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ORIGINAL PAPER

Characterization of unstable enzyme systems which evolve according to a three-exponential equation

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Abstract The time course of an enzyme catalyzed reaction is normally followed either by monitoring the instantaneous concentration or velocity of an enzyme species or a product. In many enzyme catalyzed reactions these time variations are multi-exponential. The accurate fit of the relevant curves to obtain the kinetic parameters involved can be difficult using conventional methods (Galvez et al. in J Theor Biol 89:37–44, 1981; Garcia-Canovas et al. in Biochim Biophys Acta 912:417–423, 1987; Tudela et al. in Biochim Biophys Acta 912:408–416, 1987; Teruel et al. in Biochim Biophys Acta 911:256–260, 1987; Garrido del Solo et al. in Biochem J 294:459–464, 1993; Varon et al. in Int J Biochem 25:1889–1895, 1993; Garrido del Solo et al. in An Quim 89:319–324, 1993; Varon et al. in J Mol Catal 83:273–285, 1993; Garrido del Solo et al. in Biochem J 303(Pt 2):435–440, 1994; Garrido del Solo and Varon in An Quim 91:13–18, 1995; Garrido del Solo et al. in Biosystems 38:75–86, 1996; Garrido del

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Solo et al. in Int J Biochem Cell Biol 28:1371–1379, 1996; Garrido del Solo et al. in Int J Biochem Cell Biol 30:735–743, 1998; Varon et al. in J Mol Catal 59:97–118, 1990). In order to circumvent such difficulties Arribas et al. (J Math Chem 44:379–404, 2008) proposed an evaluation method which is applicable regardless of the complexity of the kinetic equation. This procedure is based on the numerical determination of statistical moments from experimental time progress curves. The fitting of these experimentally obtained moments to the corresponding theoretical symbolic expressions allows, in most cases, all the individual rate constants involved to be evaluated. In this paper we perform a general analysis that can be applied to any unstable enzyme system described by a three-exponential equation and apply it to a substrates induced enzyme inactivation process that is described by this type of equation. To verify the goodness of the method we have simulated time progress curves and applied the suggested procedure to these curves, obtaining kinetic parameters values very close to those used to obtain simulated curves. Finally, we compare our results with those obtained in previous contributions in which other procedures were used.

Keywords Three-exponential equations · Statistical moments · Enzyme · Kinetics · Simulated progress curves · Numerical integration

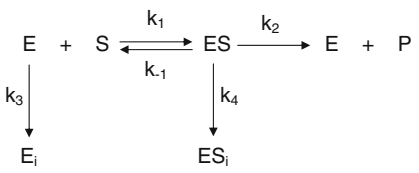
1 Introduction

Many enzyme reactions exist in which some of the reaction species are unstable, i.e. tend to impurities or irreversible inhibitors. In some other cases they may degrade spontaneously due to the assay conditions used (pH, temperature, metal ions, etc.). The case in which both the free enzyme and the enzyme-substrate complex or complexes are unstable, e.g. that shown in Scheme 1, has been studied theoretically [2,4,5,7,8,11–13,16,17] as well as experimentally [18]. Another, more complex example of a mechanism corresponding to unstable enzyme systems is shown in Schemes 2 and 3 [16].

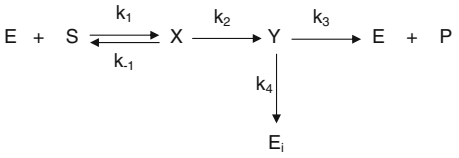
Sometimes the instability of an enzyme form is induced by the presence of an irreversible inhibitor [19–27]. There are enzyme reactions in which the product [9–11,13,28–31], the substrate [32–35] or both the product and the substrate [11] of the enzyme reaction may also be unstable, regardless of whether the free enzyme or the enzyme-substrate complex(es) are stable. Sometimes, the inhibitor involved in an enzyme reaction can be unstable [6,22].

A very interesting type of unstable enzyme system is those in which the inactivation of the enzyme is induced by the substrate. This type of inactivation is known as "substrate inactivation" or "suicide inhibition" and takes place in enzymes which act on a substrate by means of a branched mechanism consisting of a cat-

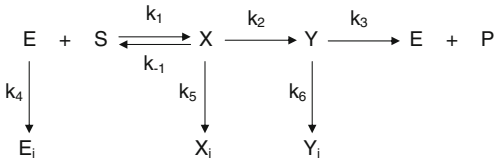
Scheme 1 Reaction mechanism in which both the free enzyme and the enzyme–substrate complex are unstable



Scheme 2 Simplest suicide inhibition reaction mechanism



Scheme 3 Reaction mechanism in which the substrate is suicide and all the enzyme forms are unstable



alytic route and an enzyme inactivating route. Such substrates are called suicide inhibitors, mechanism-based inhibitors, inactivating substrates and suicide substrates [3,14,34,36–42]. Scheme 2 [3,36,37,41,42] shows the simplest suicide inhibition mechanism, where E and E_i are the active and inactive enzyme forms, X and Y are intermediates and S and P are the substrate and the product, respectively:

There are enzyme reactions in which the substrate is suicide and, furthermore, the enzyme is unstable, such as that shown in Scheme 3 [43] consisting in Scheme 2 but where the free enzyme, E, and the complex enzyme-substrate, X, are unstable:

The importance of enzyme suicide inactivation is gaining increased recognition for both naturally occurring and totally synthetic suicide substrates [44–47], and there is a wide range of enzymes of great physiological interest which act on suicide substrates [48–55].

The first step of the strategy to evaluate the kinetic parameters is the derivation of approached analytical, giving the concentration of one or more of the evolved species and experimentally monitoring (through the corresponding progress curves) the concentration of the same species. The analytical solutions are generally multi-exponential equations. Hence, the experimental data are fitted to the corresponding theoretical symbolic equation and the kinetic parameters are evaluated.

In contributions about specific unstable enzyme systems, the above general procedure has been particularized in the corresponding experimental design and kinetic data analysis [3,5,9,11–13,16,38,53,56–63]. The suggested corresponding method in each of the above contributions is either valid only for the specific mechanism studied or, in most cases, the mathematical procedure is very laborious. Recently, a general procedure [15] valid for any unstable enzyme system, regardless of its complexity and whether the enzyme is unstable or irreversibly reacting with an inhibitor has been proposed. This procedure is based on the statistical moments of any function giving the time course concentration corresponding to an unstable enzyme system, which can be expressed as a sum of exponential terms.

The main aims of this paper are: (1) To particularize the above mentioned general procedure to triexponential kinetic equations corresponding to unstable enzyme systems; (2) To apply the results in (1) to the suicide inactivation induced by substrates described by a three-exponential equation corresponding to Scheme 2; (3) To verify

the goodness of the method using progress curves obtained by numerical integration; (4) To compare our results with those obtained in previous contributions in which other procedures were used.

2 Materials and methods

Simulated progress curves were obtained by numerical integration of the set of differential equations describing the kinetics of the reaction evolving according to the corresponding mechanism under study, using values of the rate constants and initial concentrations, which have either been published in the literature or chosen arbitrarily, but realistically. This numerical solution was found by using the classical fourth-order Runge–Kutta formula, but applying an adaptative stepsize control that was originally invented by Fehlberg [64–66] using the software WES implemented in Visual C++ 6.0 and other computer programs developed by our group [67–70]. The WES program was run on a PC compatible computer based on a Pentium IV/2 GHz processor with 512 MB of RAM.

The plots of the data obtained from the numerical integration, as well as the plots of equations made in Figs. 1, 2, 3, 4, 5, 6, 7, were carried out using the Sigma Plot Scientific Graphing System program, version 8.02 (2002, SPSS Inc).

To obtain the time course equation of the product P involved in Scheme 2 we used the software TRAPHAER implemented by Varon et al. [71].

The statistical moments from the experimental curves have been obtained by numerical evaluation of the area below the corresponding curve between $t = 0$ and $t \to \infty$. Obviously the condition $t \to \infty$ is not reachable and, therefore, an error, generally very small, is always committed when one chooses a finite time, $t \to \infty$. In our numerical example the criterion we have used to chose a finite time as $t \to \infty$ was the higher

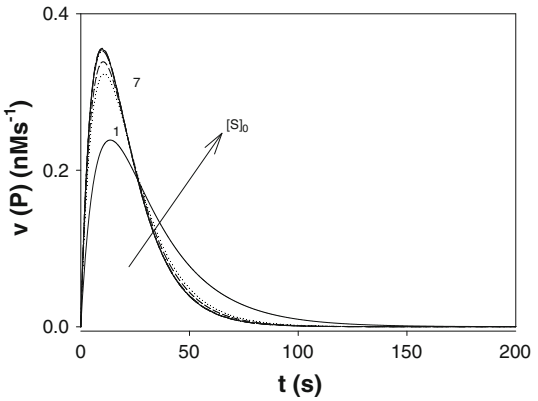


Fig. 1 Simulated time progress curves of $v(t)$ obtained from numerical integration of the set of differential equations corresponding to Scheme 2. In each of the seven curves the values of the initial enzyme concentration and rate constants were: $[E]_0 = 100\,\text{nM}$, $k_1 = 10^8\,\text{M}^{-1}\,\text{s}^{-1}$, $k_{-1} = 1{,}000\,\text{s}^{-1}$, $k_2 = 0.1\,\text{s}^{-1}$, $k_3 = 0.01\,\text{s}^{-1}$ and $k_4 = 0.1\,\text{s}^{-1}$. The different initial substrate concentration used in each curve were $[S]_0 = 10$, 50, 100, 500 μM, 1, 5 and 10 mM

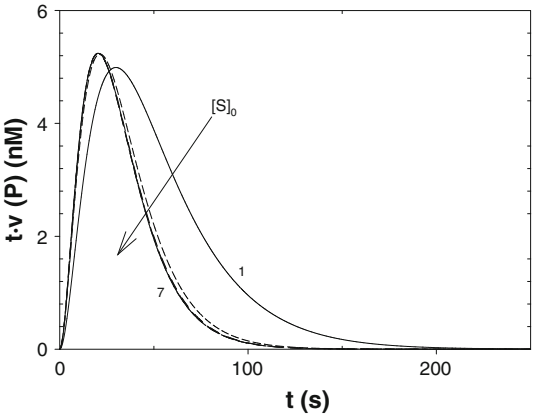


Fig. 2 Simulated time progress curves of $t \cdot v(t)$ obtained from numerical integration of the set of differential equations corresponding to Scheme 2. The values of initial enzyme concentration, the rate constants and the initial substrate concentrations were the same as in Fig. 1

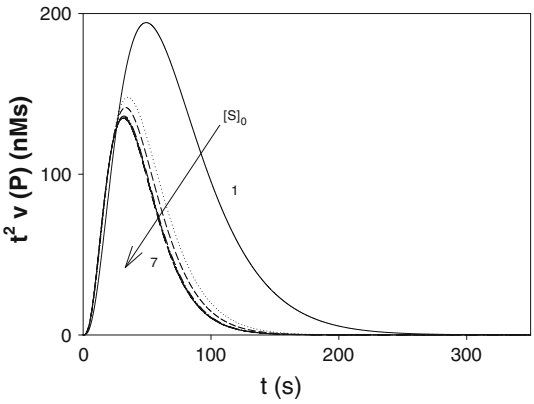


Fig. 3 Simulated time progress curves of $t^2 \cdot v(t)$ obtained from numerical integration of the set of differential equations corresponding to Scheme 2. The values of initial enzyme concentration, the rate constants and the initial substrate concentrations were the same as in Fig. 1

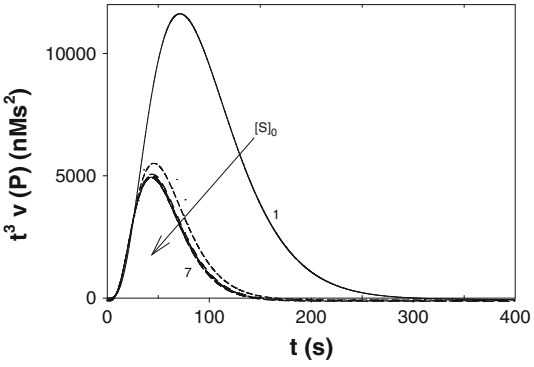


Fig. 4 Simulated time progress curves of $t^3 \cdot v(t)$ obtained from numerical integration of the set of differential equations corresponding to Scheme 2. The values of initial enzyme concentration, the rate constants and the initial substrate concentrations were the same as in Fig. 1

of these two times: the time needed to achieve a residual activity, i.e. $[E]+[X]+[Y]$, of 10^{-6} times the initial enzyme concentration, $[E]_0$, or the time needed to achieve the rate of product formation equal to 10^{-4} times its maximum value.

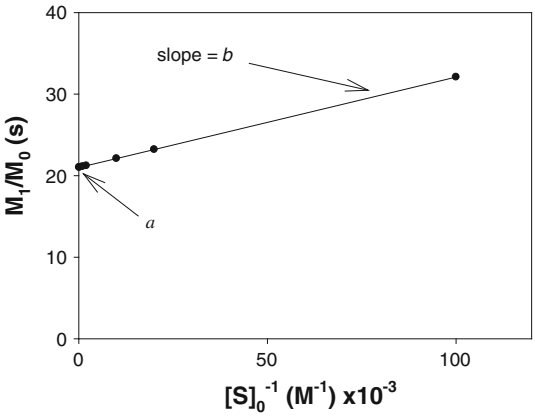


Fig. 5 Straight line resulting from the fit by linear regression of the experimental data M_1/M_0 and $[S]_0^{-1}$ to Eq. (25). From this fitting a and b are immediately obtained

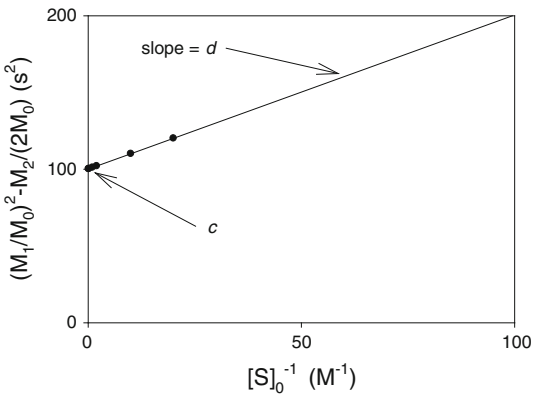


Fig. 6 Straight line resulting from the fit by linear regression of the experimental data $(M_1/M_0)^2 - M_2/(2M_0)$ and $[S]_0^{-1}$ to Eq. (28). From this fitting c and d are immediately obtained

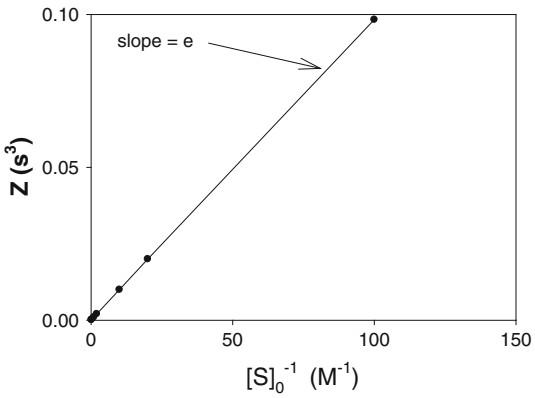


Fig. 7 Straight line resulting from the fit by linear regression of the experimental data Z and $[S]_0$ to Eq. (31). For simplicity we called Z the left side of this equation. From this fitting e is immediately obtained

3 Three-exponential time course equations for the concentrations of the species involved in unstable enzyme systems

The time-dependent function, $f(t)$, furnishing the instantaneous concentration at time t of any of the species involved in several unstable enzyme systems is given by a three-exponential equation [1,71]:

$$f(t) = \beta + \sum_{h=1}^{3} \gamma_h e^{\lambda_h t} \tag{1}$$

where $f(t)$ is the instantaneous concentration at time t of any of the species involved.

The arguments $\lambda_h (h = 1, 2, 3)$ in Eq. (1) are the roots of an equation like

$$\lambda^3 + F_1\lambda^2 + F_2\lambda + F_3 = 0 \tag{2}$$

arising from the derivation of Eq. (1).

According to the polynomial theory, among the arguments λ_h $(h = 1, 2, 3)$ the following very useful relationships are observed:

$$\left.\begin{array}{l} \lambda_1 + \lambda_2 + \lambda_3 = -F_1 \\ \lambda_1\lambda_2 + \lambda_1\lambda_3 + \lambda_2\lambda_3 = F_2 \\ \lambda_1\lambda_2\lambda_3 = -F_3 \end{array}\right\} \tag{3}$$

We denote as $P_q (q = 1, 2, 3)$ the sum of all of the q-nary products of the arguments λ_h $(h = 1, 2, 3)$. For completeness, we set $P_0 = F_0 = 1$. The following relationship between P_q and F_q, which will be useful below, is observed:

$$P_q = (-1)^q F_q \qquad (q = 0, 1, 2, 3) \tag{4}$$

The amplitudes γ_h $(h = 1, 2, 3)$ in Eq. (1) are explicit functions of λ_h $(h = 1, 2, 3)$, of the rate constants involved in the process and of the initial concentrations of the enzymes and ligand species present at the onset of the reaction. Finally, β is a time-independent, non-negative quantity, the meaning of which is the value of $f(t)$ when t goes to infinite, i.e.:

$$\beta = \lim_{t\to\infty} f(t) \tag{5}$$

There may be two different situations for the arguments $\lambda_h (h = 1, 2, 3)$: (a) Three are real and negative and (b) one is real and negative and the other two are complex conjugate with negative real part. In both cases the sum of three exponential terms goes to zero when $t \to \infty$ and, therefore, Eq. (5) is observed.

Note that at $t = 0$, the exponential terms in Eq. (1) reduce to the corresponding amplitudes $\gamma_h (h = 1, 2, 3)$ and, therefore, the relationship yields:

$$\beta = f(0) - \sum_{h=1}^{3} \gamma_h \tag{6}$$

Equation (5) can be considered as the conceptual definition of β. According to this equation, β means the value of the concentration of the species corresponding to $f(t)$ at $t \to \infty$, i.e. at high t-values. This is the reason for sometimes denoting β, according to the specific case, as $[P]_\infty$, $[E_i]_\infty$, etc. Equation (6) can be considered as the operational definition of β, indicating that it is obtained by subtracting from $f(0)$ the sum of the 3 amplitudes γ_h $(h = 1, 2, 3)$. Therefore, β depends on $f(0)$ [when $f(0) \neq 0$] and on the same parameters and initial concentrations on which the amplitudes $\gamma_h (h = 1, 2, 3)$ depend.

3.1 Example of an unstable enzyme system in which product accumulation fits a three-exponential equation like Eq. (1)

The simplest reaction mechanism for the action of an enzyme, E, on a suicide substrate, S, is that indicated in Scheme 2 above. Under conditions of limiting enzyme and considering a reaction time for which the concentration of the product accumulated is much less than the initial substrate concentration, i.e. in conditions under which the instantaneous substrate concentration, $[S]$, remains approximately constant and equal to its initial concentration, $[S]_0$, it is possible to express the accumulation of product with a corresponding approximate analytical solution of differential equations system which, under these conditions, becomes linear.

The above analytical solution can be obtained either manually or, more easily, by using the software TRAPHAER [71]. The result is:

$$[P] = [P]_\infty + \gamma_1 e^{\lambda_1 t} + \gamma_2 e^{\lambda_2 t} + \gamma_3 e^{\lambda_3 t} \tag{7}$$

where λ_1, λ_2 and λ_3 are the roots of general Eq. (2), that satisfy Eq. (3). The coefficients F_1, F_2 and F_3 for this particular case can be expressed as follows:

$$F_1 = k_1[S]_0 + k_{-1} + k_2 + k_3 + k_4 \tag{8}$$

$$F_2 = k_1 (k_2 + k_3 + k_4)\,[S]_0 + (k_{-1} + k_2)\,(k_3 + k_4) \tag{9}$$

and

$$F_3 = k_1 k_2 k_4\,[S]_0 \tag{10}$$

In turn, $[P]_\infty$ is given by:

$$[P]_\infty = r[E]_0 \tag{11}$$

where r is the so called [3] partition ratio defined as:

$$r = \frac{k_3}{k_4} \tag{12}$$

The amplitudes of Eq. (7) are:

$$\gamma_h = \frac{k_1 k_2 k_3 [S]_0 [E]_0}{\lambda_h \prod_{\substack{p=1 \\ p \neq h}}^{3} (\lambda_p - \lambda_h)} \quad (h = 1, 2, 3) \tag{13}$$

Derivatizing $[P]$, given by Eq. (7), with respect to time gives the expression in the following Eq. (14) for the time course of the velocity, $v(t)$, of the product accumulation:

$$v(t) = \sum_{h=1}^{3} \delta_h e^{\lambda_h t} \qquad (h = 1, 2, 3) \tag{14}$$

being

$$\delta_h = \lambda_h \gamma_h \qquad (h = 1, 2, 3) \tag{15}$$

Equation (14) will be used later in the kinetic data analysis we propose.

4 Statistical moments of $v(t)$

The statistical moments of $v(t)$ are the starting point for the novel method of evaluation of all of the rate constants, involved in Scheme 2, that we suggest in this contribution and which is a specific application of the general method described by Arribas et al. [15].

The statistical moments of $v(t)$ are those of order 0, 1, 2 and 3, which we denote as M_0, M_1, M_2 and M_3, respectively. The expression of the statistical moment jth ($j = 0, 1, 2, 3$) is:

$$M_j = \int_0^{\infty} t^j v(t) dt \quad (j = 0, 1, 2, 3) \tag{16}$$

Inserting Eq. (14) in Eq. (16) and integrating:

$$M_j = (-1)^{j+1} j! \sum_{h=1}^{3} \frac{\gamma_h}{\lambda_h^j} \quad (j = 0, 1, 2, 3) \tag{17}$$

4.1 Analytical determination of statistical moments M_0, M_1, M_2 and M_3

If in Eq. (17) we insert the expression of γ_h $(h = 1, 2, 3)$ given by Eq. (13) and we make the corresponding calculations, the expressions of the moments become those in Eqs. (18)–(21). To determine these sums it is useful to take into account the results in Appendix A in the above mentioned contribution of Arribas et al. [15].

$$M_0 = r[E]_0 \tag{18}$$

$$M_1 = M_0 \frac{G_2}{G_3} \tag{19}$$

$$M_2 = 2M_0 \frac{G_2^2 - G_1 G_3}{G_3^2} \tag{20}$$

$$M_3 = 6M_0 \frac{G_2^3 - G_3^2 - 2G_1 G_2 G_3}{G_3^2} \tag{21}$$

where the expressions of G_i $(i = 1, 2, 3)$ in Eqs. (18)–(21) are:

$$G_1 = k_{-1} + k_2 + k_3 + k_4 + k_1[S]_0 \tag{22}$$

$$G_2 = (k_{-1} + k_2)(k_3 + k_4) + k_1 (k_2 + k_3 + k_4)[S]_0 \tag{23}$$

$$G_3 = k_1 k_2 k_4 [S]_0 \tag{24}$$

Note that G_1, G_2 and G_3 coincide with F_1, F_2 and F_3 and that according to Eqs. (11) and (18), $[P]_\infty = M_0$.

Some useful relationships between M_0, M_1, M_2 and M_3 arising from their analytical expressions.

From Eqs. (18) and (19) we obtain

$$\frac{M_1}{M_0} = a + \frac{b}{[S]_0} \tag{25}$$

where

$$a = \frac{k_2 + k_3 + k_4}{k_2 k_4} \tag{26}$$

$$b = \frac{(k_{-1} + k_2)(k_3 + k_4)}{k_1 k_2 k_4} \tag{27}$$

From Eqs. (18)–(20) we obtain

$$\left(\frac{M_1}{M_0}\right)^2 - \frac{M_2}{2M_0} = c + \frac{d}{[S]_0} \tag{28}$$

being

$$c = \frac{1}{k_2 k_4} \tag{29}$$

$$d = \frac{k_{-1} + k_2 + k_3 + k_4}{k_1 k_2 k_4} \tag{30}$$

And, finally, from Eqs. (18)–(21) we obtain

$$\left(\frac{M_1}{M_0}\right)^3 - \frac{M_1 M_2}{M_0^2} + \frac{M_3}{6M_0} = \frac{e}{[S]_0} \tag{31}$$

being

$$e = \frac{1}{k_1 k_2 k_4} \tag{32}$$

4.2 Graphic determination of the statistical moments M_0, M_1, M_2 and M_3

The jth ($j = 0, 1, 2, 3$) statistical moment M_j can be obtained either as a symbolic form from the expression of $v(t)$ and the analytical integration indicated in Eq. (16), as above, or numerically from the experimental time course of $v(t)$ and taking into account that the integral on the right hand side of Eq. (16) coincides with the area below the curve $t^j \cdot v(t)$ between $t = 0$ and $t \to \infty$ ($t \to \infty$ must be interpreted as a time, arbitrarily chosen by the research, at which $t^j \cdot g(t) \to 0$). The time we take as $t \to \infty$ is explained in the Material and Methods section.

5 Use of the statistical moments to evaluate all of the individual rate constants and the initial enzyme concentration

In this section we use the simulated progress curves $v(t)$ as experimental progress curves to explain the method proposed here, based on the symbolic and experimental determination of its moments of order 0, 1, 2 and 3, checking the goodness of the method used. To better illustrate this procedure we apply it to the enzyme reaction shown in Scheme 2. The experimental design and kinetic data analysis proposed here consists of the following steps:

Step 1. To determine the analytical expressions of the moments M_0, M_1, M_2 and M_3. These expressions are given by Eqs. (18)–(21) for any enzyme system evolving according to an equation like Eq. (7) corresponding to Scheme 2.

Step 2. To determine graphically the values of previous moments for different values of $[S]_0$, satisfying the condition of limiting enzyme and considering a reaction time for which the concentration of the product accumulated is much less than the initial substrate concentration, at a fixed $[E]_0$-value. In the example, the time progress curves $t^j \cdot v(t)$ have been plotted, in Figs. 1, 2, 3, 4, for

Table 1 Values of the statistical moments M_0, M_1, M_2 and M_3 of $v(t)$ obtained, graphically, from the curves in Figs. 1, 2, 3, and 4 for the different $[S]_0$—values indicated in the first column

$[S]_0$ (mM)	M_0 (nM)	M_1 (μM s)	M_2 (μM s^2)	M_3 (mM s^3)
0.01	9.99900	0.320890	16.5856	1.211085
0.05	9.99838	0.232044	8.36816	0.415442
0.1	9.99823	0.221012	7.56906	0.356007
0.5	9.99812	0.212200	6.96584	0.313620
1	9.99808	0.211100	6.89268	0.308628
5	9.99804	0.210220	6.83452	0.304683
10	9.99803	0.210110	6.82727	0.304193

Table 2 Values of $\frac{M_1}{M_0}$, $\left(\frac{M_1}{M_0}\right)^2 - \frac{M_2}{2M_0}$ and $\left(\frac{M_1}{M_0}\right)^3 - \frac{M_1 M_2}{M_0^2} + \frac{M_3}{6M_0}$ obtained from the values of M_0, M_1, M_2 and M_3 given in Table 1 for the different values of $[S]_0$ shown on the first column

$[S]_0$ (mM)	$\frac{M_1}{M_0}$ (s)	$\left(\frac{M_1}{M_0}\right)^2 - \frac{M_2}{2M_0}$ (s^2)	$\left(\frac{M_1}{M_0}\right)^3 - \frac{M_1 M_2}{M_0^2} + \frac{M_3}{6M_0}$ (s^3)
0.01	32.0921	200.546	0.0999833
0.05	23.2081	120.142	0.0199833
0.1	22.1051	110.114	0.0100167
0.5	21.2240	102.101	0.00203333
1	21.1140	101.102	0.000966667
5	21.0261	100.305	0.000216667
10	21.0151	100.205	0.000125000

seven different arbitrary values of $[S]_0$ shown in Table 1 at a fixed $[E]_0$-value of 100 nM. For the construction of these curves the corresponding $v(t)$ progress curves, obtained by numerical integration, and the values of time, t, for each of the values of $v(t)$ were used. To perform the integration for each $[S]_0$ we have used, in all cases, the values of the rate constants given in Fig. 1. Table 1 shows the values of the statistical moments of $v(t)$, M_0, M_1, M_2 and M_3, obtained in this way.

Step 3. From the numerical values obtained for M_0, M_1, M_2 and M_3 for each different $[S]_0$-value we determine the corresponding numerical values for $\frac{M_1}{M_0}$, $\left(\frac{M_1}{M_0}\right)^2 - \frac{M_2}{2M_0}$ and $\left(\frac{M_1}{M_0}\right)^3 - \frac{M_1 M_2}{M_0^2} + \frac{M_3}{6M_0}$. In Table 2 we summarise the above values generated from Table 1 for the example used.

Step 4. The values of $\frac{M_1}{M_0}$ are represented versus the corresponding $\frac{1}{[S]_0}$ and, in accordance with Eq. (25) linear regression gives a straight line with the intercept a and the slope b. Figure 5 shows the above straight line for our example and in Table 3 we indicate the values of a and b obtained.

Step 5. To represent the values of $\left(\frac{M_1}{M_0}\right)^2 - \frac{M_2}{2M_0}$ versus the corresponding $\frac{1}{[S]_0}$-values. According to Eq.. (28), this plot gives, by linear regression, a straight line with

Table 3 Values of the parameters obtained from the procedure proposed in the main text for the reaction mechanism in Scheme 2

Parameter	Value
a (s)	21.002 ± 0.003
b (M s)	$(1.1086 \pm 0.0007) \times 10^{-4}$
c (s^2)	100.080 ± 0.014
d (M s^2)	$(1.0041 \pm 0.0004) \times 10^{-3}$
e (M s^3)	$(9.829 \pm 0.015) \times 10^{-7}$

Table 4 Values of the rate constants obtained from the procedure proposed in the main text for the reaction mechanism in Scheme 2

Rate constant	True values	Determinated values
k_1 (M^{-1} s^{-1})	10^8	$(1.0182 \pm 0.0016) \times 10^8$
k_{-1} (s^{-1})	1,000	$1,021.4 \pm 2.2$
k_2 (s^{-1})	0.1	0.0995 ± 0.0009
k_3 (s^{-1})	0.01	0.0100 ± 0.0013
k_4 (s^{-1})	0.1	0.1004 ± 0.0009
$K_M(M)$	1.0001×10^{-5}	$(1.0032 \pm 0.0027) \times 10^{-5}$

the intercept c and the slope d. In Fig. 6 the above straight line is indicated for our example and in Table 3 we indicate the values of c and d obtained.

Step 6. To represent the values of $\left(\frac{M_1}{M_0}\right)^3 - \frac{M_1 M_2}{M_0^2} + \frac{M_3}{6M_0}$ versus the corresponding $\frac{1}{[S]_0}$-values. In accordance with Eq. (31), this plot yields, by linear regression, a straight line through the origin with slope e. Figure 7 shows the above straight line for our example and in Table 3 we indicate the value of e obtained.

Step 7. Once the values of the parameters a, b, c, d and e have been obtained and taking into account their expressions given by Eqs. (26), (27), (29), (30) and (32) we obtain, through a straightforward algebraic calculation, the rate constants involved in the reaction mechanism under study. In Table 4 we show the values of the rate constants obtained from the simulated progress curves for the example studied using the procedure here suggested. Note the good concordance with the rate constants used for the simulation of the progress curves. From the values obtained for k_1, k_{-1} and k_2 we obtain the value $K_M = (1.0032 \pm 0.0027) \times 10^{-5}\, M$, which is the value obtained from the true values of the constants used in the numerical integration $1.0001 \times 10^{-5}\, M$. Likewise, from the values obtained for rate constants k_3 and k_4 we obtain the value of the dimensionless partition ratio $r = 0.100 \pm 0.013$, which is the true value obtained from the values of k_3 and k_4 used in the numerical integration, $r = 0.1000$.

6 Results and discussion

The calculation of each of the rate constants involved in any mechanism of enzymatic reactions requires a experimental monitoring of the time course of the concentration or the velocity of the product or of any other enzyme species. These experimental curves must be fitted to the corresponding symbolic equations obtained from the analytical integration of the set of differential equations (once linearized by using certain assumptions) that describe the kinetic behaviour of the enzymatic system under study. The rate constants, in principle, must be obtained from these fits. Generally, the symbolic equations that describe the time evolution of a concentration or of a velocity of formation are often too complicated for a suitable fit of the experimental data. For example, when the equation involves two or more exponential terms, the fit is not easy and, in many cases, impossible. The values of the rate constants obtained from these fits, when they are possible, are affected in a substantial error due to error in the parameters arising from the fit.

The simplification of the equation is often carried out assuming that the whole system has reached the steady state, or assuming that some segment of the reaction scheme is initially at a steady state (initial steady-state approach), or assuming rapid equilibrium in one or more of the reversible steps (rapid equilibrium approach). Obviously, if equations are simplified we can determine either fewer individual rates constant or determine only global kinetic parameters that are algebraic combinations of individual constants. It is clear that both the individual rate constants and the global kinetic parameters may significantly differ from actual values, due to the approximations made.

Recently Arribas et al. [15] proposed a general method to analyse the kinetic data of unstable enzymatic systems that avoids the problems mentioned above. The method is based on the analytical and numerical determination of various statistical moments, starting with that of zero order of a function of the concentration or the velocity of any species involved in the enzyme system under study, that can be followed experimentally. The number of statistical moments that we must calculate depends on the number of rate constants to be determined.

In this contribution the method has been applied to unstable enzyme systems in which the concentration or velocity of some of the species involved in the system is described by a three-exponential equation like Eq. (7), using the most simple of the suicide inhibition systems, shown in Scheme 2, which has been widely studied by other methods [3,11,14,36,37,39]. All the above methods perform simplifying assumptions in the analytical method that are not needed here.

The method suggested here offers, in our opinion, the following advantages over other methods used:

(1) No simplification of symbolic equations that express the different moments is required.
(2) The graphical determination of the moments from the experimental curves is easily obtained using numerical methods and the software indicated in the Materials and Methods section.

Table 5 Values of M_0/r, i.e. $[E]_0 = 100\,\text{nM}$, r being k_3/k_4

$[S]_0$ (mM)	M_0/r (nM)
0.01	100 ± 13
0.05	100 ± 13
0.1	100 ± 13
0.5	100 ± 13
1	100 ± 13
5	100 ± 13
10	100 ± 13

The values of M_0 are in Table 1 and the values of k_3 and k_4 on third column in Table 4

(3) The symbolic equations that express the moments or combinations of them are polynomials and they can be conveniently fitted to the values of the statistical moments obtained experimentally.
(4) It is possible to determine the five individual rate constants (Table 4).
(5) There is a good agreement between the values of the rate constants used to obtain the simulated progress curves of $v(t)$ and the values of the constants obtained by applying the proposed method, as shown in Table 4.
(6) The fit of experimental data to rational equations, such as those corresponding to the jth statistical moments, M_j ($j = 0, 1, 2, 3$), is much more reliable than fitting to a three-exponential time course equation like Eqs. (1) or (7) as is usually done [5,72].
(7) The method does not require the initial concentration, $[E]_0$, of the free enzyme to be known.
(8) This method allows the estimation of $[E]_0$ as indicated below. Indeed, after obtaining the rate constants k_3 and k_4, and therefore the partition ratio, r equal to k_3/k_4, we can estimate the initial concentration of the free enzyme, $[E]_0$, since, according to Eq. (18), it coincides with the quotient M_0/r. Therefore, according to Table 5, the estimated value for $[E]_0$ is $100 \pm 13\,\text{nM}$, while the value used in the simulations was 100 nM. Note the good agreement between these numbers. Therefore, this procedure can be regarded as an analytical method for estimating the initial concentration of the free enzyme in enzyme reactions evolving according to Scheme 2.
(9) The suggested method is independent of the relative values of the rate constants.

6.1 Assumption of a uni-exponential kinetic behaviour of unstable enzyme systems evolving according to Scheme 2

The accumulation of product in the enzyme system indicated in Scheme 2 is given by the three-exponential Eq. (7). The fit of the experimental kinetic data to a three-exponential equation, and even to a bi-exponential one, is very difficult and prone to considerable error, sometimes very large, in the values of λ_1, λ_2 and λ_3, which makes the results obtained by these fits, when they are possible, unreliable. For this reason, some authors [1,3] consider the uni-exponential behaviour to characterize the enzyme

system indicated in Scheme 2. This uni-exponential behaviour is justified when the partition ratio, r, defined in Eq. (12), is much higher than unity and when the reversible step in Scheme 2 is in rapid equilibrium. On this basis the authors can evaluate only the partition ratio r (whenever the initial enzyme concentration is known) from $[P]_\infty = r[E]_0$ and the following global kinetic parameters λ_{max} and K_λ given by:

$$\lambda_{max} = \frac{k_2 k_4}{k_2 + k_3} \tag{33}$$

$$K_\lambda = \frac{K_1 k_3}{k_2 + k_3} \tag{34}$$

being K_1 the equilibrium constant defined as k_{-1}/k_1.

The procedure suggested by us in the present contribution has the advantage that all of the individual rate constants, as well the initial enzyme concentration, can be estimated irrespective of the r value and whether rapid equilibrium conditions prevail or not.

6.2 Extrapolation of the method to other enzyme systems

The procedure suggested can by extrapolated for application to any, stable or unstable, enzyme system described by a three-exponential equation as we now summarily point out. For these systems the time course equation of the concentration of any species has the general form [73]:

$$C = \beta + \alpha_1 t + \alpha_2 t^2 + \cdots \alpha_r t^r + \sum_{h=1}^{3} \gamma_h e^{\lambda_h t} \tag{35}$$

If we take the (r+1)th time derivative in Eq. (35), and we denote $d^{r+1}C/dt^{r+1}$ as $g(t)$, we obtain:

$$g(t) = \frac{d^{r+1}C}{dt^{r+1}} = \sum_{h=1}^{3} \delta_h e^{\lambda_h t} \tag{36}$$

where

$$\delta_h = \lambda_h^{r+1} \gamma_h \tag{37}$$

Note that Eq. (36) is identical to Eq. (7) and, therefore the method can be used (whenever all of the arguments λ_h are real negative or complex with a negative real part, as it is usual in most enzyme systems).

6.3 Limitation of the method

This method has the following limitation: The statistical moment obtained from the experimental curve $g(t)$ supposes the numerical evaluation of the area below the curve between $t = 0$ and $t \to \infty$. Obviously the condition $t \to \infty$ is not reachable and so an error, albeit very small, always occurs when one chooses a finite time as $t \to \infty$. In our numerical example the criterion used to choose a finite time as $t \to \infty$ was the higher of these two times: the time needed for the residual activity, i.e. $[E]+[X]+[Y]$, to become $10^{-6} \cdot [E]_0$ or the time needed for the rate of product formation to reach a value equal to 10^{-4} times its maximum value. However any other criterion may also be acceptable whenever $t^j \cdot g(t) \to 0$ at the time chosen as $t \to \infty$. Another limitation is that the set of algebraic equations to be solved to obtain the rate constants cannot be linear and, a quadratic, cubic or higher degree equation might appear in the solution or some absurd solutions may be obtained which must be discarded. Nevertheless, the current Mathematica symbolic software packages could help considerably in this mechanical task.

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