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EXPERIMENTAL METHODS FOR KINETIC STUDY OF SUICIDE SUBSTRATES

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The kinetic study of the enzymatic inactivation originated by suicide substrates can be carried out by means of two alternative approaches. One method considers the substrate concentration as practically constant during the assay time and provides explicit equations of product concentration vs. time. The other method involves the significant consumption of the substrate, yielding implicit equations of time vs. product concentration. The utility of both methods is discussed and adequate experimental conditions for their correct application are established.

KEY WORDS: Enzyme inactivation, suicide inactivation, suicide substrates, enzyme kinetics, transient phase.

INTRODUCTION

The inactivation of enzymes induced by a substrate, takes place in enzymes acting on a substrate following a branched mechanism consisting of a catalytic route and an enzyme inactivation route. These substrates are called suicide inhibitors, mechanism-based inhibitors, inactivating substrates and suicide substrates.[1]

The relevance of the enzymatic inactivation by suicide substrates is gaining increased recognition, for both naturally ocurring and totally synthetic inactivating reagents.[1]

The reaction scheme depicted in Scheme I as proposed by Walsh *et al.*[2] provides the following minimal kinetic model

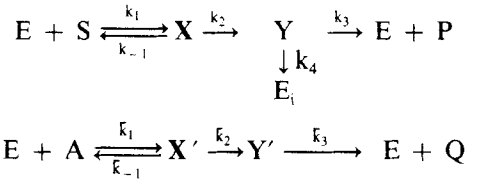
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_2} Y \xrightarrow{k_3} E + P$$
$$\downarrow k_4$$
$$E_i$$

SCHEME I

where E, S, X, Y, P and E_i denote enzyme, suicide substrate, first intermediate, second intermediate, product and inactivated enzyme, respectively.

For the cases in which the product (P) is not detectable an auxilliary substrate, A, may be used according to Scheme II

§ Correspondence
Abbreviations; MCPA-CoA: Methylenecyclopropaneacetyl-CoA



SCHEME II

where A and Q represent the auxiliary substrate and product respectively. The other components have the same significance as those of Scheme I.

The kinetics of inactivation, when following the reaction mechanism shown in Scheme I, can be studied by varying significantly the substrate concentration during the course of the reaction (in the text "variable substrate method"). This system has been studied by the steady state approach[3,4] obtaining implicit equations for time vs. [P], [S] and [E_i].

We have studied the kinetic behaviour of the reaction mechanisms described in Schemes I and II, maintaining the substrate concentration practically constant during the reaction time (in the text "constant substrate method"). Thus, the transient phase approach has been applied to obtain explicit solutions for [P] vs. time, from which the suicide substrate under study can be kinetically characterized.[5,6] An equivalent expression has been obtained from the implicit equation of time vs. [Q], by considering as negligible the consumption of suicide and auxiliary substrates during the assay time.[7]

The reaction mechanism described in Scheme I involves several particular cases which are shown in Table I. The general solution for all the cases, with respect to the transient phase, is biexponential (Table II). However, there are some cases where the values of the rate constants make the contribution of one exponential term negligible with respect to the other. In the cases corresponding to a biexponential behaviour (cases 1 and 6 in Table I and Table II), the characterization of the substrate could be obtained by determining the rate constants involved in Scheme I ($K_S = k_{-1}/k_1$, k_2, k_3 and k_4). Nevertheless, for the uniexponential behaviour the following must be determined: K_i (K_S or K_M, see Table II), k_{cat}, λ_{max}, V_{max} and r. From these values the relationships λ_{max}/K_M and $\lambda_{max} \cdot r/K_M$ could be defined.[6]

TABLE I
Particular cases of the general mechanism described by Schemes I and II

Case	$r = k_3/k_4$	$k_3/(k_2 f_x)^*$	Number of significant exponential terms
1	$\simeq 1$	$\simeq 1$	2
2	$\simeq 1$	$\gg 1$	1
3	$\simeq 1$	$\ll 1$	1
4	$\ll 1$	$\simeq 1$	1
5	$\ll 1$	$\gg 1$	1
6	$\ll 1$	$\ll 1$	2
7	$\gg 1$	$\simeq 1$	1
8	$\gg 1$	$\gg 1$	1
9	$\gg 1$	$\ll 1$	1

$^*f_x = \dfrac{S_0}{S_0 + K_S}$

TABLE II
Expressions for arguments of the exponential terms of explicit solutions here corresponding to the particular cases of Table I.

Case	$\lambda_1^{(*)}$	λ_2	Determinable constants
1	$(F_1 - F_2)/2$	$(F_1 + F_2)/2$	K_S, k_2, k_3, k_4, r
2	$\{k_2/(1 + r)\}S_0/F_S$	$k_3 + k_4$	K_S, k_2, r
3	k_4	k_2S_0/F_S	k_3, k_4, r, $(k_2/K_S)^{(***)}$
4	k_2S_0/F_S	k_4	K_S, k_2, r
5	k_2S_0/F_S	k_4	K_S, k_2, r
6	k_4	k_2S_0/F_S	K_S, k_2, k_3, k_4, r
7	$(k_{cat}/r)S_0/F_M$	$k_{cat} + S_0/F_M + k_3$	K_M, k_{cat}, r
8	$(k_2/r)S_0/F_S$	k_3	$K_M = K_S$, $k_{cat} = k_2$, r
9	k_4	$k_{cat} + S_0/F_M$	$k_{cat} = k_3$, k_4, r, $K_M^{(***)}$

(*) The expressions of λ_{max} and K_i^A are evident by contrast with eqn. 9 or 10.
(***) Only in presence of the auxiliary substrate A.
$F_1 = k_2f_x + k_3 + k_4$; $F_2 = \{(k_2f_x + k_3 + k_4)^2 - 4k_2k_4f_x\}^{1/2}$; $F_S = K_S\{1 + (A_0/K_M)\} + S_0$; $F_M = K_M\{1 + (A_0/K_M)\} + S_0$; $f_x = S_0/F_S$.

Recently, a paper has been published on the suicide inactivation kinetics of general acyl-CoA dehydrogenase from pig kidney by MPCA – CoA[8], using the variable substrate method and applying for the first time, one implicit equation previously described for the residual activity of the enzyme. In this paper (see Table I in Reference[8]) the initial concentration of reagents (S_0 and E_0) used was $S_0/E_0 = 2$ to 11. Thus, the steady state conditions are not reached, but non-linear kinetics with no analytical solution appear.

This encouraged us to study in more detail the kinetic analysis of the reaction mechanisms expressed in Schemes I and II, using methods with two different experimental approaches: constant substrate and varying substrate. In addition, both approaches can be applied in a continuous or discontinuous way. The particular cases where it is possible to use each approach and their corresponding experimental conditions are discussed.

METHODS

The corresponding calculus of numerical integration shown in the Results and Discussion Section have been carried out with an Olivetti M-24 computer, using a fourth-order Runge-Kutta method.[11] The integration step was varied in the programme by means of a 1–10% flow tolerance method.[12] The listing of the programme is available on request to the authors. The values of E_0, S_0 and k_i(i = 1–4) used to verify the analytical solutions are indicated in the legends to the Figures.

NOTATION AND SYMBOLS

The equivalence between the nomenclature here and that of other authors is indicated.

r Partition ratio ($r = k_3/k_4$)
K_i Half-saturation constant of E by S; thus, $K_i = K_S$ in cases with $r \ll 1$

and $r \simeq 1$, $K_i = K_M$ in case $r \gg 1$. (B in References[3] and [4]).

K_i^A Half-saturation constant of E by S in the presence of the auxiliary substrate, thus $K_i^A = K_i[1 + (A_0/\bar{K}_M)]$.

$\bar{K}_M$ Michaelis constant for the substrate A.

$\bar{k}_{cat}$ Catalytic constant for the substrate A.

k_{cat} Catalytic constant for the substrate S. (A.r in Reference[3] and C in Reference[4]).

λ_{max} Maximum value of the apparent inactivation constant (A in Reference[3] and C/r in Reference[4]).

λ_{max}/K_M Inactivation efficiency.

$\lambda_{max}.r/K_M$ Catalytic efficiency.

V_{max} Initial maximum velocity, $\lambda_{max}.r.E_0$ (A..r.E_0 in Reference[3] and C.E_0 in Reference[4]).

RESULTS AND DISCUSSION

A) Constant substrate approach

The reaction mechanism depicted in Schemes I and II has been studied by keeping the substrate concentration practically constant during the assay time ($S_0 \gg E_0$ and $P_\infty \ll S_0$). Thus, explicit solutions for [P] vs. t are obtained which are the basis for the method described below.[6]

1) Measurement of reaction progress In Table I the different cases corresponding to the reaction mechanisms described in Schemes I and II are indicated. It should be noted that the experimental conditions of $P_\infty = S_0 - S_\infty \ll S_0$ and $Q_\infty \ll A_0$ can always be maintained by choosing the appropriate enzyme concentration (see eqns. 11 and 12).

The methodology[6] provides the analytical expressions of concentration vs. time for the species which are useful for the measurement of the reaction progress. Thus, for the reaction mechanism depicted in Scheme I:

$$[P] = P_\infty - \gamma_{P1}e^{-\lambda_1 t} - \gamma_{P2}e^{-\lambda_2 t} \quad (1)$$

$$[S] = S_\infty + \gamma_{P1}e^{-\lambda_1 t} + \gamma_{P2}e^{-\lambda_2 t} \quad (2)$$

$$[E_i] = E_0 - \gamma_{E1}e^{-\lambda_1 t} - \gamma_{E2}e^{-\lambda_2 t} \quad (3)$$

and, for the reaction mechanism corresponding to Scheme II:

$$[Q] = Q_\infty - \gamma_{Q1}e^{-\lambda_1 t} - \gamma_{Q2}e^{-\lambda_2 t} \quad (4)$$

In both cases, the γ coefficients are functions of the apparent rate constants (λ_1 and λ_2 for Scheme I; λ'_1 and λ'_2 for Scheme II)[6]. In addition, the expression of the sum of γ coefficients is evident from eqns. 1–4 making $t = 0$.

When $|\lambda_1| \ll |\lambda_2|$ or $|\lambda'_1| \ll |\lambda'_2|$:

$$[P] = P_\infty(1 - e^{-\lambda_1 t}) \quad (5)$$

$$[S] = S_\infty(1 - e^{-\lambda_1 t}) + S_0e^{-\lambda_1 t} \quad (6)$$

$$[E_i] = E_0(1 - e^{-\lambda_1 t}) \quad (7)$$

$$[Q] = Q_\infty(1 - e^{-\lambda_1' t}) \quad (8)$$

λ_1 and λ_1' being:

$$\lambda_1 = \frac{\lambda_{max} S_0}{K_\lambda + S_0} \quad (9)$$

$$\lambda_1' = \frac{\lambda_{max} S_0}{K_\lambda^A + S_0} \quad (10)$$

and

$$P_\infty = S_0 - S_\infty = rE_0 \quad (11)$$

$$Q_\infty = \frac{(k_{cat}/K_M)A_0}{(k_2/K_S)/(1 + r)} \frac{E_0}{S_0} \quad (12)$$

In Table II the expressions of λ_1' and λ_2' (Scheme II) for the cases considered in Table I are shown. By making $A_0 = 0$ the expressions corresponding to λ_1 and λ_2 (Scheme I) are obtained. Moreover, the kinetic constants to be determined in every case are indicated.

The fitting of the progress curves ([P], [S], [E$_i$] or [Q] vs. t), to eqns. 5–8, respectively, by non-linear regression and their subsequent fitting to eqns. 1–4 allows discrimination between the different cases in Table I. If the second fitting improves with respect to the first one, this means that the system evolves with two significant exponential terms (cases 1 or 6 of Table I). The cases can be distinguished by the independence of λ_1 with regard to S_0 in case 6. In case 1 all mechanism information can be obtained measuring [P], [S], [E$_i$] or [Q], even without knowing E_0. In case 6, however, the information that may be obtained from λ_1 and λ_2 is K_S, k_2 and k_4. It is only possible to determine the values of r and k_3 ($r = k_3/k_4$) when the enzyme concentration is known. Measuring [P] and [S] (eqns. 1 and 2) allows determination of P_∞ and S_∞ and, therefore, r (eqn. 11). When measuring [E$_i$] or [Q] a discontinuous method has to be turned to, which uses the equation:

$$\frac{V_A}{V_0} = \frac{E_A}{E_0} = 1 - \frac{1}{1 + r} \frac{S_0}{E_0} \quad (13)$$

Thus, the enzyme may be incubated with different concentrations of substrate (S_0) and the corresponding residual activity at final time, V_A, with one substrate A can be measured separately.

If the fitting of the progress curves ([P], [S], [E$_i$] or [Q]) with respect to eqns. 5–8 does not improve using two exponential terms (eqns. 1–4) the information of the non-linear regression[13,14] gives: P_∞, λ_1; S_∞, E_0, λ_1 and Q_∞, λ_1', respectively. Assays with different S_0 may give: λ_1 constant (cases 3 and 9) or variable (cases 2, 3, 5, 7 and 8). A new fitting by non-linear regression of λ_1 vs. S_0 data to eqns. 9 or 10 gives the λ_{max} and K_λ constants or λ_{max} and K_λ^A. If the enzyme concentration is not known, the r values cannot be determined, although, if measuring [P] or [S], it is easy to show[6] that:

$$P_\infty = S_0 - S_\infty = \frac{V_{max}}{\lambda_{max}} \quad (14)$$

so that V_{max} (maximum initial rate can be determined).

If E_0 is known, the measurement of [P] and [S] allows calculation of r and, therefore, solving of the ambiguity between cases 3 and 9 and also that of cases 2, (4,5) and (7,8), but the ambiguity remains between (4,5) and (7,8). In order to overcome the ambiguity between cases 7 and 8 the working conditions[6] $E_0 \gg S_0$ are turned to. If $[E_i]$ or [Q] are measured to follow the reaction the discontinuous methods described by eqn. 13 has to be applied in order to determine r.

In short, Table II shows the kinetic constants that can be determined in the different possible cases under the best conditions, which involves knowing the enzyme concentration and in some cases the use of auxiliary substrate. From these values the relationships of catalysis efficiency and inactivation for the uniexponential behaviour cases can be determined.

2) Measurement of residual activity From eqn. 3 a discontinuous method can be established by a rearrangement of the equation, so that:

$$\frac{E_0 - [E_i]}{E_0} = \gamma'_{E1} e^{-\lambda_1 t} + \gamma'_{E2} e^{-\lambda_2 t} = \frac{A_r}{A_0} \qquad (15)$$

A_r = residual activity at time t; A_0 = initial activity. The information obtained by this method is equivalent to the measurement of $[E_i]$ in the continuous procedure described above.

B) Variable substrate approach

There are experimental cases where it is not possible to maintain the condition $P_i \ll S_0$ because:

(a) necessary concentration to detect P has to be raised.

(b) if the disappearance of the substrate (S) is measured, it may be necessary to monitor high variations to improve the sensitivity of the assay.

(c) when the K_i is very small, the variation of S_0 can lead to a constant response (saturation range) except at very low values of S_0. In this case, it may be necessary to monitor high variations of S_0 for their experimental detection.

1) Measurement of the reaction progress In the preceding three cases it is necessary to use implicit integrated equations like those previously described.[3,4,7]

$$t = \frac{1}{A}\left[\frac{B}{S_0 - rE_0} \ln \frac{\frac{[S]}{S_0}}{1 - \frac{S_0 - [S]}{rE_0}} - \ln\left(1 - \frac{S_0 - [S]}{rE_0}\right)\right] \qquad (16)$$

$$t = \frac{1}{A}\left[\frac{B}{S_0 - rE_0} \ln \frac{1 - \frac{[P]}{S_0}}{1 - \frac{[P]}{rE_0}} - \ln\left(1 - \frac{[P]}{rE_0}\right)\right] \qquad (17)$$

$$t = \frac{1}{A}\left[\frac{B}{S_0 - rE_0} \ln \frac{E_0\left(S_0 - \frac{r[E_i]}{E_0}\right)}{S_0\left(1 - \frac{[E_i]}{E_0}\right)} - \ln\left(1 - \frac{[E_i]}{E_0}\right)\right] \qquad (18)$$

where

$$r = k_3/k_4 \tag{19}$$

$$A = \frac{k_2 k_4}{k_2 + k_3 + k_4} \tag{30}$$

$$B = \frac{(k_{-1} + k_2)(k_3 + k_4)}{k_1(k_2 + k_3 + k_4)} \tag{21}$$

$$P_\infty = rE_0 \tag{22}$$

These equations are very useful when [S], [P] or $[E_i]$ are measured in the continuous procedure, eqns. 16–18.

Alternatively, it is possible to measure the residual activity at several time intervals ($[E]_{active} = E_0 - [E_i]$), obtaining the respective $[E_i]$ values, which can then be fitted to eqn. 18.

The r value can be determined by the discontinuous method described above, eqn. 13, when enzyme concentration E_0 is known. The data analysis described previously[7] can be used.

It is interesting to indicate the equivalence between the equations proposed by Waley[3,7] and Tatsunami *et al.*[4] for the conditions $S_0 \gg E_0$. These initial conditions should be experimentally applied to use integrated equations. So, the equations initially proposed by Waley[3] are completely valid. Both equations overlap the numerical integration, when a significant substrate consumption takes place (Figure 1A). In the uniexponential cases of Table I, Figure 1B reveals that both our explicit equation, eqn. 5, and the implicit equations overlap the numerical integration, since the implicit equations are transformed into explicit eqn. 5, when $S_0 \gg E_0$ and the substrate is practically constant. In the biexponential cases of Table I, only our analytical solution[6] overlaps the numerical integration.

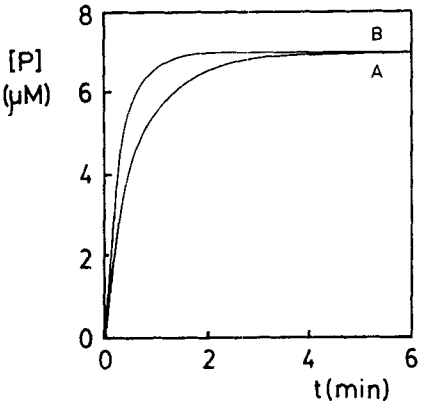


FIGURE 1 Dependence of product concentration vs. time. $k_1 = 10^8 M^{-1}s^{-1}$, $k_{-1} = 10^3 s^{-1}$, $k_2 = 10 s^{-1}$, $k_3 = 10 s^{-1}$, $k_4 = 0.1 s^{-1}$. (A) Condition $S_0 \gg E_0$ with significant substrate consumption. $S_0 = 10^{-5} M$, $E_0 = 7 \times 10^{-8} M$. (-----) Values calculated from implicit eqns. 7, 27 and 6 of References 3, 4 and 7, respectively, which coincide with the values obtained by numerical integration. (B) Condition $S_0 \gg E_0$ with substrate practically constant. $S_0 = 10^{-3} M$, $E_0 = 7 \times 10^{-8} M$. (——) Values calculated from the explicit eqn. 5 and from implicit eqns. 7, 27 and 6 of References 3, 4 and 7, respectively, which coincide with the values obtained by numerical integration.

However, the equations described by Tatsunami *et al.*[4] are proposed as valid for any condition, i.e.: $S_0 \gg E_0$; $S_0 \simeq E_0$ and $S_0 \ll E_0$ based on the fact that they used the material balance for the substrate $S_0 = [S] + [P] + [E_i]$. In fact, this is only necessary if $S_0 \leqslant E_0$, although under these conditions the steady state is never reached, and the steady state is an initial requirement for the subsequent integration.

Thus, as it was clear from Figure 3 of Reference 4, the analytical solution of Tatsunami *et al.* does not overlap the numerical integration when $E_0 \simeq S_0$, except at final time where $S_0 \simeq [S] + [P] + [E_i]$ is verified. An equivalent result is shown with values of constants similar to those used in Figure 1, but with $S_0 \simeq E_0$(Figure 2A). Under conditions $E_0 \gg S_0$ a similar result can be observed with the analytical solution of Tatsunami *et al.*,[4] whereas the corresponding solution proposed by us[6] overlaps the numerical solution (Figure 2B).

The ambiguity introduced[4] concerning the validity of those implicit equations for any ratio enzyme/substrate, led other authors[8] to use experimental conditions of S_0 slightly higher than the enzyme concentration (from 2.2 to 11) used in the study of the kinetics of suicide inactivation of general acyl-CoA dehydrogenase from pig kidney by MPCA-CoA. Thus, they calculate the value of the paramter r in assays where the ratio S_0/E_0 varies[15] from 0 to 2.2 by following the decrease in absorbance of the flavine associated with the enzyme at 446 nm which is proportional to the loss of enzyme activity at final time; they represent the data[15] (Table I) in a similar form to our eqn. 13 obtaining a value[15] of r = 4. The same information has been obtained in Figure 2 of Reference 16 (insert). Note, however, the appearance of a deviation of the linearity which can be due to the asymptotic zone of the first order kinetics, which means experimental measurements must be carried out over a longer assay time. A similar behaviour has been reported with other experimental data (see below).

Thus, it is correct to use the values of the ratio S_0/E_0 to determine r, since eqn. 13

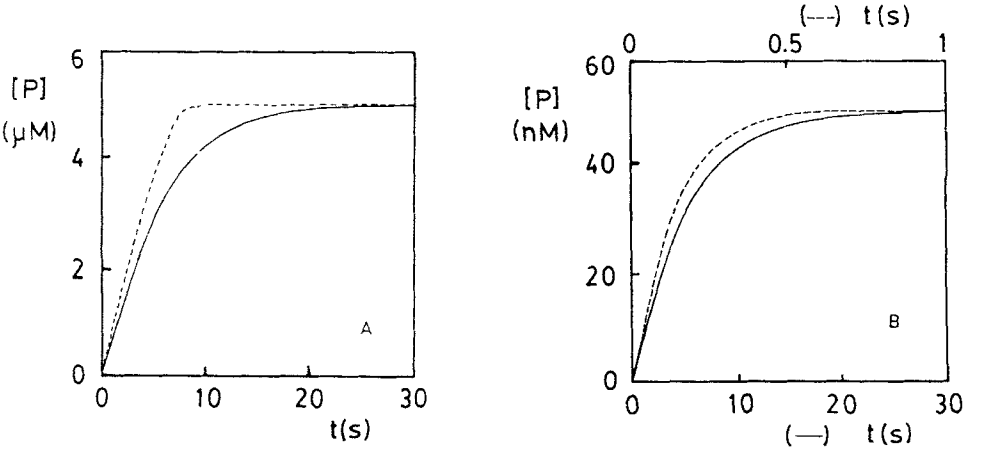


FIGURE 2 Dependence of product concentration vs. time. $k_1 = 10^8 M^{-1}s^{-1}$, $k_{-1} = 10^3 s^{-1}$, $k_2 = 10^{-1} s^{-1}$, $k_3 = 0.1 s^{-1}$, $k_4 = 0.1 s^{-1}$. (A) Condition $S_0 = E_0 = 10^{-5} M$. (-----) Values calculated from implicit eqn. 27 of Reference 4. (——) Values obtained by numerical integration. (B) Condition $E_0 \gg S_0 \cdot S_0 = 10^{-7} M$, $E_0 = 10^{-5} M$. (-----) Values calculated from implicit eqn. 27 of Reference 4. (——) Values calculated from explicit eqns. 17 and 23 of Reference 6 which coincide with the values obtained by numerical integration.

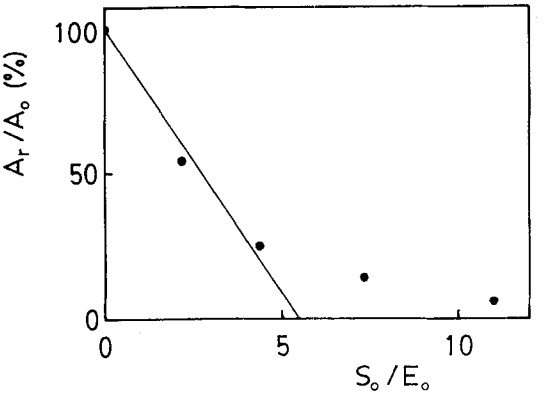


FIGURE 3 Determination of the r value for suicide inactivation of general acyl-CoA dehidrogenase by MCPA-CoA. Fractional enzymic activity vs. [MPCA-CoA]/[enzyme], according to eqn. 13. The enzyme (5.1 μM) and the MCPA-CoA (11.3–56.3 μM) in phosphate buffer, pH 7.6, were incubated and portions were withdrawn for assay. Data obtained from Reference 8 in Table I.

only considers the final state of the reaction and the value of r (with no dimension) indicates the distribution between the catalytic and the inactivation route. The same procedure, however, is not correct for studying the kinetics of the enzyme inactivation, since in the conditions detailed in Table I of Reference 8, the steady state from which the kinetics of the substrate consumption has to start is not reached. In this case, the application of the corresponding integrated equation[8] (eqn. 2) is not correct. Nevertheless, the apparent fitting of the experimental data shown in Table I to this equation may be due to the small number of the experimental points and their experimental error[8] (see Figure 1); the same occurs with data taken from Reference 16 in which the relation S_0/E_0 is 4. Note that the value $r = 4$ may correspond to one of the first three cases of Table I.

The approximate values obtained by these authors[8] for the parameters B, C and r seem to indicate that they are dealing with case 2 of Table I here. So, C is equivalent to our $k_{cat} = \lambda_{max} \cdot r = k_2k_3/(k_3 + k_4) \simeq 4k_2/5$ and B is equivalent to K_S, since the experimental data apparently fit to one exponential term whose argument (λ_1) depends on S_0. Case 1 corresponds to a biexponential behaviour while case 3 can be fitted to a uniexponential term which is independent of S_0. For a more rigorous determination of these parameters it would be convenient to study the inactivation kinetics at $S_0 \gg E_0$.

The data shown in Table I of Reference 8 have the same application as those shown in Table I of Reference 15, i.e., they are useful for calculating the value of r. In fact, the plot of relative residual activity vs. S_0/E_0 according to eqn. 13 here can be poorly fitted to a straight line (Figure 3), due to the asymptotic zone corresponding to the first order kinetics. This allows us to estimate the value of r.

CONCLUSIONS

From the above discussion it is clear that there are two useful experimental approaches for the kinetic study of the enzyme inactivation induced by suicide substrates, follow-

ing Schemes I or II. On the one hand, there is the approach proposed here, based on keeping the substrate concentration practically constant and using explicit equations for the data analysis.[6] This method has been applied successfully to the study of suicide inactivation of mushroom tyrosinase by catechol[17] and that of tyrosinase from frog epidermis by dopamine.[18] On the other hand, there is the approach that uses a significant variation of the substrate concentration during the reaction time of assay and which uses implicit eqns. 3 and 4. Both methods are only applicable under the experimental condition $S_0 \gg E_0$.

Taking into account the cases described in Table I, we propose the constant substrate method for the kinetic study of the enzyme inactivation originated by suicide substrates, as indicated above. This method was used in the first six cases of Table I, since conditions of $S_0 \gg E_0$, $r \simeq 1$ and $r \ll 1$ imply $P_x \leqslant E_0$ and $P_\infty \ll S_0$. Moreover, it has to be remembered that in these conditions the integrated equation (cases 2, 3, 4 and 5) are simplified into explicit equations. Cases 1 and 6, due to the biexponential behaviour, can be studied only in transient phase with the constant substrate method. In the cases where $r \gg 1$, the experimental condition $P_\infty \ll S_0$ may be reached by modifying the enzyme concentration. Thus, the experimental situations described as (a), (b) and (c) in Section (B) of Results and Discussion, would be the only situations where the variable substrate method should be used. In these cases, several implicit equations can be used,[3,4,7] but it should be taken into account that the expressions described in Reference 4 are only valid under the experimental condition $S_0 \gg E_0$ and in this case they become the ones previously proposed.[3,7]

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