A kinetic study of the suicide inactivation of an enzyme measured through coupling reactions

Application to the suicide inactivation of tyrosinase

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A systematic procedure for the kinetic study of reaction mechanisms with enzyme inactivation induced by a suicide substrate in the presence or in the absence of an auxiliary substrate, when the enzyme activity is measured through coupling reactions, enzymically catalysed or not, was developed and analysed by using the transient-phase approach. The methodology is established to determine the parameters and kinetic constants corresponding to the enzyme suicide inactivation and the coupling reactions. This approach is illustrated by a study of the suicide inactivation of tyrosinase by catechol in the presence of L-proline. Treatment of the experimental data was carried out by non-linear regression.

INTRODUCTION

Kinetic studies of coupled reactions enzymically catalysed or not have been carried out under different experimental conditions in the last few years (McClure, 1969; Easterby, 1973, 1981; Storer & Cornish-Bowden, 1974; Garcia-Carmona *et al.*, 1982; Escribano *et al.*, 1984, 1985).

The studies mentioned above consider that the first coupled enzyme rapidly reaches its steady state and that this is maintained during the time of the experiment, in which, after a transient phase due to the coupling reactions, the system reaches an overall steady state.

There is a wide range of enzymes that undergo an inactivation process when acting on a substrate (suicide inactivation), following a branched mechanism consisting of a catalytic route and an enzyme inactivation route that gives rise to a transient phase, until total loss of activity is reached (Ingraham, 1955; Tokuyama & Dawson, 1962;

Fernandez-Belda *et al.*, 1982; Frère *et al.*, 1982; Walsh, 1984; Knight & Waley, 1985; Faraci & Pratt, 1985). Kinetic studies of these reaction mechanisms have been carried out under approaches of significant (Waley, 1980, 1985; Tatsunami *et al.*, 1981) and negligible (Galvez *et al.*, 1981; Tudela *et al.*, 1987a) substrate consumption during the assay time. When the activities of these types of enzymes are measured through coupling reactions, the whole system has an overall transient phase originated from both the inactivation route and the coupling reactions.

The kinetic study of this type of process may be described by one of the mechanisms shown in Schemes 1 and 2. Scheme 1 is applicable to a suicide substrate (S_0) whose product (I_1) can be transformed by the coupling reactions to yield the detectable product (P). Scheme 2 is applicable to enzymes that can be measured through an auxiliary substrate (A_0) whose product (I'_1) originates the detectable species (Q). Note that coupling reactions can

Scheme 1.

Scheme 2.

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be enzymically catalysed or not (see the Notation and definitions section).

The kinetic study of suicide inactivation in the presence of coupling reactions has not been undertaken in the literature hitherto. The aim of the present paper is the kinetic analysis of these systems by using the transient-phase approach, considering as negligible the substrate consumption during the assay time. These conditions allow us to obtain explicit equations for product concentration as a function of time, which lead to an experimental design that yields the kinetic constants characterizing suicide inactivation and the coupling reactions of the system. The application of the corresponding experimental design is illustrated by means of a study of the suicide inactivation of mushroom tyrosinase by catechol in the presence of L-proline. The *o*-quinone product of the enzyme reaction is converted by non-enzymically catalysed reactions into 4-*N*-prolyl-*o*-benzoquinone, a chromophoric reagent stable during the assay time, used as final and detectable product (Scheme 4).

NOTATION AND DEFINITIONS

Species

E_0 and E_i , enzyme under study and inactive enzyme respectively.

S_0 and A_0 , suicide substrate and auxiliary substrate respectively.

I_i and I'_i , i th substrate intermediates from S_0 and A_0 respectively.

X and Y , second intermediates from S_0 and A_0 respectively.

I_1 and I'_1 , direct products from S_0 and A_0 respectively.

P and Q , detectable products originated from coupling reactions in the absence or in the presence of auxiliary substrate (A_0) respectively.

$[Pro]_0$, initial concentration of the nucleophilic form of L-proline.

Kinetic parameters

V_0 and V'_0 , early steady-state rates for S_0 and for A_0 respectively in the presence of S_0 .

λ_0 , apparent inactivation rate constant for the first enzyme (E_0).

λ_i , first-order or pseudo-first-order rate constant for the coupling reactions. For coupling enzymes $\lambda_i = V_i/K_i$. For non-enzymically catalysed reactions $\lambda_i = k_i$ or $\lambda_i = \bar{k}_i[R_i]$, R_i being a reagent present in sufficient excess to be considered as constant during the assay time.

$[P]_\infty$ and $[Q]_\infty$, concentrations of product obtained at the final time of the reaction.

Kinetic constants

k_i and k_i^A , specific rate constants for S_0 ($i = -1, +1, +2, +3, +4$) and A_0 ($i = -1, +1, +2, +3$) respectively.

r , partition ratio ($r = k_3/k_4$), equivalent to the number of catalytic events (turnovers) per inactivation event.

K_m and K_m^A , Michaelis constants for S_0 and A_0 respectively.

K_i , Michaelis constant for the i th coupling enzyme.

$k_{cat.}$ and $k_{cat.}^A$, catalytic constants for S_0 and A_0 respectively.

$V_{max.}$, maximum value of V_0 for saturating S_0 .

V_i , maximum velocity of the i th coupling enzyme [(units/ml)/min or mM/min].

$\lambda_{max.}$, maximum value of the apparent inactivation

constant (λ_0), equivalent to the number of inactivation events per time unit. Note that $\lambda_{max.} = k_{cat.}/r$.

$k_{cat.}/K_m$, efficiency of catalysis originated by S_0 .

$\lambda_{max.}/K_m$, efficiency of inactivation induced by S_0 .

KINETIC ANALYSIS

Mechanism of Scheme 1

The kinetic study for this mechanism was performed keeping in mind the following considerations.

(a) $[S_0] \gg [E_0]$ and $[P]_\infty \ll [S_0]$. This implies that the rate of the reaction catalysed by the first enzyme, at any time of reaction, V_i , must be independent of changes in the substrate concentration.

(b) The partition ratio $r = (k_{+3}/k_{+4}) \gg 1$. This case is very common at the experimental level (Walsh, 1984) and implies the attainment of an instantaneous steady state restricted to the catalytic route (E_0, X, Y), whereas there is a slow transition state between Y and E_i . In addition, there is another slow transition phase due to the accumulation of intermediate substrates, I_i , belonging to the coupling reactions. Thus the final accumulation of product (P) will depend on an overall transient phase including all the previous processes.

(c) The coupling reactions can be enzymically catalysed or not, being first-order or pseudo-first-order. Kinetics of coupling enzymes can be under first-order conditions ($\lambda_i = V_i/K_i$) with the use of appropriate enzyme concentrations.

Dependence of the concentrations of species of Scheme 1 on time can be described by the following differential equations:

$$\left. \begin{aligned} \dot{[I_1]} &= V_i - \lambda_1[I_1] = V_0 e^{-\lambda_0 t} - \lambda_1[I_1] \\ \dot{[I_i]} &= \lambda_{i-1}[I_{i-1}] - \lambda_i[I_i] \\ \dot{[P]} &= \lambda_n[I_n] \end{aligned} \right\} \quad (1)$$

These equations can be solved by using the initial conditions $[I_i] = [P] = 0$ at $t = 0$, yielding:

$$[P] = \sum_{j=0}^n \gamma_j (1 - e^{-\lambda_j t}) \quad (2)$$

$$\text{where: } \gamma_j = V_0 \prod_{k=1}^n \lambda_k \frac{1}{\lambda_j \prod_{\substack{k=0 \\ k \neq j}}^n (\lambda_k - \lambda_j)} \quad (3)$$

Experimental plots of $[P]$ versus time can be fitted to eqn. (2), in order to obtain the apparent rate constants corresponding to suicide inactivation of the first enzyme (λ_0) and to each coupling reaction (λ_i) (see the Notation and definitions section).

At experimental level, it frequently occurs that the best fit of the plots of $[P]$ versus time are to uni- or bi-exponential equations (Garcia-Canovas *et al.*, 1987; Tudela *et al.*, 1987b). This phenomenon can occur when there are one or two exponential terms much slower (i.e. significant) than the others in eqn. (2).

The kinetic parameters for the suicide inactivation reaction described in Scheme 1 have the following expressions:

$$V_0 = k_{cat.}[E_0][S_0]/(K_m + [S_0]) \quad (4)$$

$$\lambda_0 = \lambda_{max.}[S_0]/(K_m + [S_0]) \quad (5)$$

$$[P]_\infty = \sum_{j=0}^n \gamma_j = V_0/\lambda_0 = r[E_0] \quad (6)$$

Mechanism of Scheme 2

The kinetic study for this mechanism was performed keeping in mind the considerations established for the study of the mechanism of Scheme 1 and the conditions $[S_0] \gg [E_0]$, $[A_0] \gg [E_0]$, $[Q]_\infty \ll [A_0]$ and $[I_1]_\infty \ll [S_0]$.

The temporal evolution of the species of Scheme 2 is described by the following differential equations:

$$\left. \begin{aligned} \dot{[I_1]} &= V'_t - \lambda_1[I_1] = V'_0 e^{-\lambda_0 t} - \lambda_1[I_1] \\ \dot{[I_t]} &= \lambda_{t-1}[I_{t-1}] - \lambda_t[I_t] \\ \dot{[Q]} &= \lambda_n[I_n] \end{aligned} \right\} \quad (7)$$

These equations can be solved by using the initial conditions $[I_t] = [Q] = 0$ at $t = 0$, leading to an expression equivalent to eqn. (2) but with replacement of V_0 by V'_0 and of λ_0 by λ'_0 .

The kinetic parameters of suicide inactivation now have the following expressions:

$$V'_0 = k_{\text{cat}}^A [E_0] [A_0] / \left\{ \left[\frac{K_m^A}{K_m} \left(1 + \frac{[S_0]}{K_m} \right) \right] + [A_0] \right\} \quad (8)$$

$$\lambda'_0 = \lambda_{\text{max}} [S_0] / \left\{ \left[K_m \left(1 + \frac{[A_0]}{K_m^A} \right) \right] + [S_0] \right\} \quad (9)$$

$$[Q]_\infty = \frac{V'_0}{\lambda'_0} = \frac{(k_{\text{cat}}^A / K_m^A) [A_0] [E_0]}{(k_{\text{cat}}^A / K_m) / r [S_0]} \quad (10)$$

However, the expressions of the apparent rate constants of the coupling reactions are equivalent to those described for the mechanism depicted by Scheme 1.

Note that, for Scheme 1, it is possible to determine the

partition ratio, r , by using eqn. (6). However, for Scheme 2, this is not possible by application of eqn. (10). In this case the value of r can be obtained from separate measurements of residual activities at the final time (V_A), with different values of $[S_0]$, by using the equation (Knight & Waley, 1985; Tudela *et al.*, 1987a):

$$\frac{V_A}{V_0} = \frac{[E_A]}{[E_0]} = 1 - \frac{1}{1+r} \cdot \frac{[S_0]}{[E_0]} \quad (11)$$

Suicide inactivation of tyrosinase

The mechanism proposed in order to explain the suicide inactivation of mushroom tyrosinase acting on catechol (Garcia-Canovas *et al.*, 1987) is shown in Scheme 3. Experimental assays carried out with saturating O_2 as well as considering the rate-limiting character of k_2 , lead easily to the simplification of Scheme 3 to the catalytic cycle shown in Schemes 1 and 2 (Garcia-Canovas *et al.*, 1987).

The *o*-quinone product (I_1) produced in the enzymic oxidation is very reactive and tends to form adducts with nucleophilic reagents, such as OH^- , amino acids etc. Furthermore, the oxidizing character of the *o*-quinones is well known, and they can react with *o*-quinone-nucleophile adducts, regenerating the initial *o*-diphenol (Cabanès *et al.*, 1987). Therefore a tentative mechanism for measuring suicide inactivation of tyrosinase by catechol in the presence of L-proline is shown in Scheme 4. Note that the reaction mechanism is equivalent to Scheme 1, with a bifurcation from the I_1 intermediate, which can easily be accommodated by involvement in expressions of λ_i belonging to eqn. (2).

Scheme 3.

Scheme 4. Pathway proposed for the oxidation of catechol by $NaIO_4$ or tyrosinase in the presence or in the absence of L-proline

Addition of L-proline to *o*-benzoquinone is shown at the right-hand side, and addition of water to *o*-benzoquinone at the left-hand side.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase (*o*-diphenol:O₂ oxidoreductase, EC 1.14.18.1) (2000 units/mg) was supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. Catechol, L-proline and other chemical reagents were of analytical grade and were purchased from E. Merck, Darmstadt, Germany.

General assay conditions

The following reagents were maintained at constant concentrations: 0.26 mM-O₂ (saturating) and 10 mM-sodium phosphate buffer, pH 5.7, at 25 °C. Other conditions are detailed in the Figure legends. Protein concentration was determined by an improved Lowry

obtained with metaperiodate oxidation (results not shown). This indicates the existence of common coupling reactions from *o*-benzoquinone onwards (Scheme 4). The appearance of an isosbestic point suggests that there is a defined stoichiometry to stable final products. The spectra were submitted to a rank matrix analysis, by using a graphic method (Coleman *et al.*, 1970), and satisfied the test of two significant species kinetically related with stoichiometric restrictions (Garcia-Canovas *et al.*, 1982).

Inactivation assays

Inactivation assays were carried out by measuring the increase of absorbance at 520 nm with time. The following equation was fitted to the experimental recordings:

$$[P] = [P]_{\infty} \left[\left(\frac{\lambda_1}{\lambda_1 - \lambda_0} \right) (1 - e^{-\lambda_0 t}) + \left(\frac{\lambda_0}{\lambda_0 - \lambda_1} \right) (1 - e^{-\lambda_1 t}) \right] + (c_1 + c_2 t) \quad (12)$$

method (Hartree, 1972), with bovine serum albumin as standard. The values of $[Pro]_0$ were calculated by using the concentration of the commercial reagent and the value of $pK_{a_2} = 10.6$. The detectable product, 4-*N*-prolyl-*o*-benzoquinone (P in Scheme 4), has $\epsilon_{520} = 5950 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 5.7. This value was calculated from oxidation assays of catechol with lower concentrations of periodate and high concentrations of L-proline, conditions yielding the stoichiometric formation of P (Scheme 4).

The assays were carried out with a Perkin-Elmer Lambda-3 spectrophotometer, interfaced on-line to a DS-3600 computer where data were recorded, stored and later analysed.

Rapid-scan assays

Catechol oxidation by NaIO₄ or by O₂ and mushroom tyrosinase was monitored by means of successive absorption spectra, for identification of absorbing species. The tracings were recorded in 25 s, with an interval of 40 s between scans.

The oxidation of catechol with consumption of O₂ in the presence of high concentrations of tyrosinase and L-proline (Fig. 1) shows an equivalent pattern to that

The coefficient c_1 represents the uncertainty on the zero-time absorbance, caused by enzyme addition at the start of reaction, and c_2 corresponds to the slow spontaneous oxidation of catechol. The effects of both experimental artifacts have been computer-subtracted in Figures showing plots of $[P]$ versus time. Good initial estimations of the parameters for each experimental recording were obtained as follows. c_1 is equivalent to the absorbance value of the first data point. c_2 is equal to the slope of the straight part of the curve attained at the final time period, whose data points are fitted by linear regression. Then the contribution of $c_1 + c_2 t$ is computer-subtracted from the experimental recordings. Thus $[P]_{\infty}$ is obtained from the mean of data points belonging to the horizontal plateau attained at the final time of corrected recordings. The initial estimations of λ_0 and λ_1 were calculated from two subsequent logarithmic linearizations, and their respective fittings by linear regression (Hiromi, 1979).

The further non-linear-regression fitting (Marquardt, 1963) yields the final estimations of c_1 , c_2 , $[P]_{\infty}$, λ_0 and λ_1 , with the respective standard deviations and the residual sum of squares (Mannervik, 1981). The use of the final estimations of the parameters in eqn. (12) provides curves that overlap the experimental recordings.

Therefore sets of plots of $([P]_{\infty}, \lambda_0, \lambda_1)$ versus $([E_0], [S_0], [Pro]_0)$ are then submitted to linear-regression or non-linear-regression fittings, in order to determine the respective kinetic constants. The weighting factor used for each data point is the inverse of the variance of each parameter. When non-linear regression (Wilkinson, 1961; Marquardt, 1963) is applied, good initial estimations are also calculated from linear regression of data to linear transformations of the corresponding equations.

RESULTS AND DISCUSSION

Inactivation assays

The first step of the experimental design, based on the above kinetic analysis, consists of assaying the suicide inactivation of the target enzyme, under several experimental conditions. Eqn. (12) can be fitted to the recordings of these assays by non-linear regression, since two exponential terms provide the best fit (Mannervik, 1981; Burguillo *et al.*, 1983). Therefore eqn. (12) was used for fitting to all inactivation curves obtained below.

Fig. 1. Spectrophotometric recordings for the oxidation of catechol (0.8 mM) by tyrosinase (8.16 nM) in the presence of 60 mM-L-proline with consumption of O₂.

Scan speed was up to 480 nm/min at 40 s intervals.

Fig. 2. Corrected recordings of suicide inactivation of tyrosinase in the presence of L-proline for different enzyme concentrations

Conditions are as described in the Materials and methods section and with 0.034–0.0476 nM-tyrosinase, 1 mM-catechol and 50 nM-L-proline (nucleophilic form). Inset: corresponding values of $[P]_{\infty}$ for different concentrations of tyrosinase.

Effect of $[E_0]$

In this step, appropriate enzyme concentrations are chosen in order to obtain $[P]_{\infty} \ll [S_0]$ and $[P]_{\infty} \ll [O_2]_0$ in further assays. Thus negligible substrate consumption during the assay time can be experimentally assured according to eqn. (6). The corrected recordings obtained (Fig. 2) show different values of $[P]_{\infty}$ attained at a similar time interval. This indicates the non-dependence of the apparent rate constants on $[E_0]$, and this was confirmed by the values of both kinetic parameters, calculated by non-linear-regression fitting of eqn. (12) to the plot of $[P]$ versus time. This behaviour is in accordance with the expressions of λ_0 (eqn. 5) and λ_i (see the Notation and definitions section).

On the other hand, the experimental plot of $[P]_{\infty}$ versus $[E_0]$ (Fig. 2 inset) may lead to determination of the partition ratio (eqn. 6), when the non-dependence of $[P]_{\infty}$ on the concentrations of other experimental reagents has been checked in the following steps.

Effect of $[S_0]$

In this step, several assays with different substrate concentrations and other conditions remaining constant were carried out. From the fitting of eqn. (12) to the plot of $[P]$ versus time the corresponding kinetic parameters can be obtained. Thus from corrected recordings (shown in Fig. 3) it is evident that $[P]_{\infty}$ does not depend on $[S_0]$ within the experimental error. The same behaviour is shown by one of the two apparent rate constants (Fig. 3 inset), indicating that one exponential term corresponds to one of the coupled reactions depicted in Scheme 4 (see the Notation and definitions section).

Fig. 3. Corrected recordings of suicide inactivation of tyrosinase in the presence of L-proline by different concentrations of catechol

Conditions are as described in the Materials and methods section and with 0.034 nM-tyrosinase, 50 nM-L-proline (nucleophilic form) and 0.04–1 mM-catechol at pH 5.7. Inset: corresponding values of λ_0 (●) and λ_1 (▲) for different concentrations of catechol.

Table 1. Kinetic constants of the suicide inactivation of mushroom tyrosinase originated by catechol, in the presence of L-proline at pH 5.7 and 25 °C

Constant (units)	Value
K_m (mM)	0.36 ± 0.03
$k_{cat.} (= \lambda_{max.} \cdot r)$ (s^{-1})	990 ± 103
$\lambda_{max.}$ (s^{-1})	$(5.82 \pm 0.26) \times 10^{-3}$
r	$(17 \pm 1) \times 10^4$
$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$(275 \pm 51) \times 10^4$
$\lambda_{max.}/K_m$ ($M^{-1} \cdot s^{-1}$)	16.17 ± 2.07
k_{+1} ($M^{-1} \cdot s^{-1}$)	$(42.15 \pm 2.02) \times 10^3$
$k_{+2}[H_2O]$ (s^{-1})	$(2.4 \pm 0.1) \times 10^{-3}$

However, the other apparent rate constant shows a hyperbolic dependence on $[S_0]$ (Fig. 3 inset), according to eqn. (5). Therefore the other exponential term corresponds to suicide inactivation of the target enzyme. The non-linear-regression fitting of eqn. (5) to the plot of λ_0 versus $[S_0]$ yields the K_m and $\lambda_{max.}$ values (Table 1).

Effect of L-proline

Furthermore, the experimental design consists of steps with variation in the concentrations of reagents involved in the coupling reactions, in order to identify and characterize their significant contributions to the overall kinetic behaviour.

Thus assays with several L-proline concentrations and keeping other conditions constant have been made (recordings not shown). The consumption of L-proline during the assay period was also negligible, as the result of choosing an appropriate enzyme concentration such that $[P]_{\infty} \ll [Pro]_0$. The fitting of eqn. (12) to the plot of $[P]$ versus time provides the values of the corresponding kinetic parameters. Thus the non-dependence of λ_0 on $[Pro]_0$ (Fig. 4 inset) supports the appearance of one

Fig. 4. Data from suicide inactivation assays of tyrosinase in the presence of different concentrations of L-proline

Conditions are as described in the Materials and methods sections and with 0.3 nM-tyrosinase, 0.2 mM-catechol and 12–150 nM-L-proline. The main Figure shows the values of $[P]_{\infty}$ for different concentrations of L-proline. Inset: corresponding values of λ_0 (●) and λ_1 (▲) for different concentrations of L-proline.

exponential term corresponding to suicide inactivation of tyrosinase. Nevertheless, λ_1 shows a linear plot with regard to $[Pro]_0$, with an intercept on the ordinate axis (Fig. 4 inset). These results suggest that steps with L-proline and water addition to *o*-benzoquinone (Scheme 4) could be responsible for the second exponential term experimentally detected. The final redox steps with substrate regeneration (Scheme 4) are not dependent on $[Pro]_0$ and appear to be relatively fast, as a consequence of which their exponential term is not significant during the assay time.

On the other hand, the $[P]_{\infty}$ values show a hyperbolic behaviour with respect to $[Pro]_0$ (Fig. 4). This response could arise as a result of the bifurcation and substrate regeneration proposed in Scheme 4, but not considered in Scheme 1 and the corresponding eqn. (6).

Therefore a kinetic analysis of the reaction system depicted in Scheme 4 leads to the following expression:

$$[P] = [P]_{\infty} \left(\frac{\lambda_1}{\lambda_1 - \lambda_0} \right) (1 - e^{-\lambda_0 t}) + \left(\frac{\lambda_0}{\lambda_0 - \lambda_1} \right) (1 - e^{-\lambda_1 t}) \quad (13)$$

where λ_0 is described by eqn. (5) and:

$$\lambda_1 = 2(\bar{k}_{+1}[Pro]_0 + \bar{k}_{+2}[H_2O]) \quad (14)$$

and:

$$[P]_{\infty} = \frac{V_0}{\lambda_0} \cdot \frac{\bar{k}_{+1}[Pro]_0}{2(\bar{k}_{+1}[Pro]_0 + \bar{k}_{+2}[H_2O])} = r[E_0] \frac{\bar{k}_{+1}[Pro]_0}{\bar{k}_{+1}[Pro]_0 + \bar{k}_{+2}[H_2O]} \quad (15)$$

Note that eqns. (14) and (15) agree with the experimental results shown in Fig. 4 inset and Fig. 4 respectively.

Eqn. (14) describes the competition between the addition of L-proline and of water to *o*-benzoquinone

(Scheme 4). The regeneration of substrate is represented by the numeric coefficient in this expression.

Eqn. (15) involves the catalytic and inactivation processes of the enzyme reaction. Thus tyrosinase generates two molecules of *o*-quinone per turnover (Scheme 3), the equation being:

$$V_0 = 2k_{cat}[E_0][S_0]/(K_m + [S_0]) \quad (16)$$

This coefficient is simplified in eqn. (15), yielding an expression similar to eqn. (6), but corrected with a distribution factor corresponding to fraction of *o*-benzoquinone molecules transformed into the chromophoric product (4-*N*-prolyl-*o*-benzoquinone). Furthermore, it must be taken into account that mushroom tyrosinase is a tetrameric protein with two active sites (Robb, 1984), and therefore:

$$[P]_{\infty} = 2r[E_0] \frac{\bar{k}_{+1}[Pro]_0}{\bar{k}_{+1}[Pro]_0 + \bar{k}_{+2}[H_2O]} \quad (17)$$

From this kinetic analysis it is evident that the values of $\bar{k}_{+1}$ and $\bar{k}_{+2}$ can be determined from a plot of λ_1 versus $[Pro]_0$ (Fig. 4 inset), by using eqn. (14). Further, the value of the partition ratio can be calculated from a plot of $[P]_{\infty}$ versus $[E_0]$ (Fig. 2 inset), by application of eqn. (17). These values are shown in Table 1.

In conclusion, the kinetic analysis of the suicide inactivation of enzymes measured through coupling reactions has been developed, by application of the transient-phase approach. The plots of product concentration versus time allow us to identify the reactions with significant contributions to the overall kinetic behaviour of the system under study, as well as the determination of their corresponding kinetic parameters and constants.

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