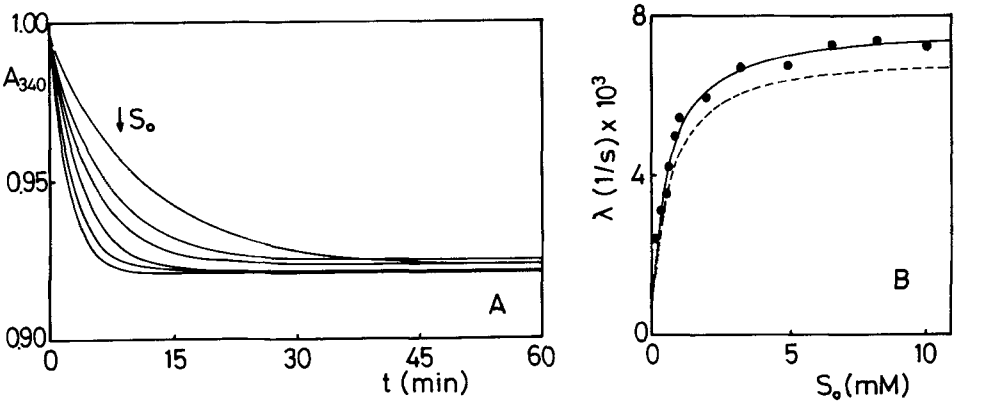


FIGURE 3 (A) Corrected recordings of suicide inactivation of tyrosinase by different concentrations of dopamine. Conditions are as described in Materials and Methods and with 5.47 nM tyrosinase and 0.1–10 mM dopamine, at pH 7.0. (B) Corresponding values of λ for different concentrations of substrate.

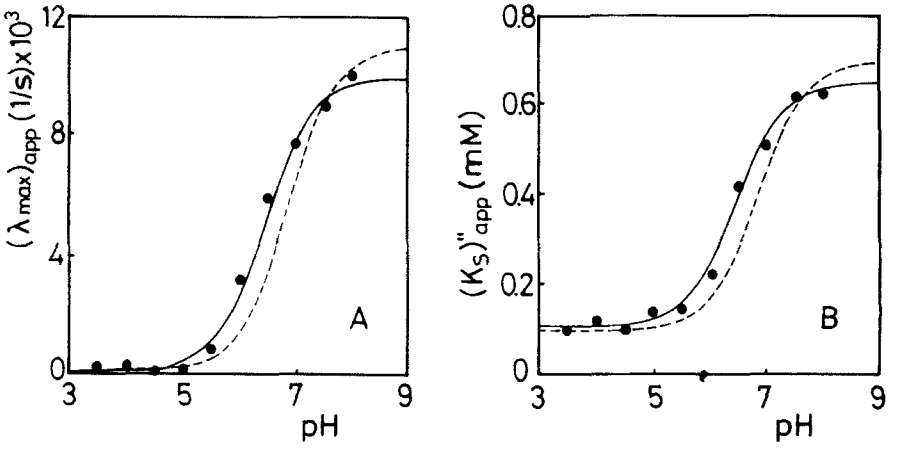


FIGURE 4 (A) Dependence of $(\lambda_{max})_{app}$ on pH. Conditions are as described in Materials and Methods and with 5.47 nM tyrosinase. (- - - -) Calculated values with initial estimations for data fitting, obtained from limits and inflexion point of eqn. 4. (——) Calculated values with final estimations from data fitting. (B) Dependence of $(K_S)''_{app}$ on pH. Conditions are as described in Materials and Methods and Figure 4A. (- - - -) Calculated values with initial estimations for data fitting, obtained from limits and inflexion point of eqn. 5. (——) Calculated values with final estimations from data fitting.

deprotonation of the enzyme. However, the affinity of tyrosinase for dopamine decreases whereas the rapidity of the inactivation process increases as the pH rises, (see Figures 4A and 4B).

Suicide inactivation of tyrosinase

In addition to the previous results the following considerations must be taken into account to provide a full picture of the events occurring during inactivation of the enzyme by dopamine hydrochloride. (a) A structure of the active site for *N. crassa* met-tyrosinase, including ligands between the two Cu(II) ions, has been proposed.[25] Protonation of frog epidermis tyrosinase has been attributed to a possible endogenous ligand.[26] (b) The oxidation of *o*-diphenol by met-tyrosinase has been proposed as the limiting step.[27] (c) Dopamine is available as the hydrochloride, and chloride inhibits the enzyme in a competitive way, an effect that increases in acidic pH.[26,28] Therefore, we propose the mechanism shown in Scheme I and analysed in detail in Appendix. Observe how eqn. 1 of the Materials and Methods corresponds to eqn. 3A where the expressions for P_x and λ are shown in eqns. 4A and 7A. Eqn. 2 of the text is equivalent to eqn. 7A.

Taking into account that the substrate dopamine is stoichiometric with the inhibitor chloride ([I] = [S]), from eqns. 7A–10A it follows that:

$$\lambda = (\lambda_{max})_{app} S_0 / ((Ks)''_{app} + S_0) \tag{3}$$

$$(\lambda_{max})_{app} + \frac{\lambda_{max} K_I K_{a2} + \lambda_{max} K_I 10^{-pH}}{K_I K_{a2} + (K'_S + K_I) 10^{-pH}} \tag{4}$$

$$(K_S)''_{app} = \frac{K'_S K_I K_{a1} + K'_S K_I 10^{-pH}}{K_I K_{a2} + (K'_S + K_I) 10^{-pH}} \tag{5}$$

An analysis by non-linear regression[20,21] of λ data *vs.* S_0 according to eqn. 3 at each

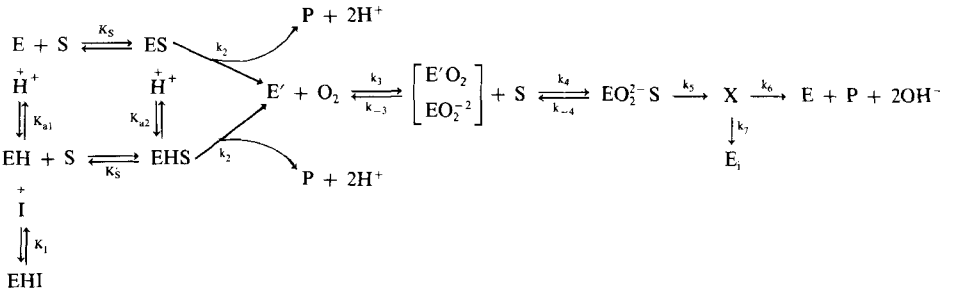
pH, yields the parameters $\lambda_{max})_{app}$ and $(K_S)''_{app}$. A new non-linear regression of these data *vs.* pH using eqns. 4 and 5 allows the calculation of λ_{max}, K_S, K'_S, K_{a1} and K_{a2} (see Table I), taking into account the relationship $K'_S/K_S = K_{a1}K_{a2}$ (Figures 4A and 4B). From λ_{max} the catalytic constant k_{cat} (see Table I) can be calculated according to eqn. 7A, since r was calculated in a previous step. From among the parameters λ_{max}, K_s and K'_s, the first one only gives information about the rapidity of the reaction, and the others about the affinity of the enzyme for the substrate. So, in order to compare two suicide substrates, the relationships λ_{max}/K_S and $\lambda_{max}\cdot r/K_S$ can be calculated to represent the efficiency of the inactivation and of the catalysis respectively (see Table I).

With regard to the molecular inactivation mechanism, the radiometric assays show the non-incorporation in the enzyme of labelled dopamine. Similar results regarding inactivation by [^{14}C] radiolabelled phenol and dopa, have been obtained for tyrosinase from *N. crassa*[29,30] and mouse melanoma,[31] respectively. Furthermore, modification of His-306 during inactivation of tyrosinase from *N. crassa* by catechol has been reported[29,30] which seems to indicate that the inactivation process does not occur in the first oxidation step (the one controlled by k_2 in Scheme I, see Appendix). This suggests that another reagent such as oxygen is necessary in order that the inactivation in the step controlled by k_7 can take place. Moreover, the addition of radical trapping agents, such as formate do not influence the inactivation. Similar results have been obtained for the inactivation of tyrosinase from mouse melanoma by L-dopa.[31] Therefore this process, if it is originated by the action of free radicals, can be internal in the active site as occurs with the *N. crassa* enzyme.[30]

In summary, the kinetic study of the transient phase described here allows determination of the kinetic parameters and constants which characterize the action of epidermis frog tyrosinase on a suicide substrate, or dopamine, either alone or in the presence of a stoichiometric inhibitor, chloride ion.

Appendix

Taking into account the experimental results obtained as a function of pH, the proposed mechanism is:



The mechanism shown in Scheme I reflects that the binding of substrate to the met-tyrosinase form is dependent on pH, this not being the case from the deoxy-tyrosinase form onwards. The inhibitor binds to the protonated form of the enzyme so that it competes with the substrate.

NOTATION AND DEFINITIONS:

Species and concentrations

E	Met-tyrosinase.
E′	Deoxy-tyrosinase.
EO_2^{2-}	Oxy-tyrosinase.
E_i	Inactive enzyme.
E_0	Initial concentration of tyrosinase.
S	*o*-Diphenol acting as suicide substrate.
S_0	Initial concentration of substrate S.
$[O_2]$	Initial concentration of molecular oxygen.
P	*o*-Quinone product originated from S.
I	Inhibitor (halide).
E_S	Set of enzyme species involved in a restricted steady state, Schemes I and II. $E_S = E + ES + E' + EO_2^{2-} + EO_2^{2-}S + X + EH + + EHS$.
f_x	Concentration factor corresponding to enzyme species X with regard to E_S.

Kinetic parameters

λ	Apparent inactivation constant.
P_∞	Concentration of P obtained at final time of reaction.
V_0	Initial rate of the catalytic route.

Kinetic constants

k_i	Rate constants of the catalytic route (i = 2–6)
k_7	Inactivation rate constant.
r	Partition ration, $r = k_6/k_7$.
K_S	Dissociation constant of the ES complex.
K_S'	Dissociation constant of the EHS complex.
K_{a1}	Dissociation constants of EH complex.
K_{a2}	Dissociation constants of ESH complex.
$(K_S)_{ap.}$	Dissociation constant of met-tyrosinase with regard to S in function of pH.
$(K_S)'_{app}$	Value of $(K_S)_{app}$ in presence of a competitive inhibitor I.
$(K_S)''_{app}$	Value of $(K_S)_{app}$ in presence of a competitive inhibitor I stoichiometric with the substrate.
K_I	Dissociation constant of complex EHI.
$(K_I)_{app}$	Dissociation constant of complex EHI in function of pH.
k_{cat}	Catalytic constant of the catalytic route.
λ_{max}	Maximum value of λ for saturating S_0.
$(\lambda_{max})_{app}$	Maximum value of λ for saturating S_0 in function of pH and with a stoichiometric inhibitor with substrate.
V_{max}	Maximum value of V_0 for saturating S_0.

From the initial step described in the Results and Discussion section it was verified that the suicide inactivation process was best fitted with only one significant exponential term. The studies on the effect of E_0 on the reaction allowed an r value to be

obtained so that $r \gg 1$, therefore, all the enzyme forms quickly reach the steady state. Moreover, it is known that the limiting step is the one controlled by k_2.[27] The assay conditions are S_0, $[O_2] \gg E_0$ and $P_\infty \ll S_0$, $[O_2]$.

Taking into account these considerations, the mechanism described in Scheme I can be schematized in the following way:

$$E_i \underset{k_7 f_x}{\longleftarrow} E_S \xrightarrow{2k_6 f_x} 2P + 2H^+ + 2OH^-$$

Scheme II

Dependence of E_S and P on time can be described by the differential equations:

$$[\dot{E}_S] = -k_7 f_x [E_S] \quad (1A)$$

$$[\dot{P}] = 2k_6 f_x [E_S] \quad (2A)$$

The integration of eqns. 1A and 2A, considering $[E_S] = E_0$ and $[P] = 0$ at $t = 0$, gives,

$$[P] = P_\infty (1 - e^{-\lambda t}) \quad (3A)$$

with,

$$P_\infty = 2 (k_6/k_7) E_0 = 2rE_0 \quad (4A)$$

$$\lambda = k_7 f_x \quad (5A)$$

The expression for f_x is derived by applying the steady state to the enzyme species in E_S:

$$f_x = (k_2/k_6) S_0/((K_S)'_{app} + S_0) \quad (6A)$$

Thus, λ can be expressed as a function of k_{cat} and r:

$$\lambda = (k_{cat}/r) S_0/[(K_S)'_{app} + S_0] = \lambda_{max} S_0/[(K_S)'_{app} + S_0] \quad (7A)$$

where,

$$(K_S)'_{app} = (K_S)_{app} [1 + (I_0/(K_I)_{app})] \quad (8A)$$

and,

$$(K_S)_{app} = K_S[1 + ([H^+]/K_{a1})]/[1 + (H^+]/K_{a2})] \quad (9A)$$

$$(K_I)_{app} = K_I[1 + (K_{a1}/[H^+])] \quad (10A)$$

From eqn. 7A, taking into account eqns. 8A–10A and that $I_0 = S_0$, eqn. (4) is obtained. Since $r \gg 1$, the catalytic route reaches an early steady state. The relationships existing between the parameters, V_0 and V_{max}, and those of the transient phase, P_∞ and λ are:

$$V_0 = \lambda P_\infty \quad (11A)$$

$$V_{max} = \lambda_{max} P_\infty \quad (12A)$$

Thus, the parallelism between V_0 and λ for conventional and suicide substrates, respectively, is evident from eqns. 11A and 12A.

Acknowledgements

This work has been partially supported by a grant from the Comisión Asesora de Investigación Científica y Técnica (Spain), project number 287 (85). The authors are grateful to Mrs. Stephanie Tischer for typing the manuscript.

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