# A method, based on statistical moments, to evaluate the kinetic parameters involved in unstable enzyme systems 

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#### Abstract

The evaluation of individual rate constants involved in any reaction mechanism of an enzymatic systems first requires experimental monitoring of the time course of the concentration or product rate creation or of any enzyme species. The experimental progress curves obtained must then be fitted to the corresponding theoretical symbolic equation. Nevertheless, in some cases, e.g. when the equation involves two or more exponential terms, this fit is not easy and sometimes impossible. Simplification of the equation is usually required by assuming, for example, that the system has reached the steady-state, assuming an initial steady-state of a segment in the scheme of the reaction mechanism or assuming rapid equilibrium in one or more of the reversible steps, if there are any. But, obviously, simplified equations produce either fewer individual rate constants or global constants consisting of algebraic associations of individual rate constants or individual rate constants or global constants that might considerably differ from the real ones due to the approaches made. In this contribution, we suggest an alternative procedure for evaluating the rate constants of enzyme reactions corresponding to enzyme systems where one or more of the species involved


[^0]is unstable or where one or more of the enzyme species is irreversibly inhibited, or both. The 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 expressions allows us, in most cases, to evaluate of all of the rate constants involved, with only a small error. To verify the goodness of the suggested procedure, it was applied to an unstable enzyme system which had previously been analysed with other methods. Finally, it is indicated how this procedure could also be extrapolated for application to any stable or unstable enzyme system.

Keywords Statistical • Moment • Enzyme • Kinetics • Unstable

## 1 Introduction

In almost all biochemistry courses, as well as in supporting textbooks [1-7], metabolic pathways are taught under the tacit assumption that the reactants, the intermediates and the products are stable or confined to the reaction shown in the metabolic scheme presented. Thus, side reactions and spontaneous decomposition of the reaction species in the scheme are usually ignored, despite the fact that they often play an important role in vivo [8-17].

At first sight, the structural stability of a metabolic or regulatory enzyme seems to represent a favourable quality, because it increases the efficiency of the pathway and saves energy, as well as the substance required to compose and maintain the system. However, metabolism must be seen in a wider perspective, since other processes must also be kept in operation. These may require resources, e.g. amino acids, high energy phosphate and coenzymes, or cofactors, which may be in short supply. Hence, the limited stability of some components of the system may serve to partition the resources to maintain the overall dynamic balance in homeostasis or growth processes, while the failure of such as integrated regulation may indirectly lead to cell degeneration or uncontrolled proliferation, e.g. autoimmunity and cancer [12,18].

Some enzyme reactions exist in which some of the reaction species are unstable, e.g. swing 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 [ $9,11,12,19-21]$ as well as experimentally [22]. Another, more complex example of a mechanism corresponding to enzyme unstable systems is shown in Schemes 2 and 3 [9].

A very interesting type of unstable enzyme system is that in which the enzyme inactivation is induced by the substrate. Although the enzyme is stable, the substrate provokes instability in the enzyme-substrate complex. 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 catalytic route and an enzyme inactivating route. Such substrates are called suicide inhibitors, mechanism-based inhibitors, inactivating substrates and suicide substrates

## Scheme 1



Scheme 2


Scheme 3


Scheme 4

[16,23-28]. Scheme $2[16,25]$ 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 [29] 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 [30-33], and there is a wide range of enzymes of great physiological interest which act on suicide substrates [34-41].

Sometimes the instability of an enzyme form is induced by the presence of an irreversible inhibitor, I, e.g. as in Scheme 4.

There are enzyme reactions in which the product [11,20,42-47], the substrate [48-51] 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 [52,53].

Characterization of unstable enzyme systems, as well as of the stable systems, consists of evaluating the individual rate constants and/or some global constants consisting of an algebraic combination of individual rate constants, such as the Michaelis constant and equilibrium constants, or algebraic combinations of rate constants and initial concentrations such as the $V_{\max }$.

The general strategy followed to evaluate the above kinetic parameters is, usually, as follows:
(a) Based on the suggested scheme of the reaction mechanism one writes the set of ordinary differential equations describing the kinetic behaviour of the species involved in the reaction. This set of differential equations is generally non-linear.
(b) To linearize the above set of non-linear differential equations some reasonable assumptions are made, such as that the initial concentration of the substrate and other possible ligands (e.g. inhibitors) are in excess compared with the free enzyme or vice versa, so that approximate analytical equations corresponding to the time course of any of the involved species can be derived.
(c) Sometimes additional assumptions are made to simplify the resulting approximate analytical solutions compared with the strict ones derived in step (b), e.g. the approach of the rapid equilibrium of one or more of the reversible steps (if any) or the assumption of an initial steady-state in the catalytic route of the reaction mechanism scheme. This step (c) should, in our opinion, be avoided if it is not absolutely justified, because the assumptions used in it are artificial and, moreover, they yield global constants instead of the individual rate constants.
(d) The time course of the same magnitude as that of which the analytical solution is experimentally monitored.
(e) Finally, the experimental data are fitted to the corresponding theoretical symbolic equation and the kinetic parameters are evaluated.

In different contributions about specific unstable enzyme systems, the above general procedure has been particularized in the corresponding experimental design and kinetic data analysis $[9,11,12,15,19,20,24,25,39,44,54-60]$. 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. No general procedure valid for any unstable enzyme system independently of its complexity and whether the involved enzyme is unstable or irreversibly reacting with an inhibitor, has been suggested.

This paper proposes a novel and general procedure, which is easy to apply and valid for any unstable system, based on the statistical moments of any function arising from the set of differential equations (once linearized) corresponding to the unstable enzyme system, which can be expressed as a sum of exponential terms. The ease of the procedure for mechanisms of any complexity is based on an algorithm developed in the Appendix A, which allows the systematic derivation of the analytical expressions of the j-th statistical moments of the function. This is especially useful for high j -values and or high number of exponential terms in the function.

## 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 the use of the classical fourth-order Runge-Kutta formula, but applying an adaptative stepsize control that was originally invented by Fehlberg [61-63] using the software WES implemented in Visual C++ 6.0 [64]. The above 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. 1-7, were carried out using the SigmaPlot Scientific Graphing System program, version 8.02 (2002, SPSS Inc).

## 3 Time course equations for the concentrations of the species involved in unstable enzyme systems

From the set of differential equations describing the kinetic behaviour of an unstable enzyme system, after linearization, approached (because the set of differential equations is only approximately linear) integrated analytical solutions giving the time course concentration of any involved species can be derived. From these results any other time course equation can be obtained, e.g. those corresponding to a sum of concentrations of enzyme forms, or to the first time derivative of a concentration or of a sum of concentrations, etc.


Fig. 1 Time progress curve of $v(t)$ corresponding to an enzyme system evolving according to reaction mechanism in Scheme 1 for the following set of values of the rate constants and initial enzyme and substrate concentrations: $k_{1}=10^{5} \mathrm{M}^{-1} \mathrm{~s}^{-1}, k_{-1}=3 \mathrm{~s}^{-1}, k_{2}=2 \mathrm{~s}^{-1}, k_{3}=1 \mathrm{~s}^{-1}, k_{4}=5 \mathrm{~s}^{-1},[\mathrm{E}]_{0}=1.7 \mu \mathrm{M}$ and $[\mathrm{S}]_{0}=0.5 \mathrm{mM}$. The area below the curve, from $t=0$ to $t \rightarrow \infty$, which can be obtained both numerically and analytically, coincides with the moment $M_{0}$


Fig. 2 Time progress curve of $t \cdot v(t)$ corresponding to an enzyme system evolving according to reaction mechanism in Scheme 1 for the same set of values of the rate constants and initial concentrations as in Fig. 1. The area below the curve, from $t=0$ to $t \rightarrow \infty$, which can be obtained both numerically and analytically, coincides with the moment $M_{1}$


Fig. 3 Time progress curve of $t^{2} \cdot v(t)$ corresponding to an enzyme system evolving according to reaction mechanism in Scheme 1 for the same set of values of the rate constants and initial concentrations as in Fig. 1. The area below the curve, from $t=0$ to $t \rightarrow \infty$, which can be obtained both numerically and analytically, coincides with the moment $M_{2}$

The derivation of the approximated analytical equations corresponding to an unstable enzyme system can be derived from the linearized set of differential equations by one of the following ways: [65-69]. The time course equations can also be derived by considering the reaction mechanism of the enzyme system under study as a derived mechanism of another, more complex, primitive enzyme system and introducing in the equations of the primitive mechanism the same changes that reduced it to a derived mechanism [70].


Fig. 4 Simulated time progress curves of $v(t)$ obtained from numerical integration of the set of differential equations in Appendix B corresponding to Scheme 1. In each of the curves the values of $[E]_{0}$ and the rate constants were the same as in Fig. 1. The $[\mathrm{S}]_{0}$-values used for each curve were the same as those shown in Table 2 in increasing order from curve 1 to curve 8. Inset: Details at the onset of the reaction


Fig. 5 Simulated time progress curves of $t \cdot v(t)$ obtained from numerical integration of the set of differential equations in Appendix B corresponding to Scheme 1. In each of the curves the values of $[E]_{0}$ and the rate constants were the same as those in Fig. 1. The $[\mathrm{S}]_{0}$-values used for each curve were the same as those shown in Table 2 in increasing order from curve 1 to curve 8. Inset: Details at the onset of the reaction

The time-dependent function, $f(t)$, furnishing the instantaneous concentration at time $t$ of any of the species involved in an unstable enzyme system and whose concentration varies with time (i.e. its concentrations is not assumed constant in the linearization process) is given by the general equation [65,68]:

$$
\begin{equation*}
f(t)=\beta+\sum_{h=1}^{n} \gamma_{h} e^{\lambda_{h} t} \tag{1}
\end{equation*}
$$

Fig. 6 Simulated time progress curves of $t^{2} \cdot v(t)$ obtained from numerical integration of the set of differential equations in Appendix B corresponding to Scheme 1. In each of the curves the values of $[\mathrm{E}]_{0}$ and the rate constants were the same as those in Fig. 1. The $[\mathrm{S}]_{0}$-values used for each curve were the same as those shown in Table 2 in increasing order from curve 1 to curve 8 . Inset: Details at the onset of the reaction



Fig. 7 Fit by linear regression of the experimental data $[S]_{0}$ and $M_{0}$ to Eq. 43. For simplicity we called $Z$ the left side of this equation. From this fitting $a^{\prime \prime}$ and $b^{\prime \prime}$ are immediately obtained. Inset: Details for low $[S]_{0}$-values
where $n$, according to the specific mechanism and the specific species in the mechanism can take any of the values $1,2, \ldots$. Generally, the more complex the reaction mechanism, the higher the $n$-value.

The arguments $\lambda_{h}(h=1,2, \ldots, n)$ in Eq. 1 are the roots of an equation

$$
\begin{equation*}
\lambda^{n}+F_{1} \lambda^{n-1}+\cdots+F_{n-1} \lambda+F_{n}=0 \tag{2}
\end{equation*}
$$

arising in the derivation of Eq. 1 and any of these roots can be either real and negative or complex with a negative real part.

According to the polynomial theory, among the arguments $\lambda_{h}(h=1,2, \ldots, n)$ the following very useful (see below) relationships are observed:

$$
\left.\begin{array}{c}
\lambda_{1}+\lambda_{2}+\cdots+\lambda_{n}=-F_{1}  \tag{3}\\
\lambda_{1} \lambda_{2}+\lambda_{1} \lambda_{3}+\cdots+\lambda_{n-1} \lambda_{n}=F_{2} \\
\vdots \\
\lambda_{1} \lambda_{2} \cdots \lambda_{n}=(-1)^{n} F_{n}
\end{array}\right\}
$$

We denote as $P_{q}(q=1,2, \ldots, n)$ the sum of all of the $q$-nary products of the arguments $\lambda_{h}(h=1,2, \ldots, n)$. 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:

$$
\begin{equation*}
P_{q}=(-1)^{q} F_{q}(q=0,1,2, \ldots, n) \tag{4}
\end{equation*}
$$

The amplitudes $\gamma_{h}(h=1,2, \ldots, n)$ of Eq. 1 are explicit functions of $\lambda_{h}(h=$ $1,2, \ldots, n)$, of the rate constants involved in the process and of the initial concentrations of the ligand species present at the onset of the reaction. Finally, $\beta$ is a timeindependent, non-negative quantity, the meaning of which is the value of $f(t)$ when $t$ tends to infinite, i.e.:

$$
\begin{equation*}
\beta=\lim _{t \rightarrow \infty} f(t) \tag{5}
\end{equation*}
$$

Due to the properties of the arguments $\lambda_{h}(h=1,2, \ldots, n)$ each of the exponential terms corresponding to a real tends to zero when $t \rightarrow \infty$ and each sum of two exponential terms corresponding to complex and conjugated $\lambda_{h}(h=1,2, \ldots, n)$, if any, goes to zero when $t \rightarrow \infty$ and, therefore, Eq. 5 is observed.

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

$$
\begin{equation*}
\beta=f(0)-\sum_{h=1}^{n} \gamma_{h} \tag{6}
\end{equation*}
$$

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

### 3.1 Examples of time course equations for the concentrations

As an example, we summarize in Table 1 the time course equation of the concentrations [E], [ES], $\left[\mathrm{E}_{\mathrm{i}}\right],\left[\mathrm{ES}_{\mathrm{i}}\right]$ and $[\mathrm{P}]$ of the enzyme forms $\mathrm{E}, \mathrm{ES}, \mathrm{E}_{\mathrm{i}}, \mathrm{ES}_{\mathrm{i}}$ and the product $P$ involved in unstable enzyme systems evolving according to the reaction mechanism in Scheme 1. The table also shows the concentration of the residual enzyme activity defined as the sum $[\mathrm{E}]+[\mathrm{ES}]$. To derive these equations we have assumed that the instantaneous substrate concentration, [S], remains approximately constant during the whole course of the reaction (i.e. until the active enzyme vanishes) in order to the corresponding set of differential equations describing the kinetic behaviour of the system becomes linear. The approximate constancy of [S] can be experimentally reached if the initial concentration of the substrate, $[S]_{0}$ is set much higher than the initial concentration of enzyme, $[\mathrm{E}]_{0}$. The strict equations $f(t)$ in Table 1 have been taken from Garrido del Solo et al. [19] and all of them have the common form:

$$
\begin{equation*}
f(t)=\beta+\gamma_{1} e^{\lambda_{1} t}+\gamma_{2} e^{\lambda_{2} t} \tag{7}
\end{equation*}
$$

where $\lambda_{1}$ and $\lambda_{2}$ are the roots of the equation:

$$
\begin{equation*}
\lambda^{2}+F_{1} \lambda+F_{2}=0 \tag{8}
\end{equation*}
$$

being:

$$
\begin{equation*}
F_{1}=k_{1}[\mathrm{~S}]_{0}+k_{3}+k_{-1}+k_{2}+k_{4} \tag{9}
\end{equation*}
$$

and

$$
\begin{equation*}
F_{2}=k_{3}\left(k_{-1}+k_{2}+k_{4}\right)+k_{1} k_{4}[\mathrm{~S}]_{0} \tag{10}
\end{equation*}
$$

Solving Eq. 8 we get:

$$
\begin{align*}
& \lambda_{1}=\frac{-F_{1}+\sqrt{F_{1}^{2}-4 F_{2}}}{2}  \tag{11}\\
& \lambda_{2}=\frac{-F_{1}-\sqrt{F_{1}^{2}-4 F_{2}}}{2} \tag{12}
\end{align*}
$$

Roots $\lambda_{1}$ and $\lambda_{2}$ are real and negative or complex conjugated with a negative real part, according to $F_{1}^{2} \geq 4 F_{2}$ or $F_{1}^{2}<4 F_{2}$, respectively. Between $\lambda_{1}$ and $\lambda_{2}$ the following relationships exist:

$$
\begin{equation*}
\lambda_{1}+\lambda_{2}=-F_{1} \tag{13}
\end{equation*}
$$

Table 1 Time course of the concentrations $[\mathrm{E}],[\mathrm{ES}],[\mathrm{E}]+[\mathrm{ES}],\left[\mathrm{E}_{\mathrm{i}}\right],\left[\mathrm{ES}_{\mathrm{i}}\right]$ and $[\mathrm{P}]$, assuming that the initial concentration of the substrate, $[\mathrm{S}]_{0}$, is much higher than the initial concentration, $[\mathrm{E}]_{0}$, of the free enzyme. During the whole course of the reaction, the substrate concentration remains approximately constant and the set of differential equations describing the kinetic behaviour of the unstable enzyme systems evolving according to Scheme 1 becomes (approximately) linear. In this case, all of the functions $f(t)$ explained in the main text have the form $f(t)=\beta+\gamma_{1} e^{\lambda_{1} t}+\gamma_{2} e^{\lambda_{2} t}$. In the first column the specific function $f(t)$ is indicated and in columns 2nd, 3rd and 4th the expressions corresponding to $\beta, \gamma_{1}$ and $\gamma_{2}$ are given. The parameter $V_{\max }$ involved in the expression of $\beta, \gamma_{1}$ and $\gamma_{2}$ is given by $k_{2}[\mathrm{E}]_{0}$. The expressions of the arguments $\lambda_{1}$ and $\lambda_{2}$ are those given in the main text

| $f(t)$ | $\beta$ | $\gamma_{1}$ | $\gamma_{2}$ |
| :--- | :--- | :--- | :--- |
| $[\mathrm{E}]$ | 0 | $-\frac{\lambda_{1}+k_{-1}+k_{2}+k_{4}}{\lambda_{2}-\lambda_{1}}$ | $-\frac{\lambda_{2}+k_{-1}+k_{2}+k_{4}}{\lambda_{1}-\lambda_{2}}$ |
| $[\mathrm{ES}]$ | 0 | $-\frac{k_{1}[\mathrm{~S}]_{0}[\mathrm{E}]_{0}}{\lambda_{2}-\lambda_{1}}$ | $-\frac{k_{1}[\mathrm{~S}]_{0}[\mathrm{E}]_{0}}{\lambda_{1}-\lambda_{2}}$ |
| $[\mathrm{E}]+[\mathrm{ES}]$ | 0 | $-\frac{\lambda_{1}+k_{-1}+k_{2}+k_{4}+k_{1}[\mathrm{~S}]_{0}}{\lambda_{2}-\lambda_{1}}$ | $-\frac{\lambda_{2}+k_{-1}+k_{2}+k_{4}+k_{1}[\mathrm{~S}]_{0}}{\lambda_{1}-\lambda_{2}}$ |
| $\left[\mathrm{E}_{\mathrm{i}}\right]$ | $\frac{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)[\mathrm{E}]_{0}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)+k_{1} k_{4}[\mathrm{~S}]_{0}}$ | $-\frac{k_{3}\left(\lambda_{1}+k_{-1}+k_{2}+k_{4}\right)[\mathrm{E}]_{0}}{\lambda_{1}\left(\lambda_{2}-\lambda_{1}\right)}$ | $-\frac{k_{3}\left(\lambda_{2}+k_{-1}+k_{2}+k_{4}\right)[\mathrm{E}]_{0}}{\lambda_{2}\left(\lambda_{1}-\lambda_{2}\right)}$ |
| $\left[\mathrm{ES}_{\mathrm{i}}\right]$ | $\frac{k_{1} k_{4}[\mathrm{~S}]_{0}[\mathrm{E}]_{0}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)+k_{1} k_{4}[\mathrm{~S}]_{0}}$ | $-\frac{k_{1} k_{4}[\mathrm{~S}]_{0}[\mathrm{E}]_{0}}{\lambda_{1}\left(\lambda_{2}-\lambda_{1}\right)}$ | $-\frac{k_{1} k_{4}[\mathrm{~S}]_{0}[\mathrm{E}]_{0}}{\lambda_{2}\left(\lambda_{1}-\lambda_{2}\right)}$ |
| $[\mathrm{P}]$ | $\frac{k_{1} V_{\max }[\mathrm{S}]_{0}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)+k_{1} k_{4}[\mathrm{~S}]_{0}}$ | $-\frac{k_{1} V_{\max }[\mathrm{S}]_{0}}{\lambda_{1}\left(\lambda_{2}-\lambda_{1}\right)}$ | $-\frac{k_{1} V_{\max }[\mathrm{S}]_{0}}{\lambda_{2}\left(\lambda_{1}-\lambda_{2}\right)}$ |

and

$$
\begin{equation*}
\lambda_{1} \lambda_{2}=F_{2} \tag{14}
\end{equation*}
$$

The expressions of $\beta, \gamma_{1}$ and $\gamma_{2}$ are given in Table 1.

### 3.2 Definition of the function $g(t)$ associated with the function $f(t)$

We will define the function $g(t)$ associated with the function $f(t)$ as follows:

$$
g(t)= \begin{cases}f(t) & \text { if } \beta=0  \tag{15}\\ \frac{\mathrm{~d} f(t)}{\mathrm{d} t} & \text { if } \beta \neq 0\end{cases}
$$

i.e.

$$
\begin{equation*}
g(t)=\sum_{h=1}^{n} \delta_{h} e^{\lambda_{h} t} \tag{16}
\end{equation*}
$$

where:

$$
\delta_{h}= \begin{cases}\gamma_{h} & \text { if } \beta=0  \tag{17}\\ \lambda_{h} \gamma_{h} & \text { if } \beta \neq 0\end{cases}
$$

## 4 Statistical moments of $g(t)$

From the well know definition of statistical moment of order j of a function [63,7173], the $j$-th $(j=0,1,2, \ldots)$ statistical moment (which, for ease, we will denote as $M_{j}$ ) of any of the above defined functions $g(t)$ is given by:

$$
\begin{equation*}
M_{j}=\int_{0}^{\infty} t^{j} g(t) \mathrm{d} t(j=0,1,2, \ldots) \tag{18}
\end{equation*}
$$

where, for completeness, we have included the value $j=0$.
The $j$-th statistical moment $M_{j}$ can be obtained either as a symbolic expression from the symbolic expression of $g(t)$ and the analytical integration indicated in Eq. 18 , or in a numerical way from the experimental time course of $g(t)$ and taking into account that the integral on the right hand side of Eq. 18 coincides with the area below the curve $t^{j} \cdot g(t)$ between $t=0$ and $t \rightarrow \infty(t \rightarrow \infty$ must be interpreted as a time, arbitrarily chosen by the worker, at which $\left.t^{j} \cdot g(t) \rightarrow 0\right)$.

If in Eq. 18 we insert Eq. 16, we have:

$$
\begin{equation*}
M_{j}=\sum_{h=1}^{n} \delta_{h}\left(\int_{0}^{\infty} t^{j} e^{\lambda_{h} t} d t\right) \quad(j=0,1,2, \ldots) \tag{19}
\end{equation*}
$$

The integral in Eq. 19 is the well known Gamma Function [63] and it is given by:

$$
\begin{equation*}
\int_{0}^{\infty} t^{j} e^{\lambda_{h} t} \mathrm{~d} t=\frac{j!}{\left(-\lambda_{h}\right)^{j+1}}\left[\operatorname{Re}(j)>-1, \operatorname{Re}\left(\lambda_{h}\right)<0\right] \tag{20}
\end{equation*}
$$

Hence, Eq. 19 can be written as:

$$
\begin{equation*}
M_{j}=(-1)^{j+1} j!\sum_{h=1}^{n} \frac{\delta_{h}}{\lambda_{h}^{j+1}} \quad(j=0,1,2, \ldots) \tag{21}
\end{equation*}
$$

If the corresponding expressions of $\delta_{h}(h=1,2, \ldots, n)$ are inserted in Eq. 21, then the sum indicated is carried out and, if the relationship between the arguments $\lambda_{h}$ ( $h=1,2, \ldots n$ ), given by Eq. 3, is taken into account, then, we obtain $M_{j}$ in terms of the kinetic parameters involved in the reaction mechanism scheme and the initial concentrations of the species present at the onset of the reaction.
4.1 Example: some statistical moments of the function $\mathrm{d}[\mathrm{P}] / \mathrm{d} t$ corresponding to the example in Scheme 1 under the condition of limiting enzyme

As an example we next derived the statistical moments $M_{0}, M_{1}$ and $M_{2}$ corresponding to the time function $\mathrm{d}[\mathrm{P}] / \mathrm{d} t$ corresponding to Scheme 1. The function $[\mathrm{P}]$ [i.e. $f(t)]$ furnishing the time product accumulation is given by Eq. 7, where the expressions of $\beta, \gamma_{1}$ and $\gamma_{2}$ are given in Table 1. If we derive [P] with respect to time, we obtain the function $g(t)$, i.e. the instantaneous rate, $v$, of product formation, $P$, given by:

$$
\begin{equation*}
g(t)=\delta_{1} e^{\lambda_{1} t}+\delta_{2} e^{\lambda_{2} t} \tag{22}
\end{equation*}
$$

where

$$
\begin{equation*}
\delta_{1}=\gamma_{1} \lambda_{1} \tag{23}
\end{equation*}
$$

and

$$
\begin{equation*}
\delta_{2}=\gamma_{2} \lambda_{2} \tag{24}
\end{equation*}
$$

Expression of $M_{0}$. From Eq. 21 with $n=2$ and $j=0$ and from Eqs. 23 to 24 and the expression of $\gamma_{1}$ and $\gamma_{2}$ in the last row of Table 1, we obtain:

$$
\begin{equation*}
M_{0}=k_{1} V_{\max }[\mathrm{S}]_{0}\left\{\frac{1}{\lambda_{1}\left(\lambda_{2}-\lambda_{1}\right)}+\frac{1}{\lambda_{2}\left(\lambda_{1}-\lambda_{2}\right)}\right\} \tag{25}
\end{equation*}
$$

where the parameter $V_{\max }$ is given by

$$
\begin{equation*}
V_{\max }=k_{2}[\mathrm{E}]_{0} \tag{26}
\end{equation*}
$$

If the indicated algebraic "operations" are carried out and Eqs. 14 and 10 are taken into account, then the following results:

$$
\begin{equation*}
M_{0}=\frac{k_{1} V_{\max }[\mathrm{S}]_{0}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)+k_{1} k_{4}[\mathrm{~S}]_{0}} \tag{27}
\end{equation*}
$$

$M_{0}$ can be obtained graphically because it coincides with the area below the time progress curve of the rate, $v$, of the product accumulation, as shown in Fig. 1 for a simulated time progress curve obtained from the arbitrary set of values of the rate constants and $[\mathrm{S}]_{0}$ indicated in the figure caption.

By dividing both numerator and denominator from the right side of Eq. 27 by $k_{3}\left(k_{-1}+k_{2}+k_{4}\right)$ we get:

$$
\begin{equation*}
M_{0}=\frac{b V_{\max }[\mathrm{S}]_{0}}{1+c[\mathrm{~S}]_{0}} \tag{28}
\end{equation*}
$$

$b$ and $c$ being:

$$
\begin{align*}
b & =\frac{k_{1}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)}  \tag{29}\\
c & =\frac{k_{1} k_{4}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)} \tag{30}
\end{align*}
$$

Expression of $M_{1}$. Hence, from Eq. 21 with $n=2$ and $j=1$ Eqs. 23 and 24 and the expression of $\gamma_{1}$ and $\gamma_{2}$ on the last row in Table 1, one obtains:

$$
\begin{equation*}
M_{1}=-k_{1} V_{\max }[\mathrm{S}]_{0}\left\{\frac{1}{\lambda_{1}^{2}\left(\lambda_{2}-\lambda_{1}\right)}+\frac{1}{\lambda_{2}^{2}\left(\lambda_{1}-\lambda_{2}\right)}\right\} \tag{31}
\end{equation*}
$$

Carrying out the sum indicated in Eq. 31 and taking into account Eqs. 13, 14, 9 and 10 we obtain:

$$
\begin{equation*}
M_{1}=\frac{k_{1} V_{\max }\left(k_{-1}+k_{2}+k_{3}+k_{4}+k_{1}[\mathrm{~S}]_{0}\right)[\mathrm{S}]_{0}}{\left\{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)+k_{1} k_{4}[\mathrm{~S}]_{0}\right\}^{2}} \tag{32}
\end{equation*}
$$

By dividing both numerator and denominator on the right side of Eq. 32 by $k_{3}^{2}\left(k_{-1}+\right.$ $\left.k_{2}+k_{4}\right)^{2}$, we obtain:

$$
\begin{equation*}
M_{1}=\frac{b V_{\max }\left(a^{\prime}+b[\mathrm{~S}]_{0}\right)[\mathrm{S}]_{0}}{\left(1+c[\mathrm{~S}]_{0}\right)^{2}} \tag{33}
\end{equation*}
$$

where $b$ and $c$ are given by Eqs. 29 and 30 and $a^{\prime}$ by:

$$
\begin{equation*}
a^{\prime}=\frac{k_{-1}+k_{2}+k_{3}+k_{4}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)} \tag{34}
\end{equation*}
$$

$M_{1}$ can be obtained graphically because it coincides with the area below the time progress curve $t \cdot v$, as shown in Fig. 2 for a simulated time progress curve obtained from the same set of values of the rate constants and initial concentration of the substrate as used in Fig. 1.

Expression of $M_{2}$. Finally, the expression for the moment $M_{2}$ can be derived. From Eq. 21 with $n=2$ and $j=2$, Eqs. 23 and 24 and the expression of $\gamma_{1}$ and $\gamma_{2}$ on the last row in Table 1, one obtains:

$$
\begin{equation*}
M_{2}=2 k_{1} V_{\max }[\mathrm{S}]_{0}\left\{\frac{1}{\lambda_{1}^{3}\left(\lambda_{2}-\lambda_{1}\right)}+\frac{1}{\lambda_{2}^{3}\left(\lambda_{1}-\lambda_{2}\right)}\right\} \tag{35}
\end{equation*}
$$

Carrying out the indicated sum in above Eq. 35, taking Eqs. 13 and 14 into account, as well as some algebraic considerations, gives:

$$
\begin{equation*}
M_{2}=2 k_{1} V_{\max }[\mathrm{S}]_{0} \frac{F_{1}^{2}-F_{2}}{F_{2}^{3}} \tag{36}
\end{equation*}
$$

where $F_{1}$ and $F_{2}$ are given by Eqs. 9 and 10 .
By dividing both numerator and denominator from the right side of Eq. 36 by $k_{3}^{3}\left(k_{-1}+k_{2}+k_{4}\right)^{3}$, one obtains:

$$
\begin{equation*}
M_{2}=\frac{2 b V_{\max }\left(a^{\prime \prime}+b^{\prime \prime}[\mathrm{S}]_{0}+b^{2}[\mathrm{~S}]_{0}^{2}\right)[\mathrm{S}]_{0}}{\left(1+c[\mathrm{~S}]_{0}\right)^{3}} \tag{37}
\end{equation*}
$$

where:

$$
\begin{gather*}
a^{\prime \prime}=\frac{1}{k_{3}^{2}}+\frac{a^{\prime}}{k_{-1}+k_{2}+k_{4}}  \tag{38}\\
b^{\prime \prime}=b\left(a^{\prime}+\frac{k_{-1}+k_{2}+k_{3}}{k_{3}\left(k_{-1}+k_{2}+k_{4}\right)}\right) \tag{39}
\end{gather*}
$$

$M_{2}$ can be obtained graphically because it coincides with the area below the time progress curve $t^{2} \cdot v$, as shown in Fig. 3 for a simulated time progress curve obtained from the same set of values of the rate constants and initial concentration of the substrate as used in Fig. 1.

Equations 25, 31 and 35 contains expressions like:

$$
\begin{equation*}
\sum_{h=1}^{2} \frac{1}{\lambda_{h}^{r}\left(\lambda_{p}-\lambda_{h}\right)}(p \neq h ; r=1,2,3, \ldots) \tag{40}
\end{equation*}
$$

which can be solved individually and simply, except, perhaps expression corresponding to Eq. 35. Nevertheless, in the analysis of statistical moments of other more complex unstable enzyme systems, the corresponding expression of Eq. 40 might be more difficult to obtain in spite of it having the same form. However, an algorithm [17,74,75] exits which allows the expressions like:

$$
\begin{equation*}
\sum_{h=1}^{n} \frac{1}{\lambda_{h}^{r} \prod_{\substack{p=1 \\ p \neq h}}^{n}\left(\lambda_{p}-\lambda_{h}\right)} \quad(n=1,2,3, \ldots ; r \text { is an integer number }) \tag{41}
\end{equation*}
$$

to be easily obtained.
In Appendix A we summarize how to express a sum of the type in Eq. 41 as a function of the coefficients $F_{1}, F_{2}, \ldots, F_{n}$ involved in polynomial $F(s)$. This expression
depends on the relative values of $n$ and $r$ and on the fact that $r$ is negative, positive or zero.

## 5 Description of the suggested method

In the description of the method proposed is assumed the existence of only one ligand species at the onset of the reaction, e.g. the substrate, S. The method is easily extrapolable to those cases in which two or more ligand species exist (e.g. a substrate and an inhibitor, two substrates, etc.). This method consists of the following steps:
(1) A species involved in the reaction mechanism scheme is chosen whose time variation can be monitored experimentally with the media available, such as the concentration of an enzyme form, the product accumulation, etc. It is also possible to choose two or more species whose sum of concentrations, e.g. the residual activity in some cases, can be monitored.
(2) The corresponding function $f(t)$ is derived, see Eq. 7, or one is taken from literature.
(3) The function $g(t)$, see Eq. 16, associated with $f(t)$ is derived.
(4) Different curves $g(t)$ at a fixed value of the initial enzyme concentration and at different values of the initial concentration of the substrate are determined experimentally.
(5) From each of the above curves (i.e. for each of the $[\mathrm{S}]_{0}$-values used) one numerically determines the area below the curve, i.e. the corresponding statistical moment $M_{0}$.
(6) Using Eqs. 21 and 17 and the corresponding expressions of Eqs. 27 and 28, the analytical expression of $M_{0}$ is determined. This will depend on $[\mathrm{S}]_{0}$ through certain parameters, each of them related with the individual rate constants involved in the scheme of reaction mechanism.
(7) The curves $t \cdot g(t)$ are determined from the different experimental curves $g(t)$ corresponding to each of the $[\mathrm{S}]_{0}$-values obtained in above point (4).
(8) From each of the above curves (i.e. for each of the $[\mathrm{S}]_{0}$-values used) one numerically determines the area below the curve, i.e. the corresponding statistical moment $M_{1}$.
(9) Using Eqs. 21 and 17 and the corresponding expressions Eqs. 32 and 33 one determines the analytical expression of $M_{1}$, which will depend on $[\mathrm{S}]_{0}$ through certain parameters, each of them related with the individual rate constants involved in the scheme of reaction mechanism by one equation.
(10) The above processes in steps (7)-(9) is repeated, obtaining the time progress curves $t^{2} \cdot g(t), t^{3} \cdot g(t), \ldots$, until a the sufficient number of equations relating the overall parameters involved in the expression of the moments $M_{j}$ ( $j=$ $0,1,2, \ldots$ ) with the individual rate constants are obtained.
(11) From here the strategy to be followed depends on the form of the specific expressions for these moments, which, in its turn, depends on the scheme of the reaction mechanism under study. But generally, the procedure to be used consists of fitting by non-linear regression the experimental data of $M_{0}$ for each $[\mathrm{S}]_{0}$-value and using the parameters obtained in this fitting in the next fitting involving

Table 2 Values of the statistical moments $M_{0}, M_{1}$ and $M_{2}$ corresponding to the time progress curve $v(t)=\mathrm{d}[\mathrm{P}] / \mathrm{d} t$ for the reaction mechanism in Scheme 1 simulated using the same values of the rate constants and initial concentration of enzyme as in Fig. 1 and the initial substrate concentration indicated in first column

| $[\mathrm{S}]_{0}(\mathrm{mM})$ | $M_{0} \times 10^{7}(\mathrm{M})$ | $M_{1} \times 10^{7}(\mathrm{Ms})$ | $M_{2} \times 10^{7}\left(\mathrm{M} \mathrm{s}^{2}\right)$ |
| :--- | :--- | :--- | :--- |
| 0.01 | 2.2037 | 1.7608 | 2.5463 |
| 0.1 | 5.6561 | 1.9864 | 1.2072 |
| 0.2 | 6.1784 | 1.7438 | 0.8719 |
| 0.5 | 6.5378 | 1.5344 | 0.6699 |
| 1 | 6.6666 | 1.4511 | 0.6056 |
| 2 | 6.7327 | 1.4066 | 0.5744 |
| 4 | 6.7662 | 1.3835 | 0.5591 |
| 8 | 6.7831 | 1.3718 | 0.5515 |

the experimental $M_{1}$-values at each $[\mathrm{S}]_{0}$-value and the corresponding symbolic expression for $M_{1}$. Then the results obtained in this fitting are used in the next fitting involving the experimental $M_{2}$-values at each $[\mathrm{S}]_{0}$-value and the corresponding symbolic expression for $M_{2}$, etc. until all of the individual rate constants can be evaluated.

### 5.1 Numerical example

Next we illustrate the general procedure above to the specific Scheme 1 of the reaction mechanism of an unstable system. The time progress curves were obtained by numerical integration of the set of differential equations in Appendix B, which describes the kinetic behaviour of the system. The values of the rate constants used in the simulation are those indicated in Fig. 1 and are the same as those used by Garrido del Solo et al. [19] in a contribution also concerning the evaluation of the rate constants involved in Scheme 1, but using a different method. In all the simulated progress curves $[E]_{0}$ was $1.7 \mu \mathrm{M}$ and the values of $[\mathrm{S}]_{0}$ used for each simulated curve are indicated in the legend to the corresponding figure.

The same steps (1)-(11) as in the general procedure are followed.
(1) Product $P$ is the species to be monitored.
(2) The function $f(t)$ is [P], given by Eq. 7, with the expressions of $\beta, \gamma_{1}$ and $\gamma_{2}$ given in Table 1 and the relationships between $\lambda_{1}$ and $\lambda_{2}$ given by Eqs. 13 and 14.
(3) Because $\beta \neq 0$, is $g(t)=\mathrm{d} f(t) / \mathrm{d} t$, see Eq. 15, i.e. $g(t)=\mathrm{d}[P] / \mathrm{d} t=v(t)$.
(4) Figure 4 shows different simulated time progress curves $v(t)$ versus $t$ at a fixed value of the initial enzyme concentration and at different values of the initial concentration of the substrate.
(5) Table 2 summarizes the areas below each of the curves in step (4), i.e. the $M_{0}$-value for each of the $[\mathrm{S}]_{0}$-values used.
(6) The analytical expression of $M_{0}$ showing its dependence on $[\mathrm{S}]_{0}$ is given by Eq. 28. The parameters $b$ and $c$ involved in this equation are related with the individual rate constants by Eqs. 29 and 30.
(7) Figure 5 shows different simulated time progress curves $t \cdot v(t)$ versus $t$ obtained from the different experimental curves $v(t)$ in step (4).
(8) Table 2 summarizes the areas below each of the curves in step (7), i.e. the $M_{1}$-value for each of the $[\mathrm{S}]_{0}$-values used.
(9) The analytical expression of $M_{1}$ showing its dependence on [ S$]_{0}$ is given by Eq. 33. The parameters $a^{\prime}, b$ and $c$ involved in this equation are related with the individual rate constants by Eqs. 34, 29 and 30.
(10) Steps (7)-(9) are repeated to obtain the progress curves $t^{2} \cdot v(t)$ versus $t$ from those of $v(t)$ versus $t$. In Fig. 6 we show different simulated time progress curves $t^{2} \cdot v(t)$ versus $t$ obtained from the different experimental curves $v(t)$ in step (4). Table 2 summarizes the areas below each of the curves in this step, i.e. the $M_{2}$-value for each of the $[\mathrm{S}]_{0}$-values used. The analytical expression of $M_{2}$ showing its dependence on $[\mathrm{S}]_{0}$ is given by Eq. 37. The parameters $a^{\prime \prime}, b^{\prime \prime}, b$ and $c$ involved in this equation are related with the individual rate constants by Eqs. $38,39,29$ and 30.
(11) From here we suggest the following procedure to provide the individual rate constants.

### 5.1.1 Procedure

The procedure consists of the following six steps:
(i) The experimental data of $M_{0}$ for each $[\mathrm{S}]_{0}$ are fitted to the two parameters rational Eq. 28 and, from this, the value of $b, V_{\max }$ and $c$ which are given in Table 3 can be estimated.
(ii) Because Eq. 33 can also be written as:

$$
\begin{equation*}
\frac{M_{1}\left(1+c[\mathrm{~S}]_{0}\right)^{2}}{b V_{\max }[\mathrm{S}]_{0}}=a^{\prime}+b[\mathrm{~S}]_{0} \tag{42}
\end{equation*}
$$

a plot of the experimental data $M_{1}\left(1+c[\mathrm{~S}]_{0}\right)^{2} /\left(b V_{\max }[\mathrm{S}]_{0}\right)$ and $[\mathrm{S}]_{0}$ to Eq. 42 allows us to estimate $a^{\prime}$ and b . Thus, steps (i) and (ii) provide the values of $b$, $V_{\text {max }}, c$ and $a^{\prime}$.

Table 3 Values of the parameters $V_{\max }, b, c, a^{\prime}, a^{\prime \prime}$ and $b^{\prime \prime}$ obtained from the procedure proposed in the main text for the reaction mechanism in Scheme 1. In the third column is indicated the equation relating the corresponding parameter with the rate constants. In the 4th column the values of these parameters obtained from the rate constants used in the simulations are shown

| Parameter | Determined value | Equation | True value |
| :--- | :--- | :--- | :--- |
| $V_{\max }\left(\mathrm{M} \mathrm{s}^{-1}\right)$ | $(3.523 \pm 0.009) \times 10^{-6}$ | 26 | $3.4 \times 10^{-6}$ |
| $b\left(\mathrm{M}^{-1} \mathrm{~s}\right)$ | $(0.9815 \pm 0.0022) \times 10^{4}$ | 29 | $10^{4}$ |
| $c\left(\mathrm{M}^{-1}\right)$ | $(4.910 \pm 0.010) \times 10^{4}$ | 30 | $5 \times 10^{4}$ |
| $a^{\prime}(\mathrm{s})$ | $1.0933 \pm 0.0007$ | 34 | 1.1 |
| $a^{\prime \prime}(\mathrm{s})$ | $1.1071 \pm 0.0024$ | 38 | 1.11 |
| $b^{\prime \prime}\left(\mathrm{M}^{-1} \mathrm{~s}\right)$ | $(1.6565 \pm 0.0008) \times 10^{4}$ | 39 | $1.7 \times 10^{4}$ |

Table 4 Values of the rate constants $k_{1}, k_{-1}, k_{2}, k_{3}$ and $k_{4}$ obtained from the procedure proposed for the reaction mechanism in Scheme 1. The value of $k_{2}$ can only be obtained if $[E]_{0}$ is known ( $[\mathrm{E}]_{0}=1.7 \mu \mathrm{M}$ in all of the simulations) using Eq. 26 and the $V_{\text {max }}$-value in Table 3. In the last column the values obtained for the rate constants by Garrido del Solo et al. are also indicated

| Rate constant | Determined values | True values | Values obtained by Garrido <br> del Solo et al. [19] |
| :--- | :--- | :--- | :--- |
| $k_{1}\left(\mathrm{M}^{-1} \mathrm{~s}^{-1}\right)$ | $(0.986 \pm 0.009) \times 10^{5}$ | $10^{5}$ | $(1.01 \pm 0.06) \times 10^{5}$ |
| $k_{-1}\left(\mathrm{~s}^{-1}\right)$ | $2.928 \pm 0.005$ | 3 | $2.85 \pm 1.93$ |
| $k_{2}\left(\mathrm{~s}^{-1}\right)$ | $2.072 \pm 0.005$ | 2 | $2.00 \pm 0.26$ |
| $k_{3}\left(\mathrm{~s}^{-1}\right)$ | $1.001 \pm 0.004$ | 1 | $0.90 \pm 0.62$ |
| $k_{4}\left(\mathrm{~s}^{-1}\right)$ | $5.003 \pm 0.015$ | 5 | $5.00 \pm 0.63$ |

(iii) Because Eq. 37 can also be written as:

$$
\begin{equation*}
\frac{M_{2}\left(1+c[\mathrm{~S}]_{0}\right)^{3}}{2 b V_{\max }[\mathrm{S}]_{0}}-b^{2}[\mathrm{~S}]_{0}^{2}=a^{\prime \prime}+b^{\prime \prime}[\mathrm{S}]_{0} \tag{43}
\end{equation*}
$$

a plot of the experimental data $M_{2}\left(1+c[\mathrm{~S}]_{0}\right)^{3} /\left(2 b V_{\max }[\mathrm{S}]_{0}\right)-b^{2}[\mathrm{~S}]_{0}^{2}$ and $[\mathrm{S}]_{0}$ to Eq. 43 allows us to estimate $a^{\prime \prime}$ and $b^{\prime \prime}$, as shown in Fig. 7.
(iv) After steps (i)-(iii) the values of $V_{\max }, b, c, a^{\prime}, a^{\prime \prime}$ and $b^{\prime \prime}$ are known, and they are shown Table 3 for our numerical example.
(v) $V_{\max }$ has already been evaluated in step (ii). If moreover the initial enzyme concentration $[\mathrm{E}]_{0}$ is known, then from Eq. $26 k_{2}$ is immediately obtained.
(vi) From the values of $b, c, a^{\prime}, a^{\prime \prime}$ and $b^{\prime \prime}$ and Eqs. 29, 30, 34, 38 and 39, the individual rate constants $k_{1}, k_{-1}, k_{3}$ and $k_{4}$ can be evaluated by solving the corresponding set of algebraic equations. These values are given in Table 4, where also the true values of these rate constants used for the numerical integration are shown. Furthermore in this table are indicated the corresponding values obtained using another more complicated method developed by Garrido del Solo et al. [19] based on the fit of the biexponential product accumulation equation. Note that the values obtained for the rate constants using our novel method are considerably more accurate than those given in the above mentioned contribution.

## 6 Results and discussion

The main purpose of any kinetic analysis of an enzyme system, unstable systems, is the evaluation of the individual rate constants. The evaluation of these kinetic parameters involves, first of all, the experimentally obtaining the time progress curves of any of the involved species or set of species (e.g. the residual activity equal to the sum of active enzyme forms). Then, the progress curves obtained are fitted to the corresponding theoretical equation, which generally consists in a polynomial part [consisting of the term $\beta$ in unstable systems] and an exponential part consisting either of one exponential term or a sum of exponential terms. From these fittings one evaluates, in principle,
the kinetic parameters involved or algebraic combinations of them [9, 25,76]. Nevertheless, in many cases, e.g. when these equations contain two or more exponential terms, fitting is not easy and indeed may be impossible. To minimize these problems there exists, as mentioned above, different strategies leading to the derivation of a simpler time course equation which the experimental data fit better. Obviously, from the simplified equations the results can quite considerably differ from the real ones.

In this paper, we propose a novel method for evaluating the individual rate constants involved in any scheme of unstable enzyme reactions, irrespective of its complexity and, therefore, of the number of exponential terms of the time-course equations corresponding to the species to be experimentally monitored. This method is based on the numerical and analytical evaluation of the $j$-th $(j=0,1,2, \ldots)$ statistical moments of the function $g(t)$ defined above. The number of moments to be taken depends on the mechanism complexity. This method has, in our opinion, the following advantages:
(1) The method is applicable to any unstable enzyme system independently of its complexity and, therefore, of the number of exponential terms of the function $f(t)$ corresponding to the enzyme species o sum of concentrations of enzyme species to be monitored.
(2) The $j$-th statistical moments corresponding to the experimental progress curves $g(t)$ are easy to evaluate, e.g. using numerical methods such as the trapezoidal method [63].
(3) The analytical expression of the $j$-th statistical moment of the function $g(t)$ is easy to derive from Eq. 21, the corresponding expressions of the amplitudes $\delta_{h}(h=1,2, \ldots, n)$ and the relationships between the arguments $\lambda_{h}(h=$ $1,2, \ldots, n)$ corresponding to the time course equation of the specific species in the unstable enzyme system under study. By obtaining the statistical moments $M_{j}(j=0,1,2, \ldots)$ to express them as a function of the individual rate constants and initial concentrations appear intermediate expressions like Eq. 25.
(4) The fit of experimental data to rational equations such as the corresponding ones to the $j$-th statistical moments, $M_{j}(j=0,1,2, \ldots)$ is much more reliable than the fit to an multiexponential time course equation as it is now usually made [19,76].
(5) The method is also applicable to the simplified time course equations.
(6) The method suggested here is independent of the relative values of the rate constants involved in the scheme of reaction mechanism of the unstable system. Thus, if these values would be $k_{1}=10^{6} \mathrm{M}^{-1} \mathrm{~s}^{-1}, k_{-1}=100 \mathrm{~s}^{-1}, k_{2}=5 \mathrm{~s}^{-1}, k_{3}=$ $4 \mathrm{~s}^{-1}, k_{4}=2 \mathrm{~s}^{-1}$, we would obtain the values of rate constants in Table 5.
(7) The procedure suggested can by extrapolated for application to any stable enzyme systems as we now summarily point out. Effectively, for a stable enzyme system the time course equation of the concentration of any species as the general form:

$$
\begin{equation*}
C=\beta+\alpha_{1} t+\alpha_{2} t^{2}+\cdots \alpha_{r} t^{r}+\sum_{h=1}^{n} \gamma_{h} e^{\lambda_{h} t} \tag{44}
\end{equation*}
$$

If we take the $(r+1)$-th time derivative in Eq. 44, and we denote $d^{r+1} C / \mathrm{d} t^{r+1}$ as $g(t)$, we obtain:

Table 5 Values of the rate constants $k_{1}, k_{-1}, k_{2}, k_{3}, k_{4}$ and $K_{M}$ obtained using the procedure proposed for the reaction mechanism in Scheme 1. The method suggested here is independent of the relative values of the rate constants involved in the reaction mechanism scheme of the unstable system The value of $k_{2}$ can only be obtained if $[\mathrm{E}]_{0}$ is known ( $[\mathrm{E}]_{0}=0.1 \mu \mathrm{M}$ in all of the simulations) using Eq. 26

| Rate constant | Determined value | True value |
| :--- | :--- | :---: |
| $k_{1}\left(\mathrm{M}^{-1} \mathrm{~s}^{-1}\right)$ | $(1.022 \pm 0.008) \times 10^{6}$ | $10^{6}$ |
| $k_{-1}\left(\mathrm{~s}^{-1}\right)$ | $99.98 \pm 0.28$ | 100 |
| $k_{2}\left(\mathrm{~s}^{-1}\right)$ | $5.0000 \pm 0.0007$ | 5 |
| $k_{3}\left(\mathrm{~s}^{-1}\right)$ | $4.0981 \pm 0.0013$ | 4 |
| $k_{4}\left(\mathrm{~s}^{-1}\right)$ | $1.9996 \pm 0.0004$ | 2 |

$$
\begin{equation*}
g(t)=\frac{d^{r+1} C}{\mathrm{~d} t^{r+1}}=\sum_{h=1}^{n} \delta_{h} e^{\lambda_{h} t} \tag{45}
\end{equation*}
$$

where

$$
\begin{equation*}
\delta_{h}=\lambda_{h}^{r+1} \gamma_{h} \tag{46}
\end{equation*}
$$

Note that Eq. 45 is identical to Eq. 16 and, therefore the method can be used (whenever all of the arguments $\lambda_{h}$ are real negative or complex with a negative real part, as it is usual in most of the enzyme systems). Nevertheless in these cases the time taken as $t \rightarrow \infty$ must be the maximum $t$-value at which approached Eq. 5 is valid.

But 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 $t \rightarrow \infty$. Obviously the condition $t \rightarrow \infty$ is not attachable and, so, an error, albeit very small, always occur when the worker chooses what is a finite time as $t \rightarrow \infty$. In our numerical example the criterion we used to choose a finite time as $t \rightarrow \infty$ was the higher of these two times: the time needed to the residual activity, i.e. $[\mathrm{E}]+[\mathrm{ES}]$, becomes $10^{-6}[E]_{0}$ or the time needed to the rate of product formation reaches a value equal to $10^{-4}$ times its maximum value but any other criterion may also be acceptable whenever at the time chosen as $t \rightarrow \infty$ is observed that $t^{j} \cdot g(t) \rightarrow 0$.

Another disadvantage is that the set of algebraic equations to be solved to obtain the rate constants could be not linear and, so a quadratic, cubic or higher degree equation appears, which must be solved and some absurd solutions must be discarded. Nevertheless, the actual Mathematica symbolic software packages may considerably help in this mechanical task.

## Appendix A

In this Appendix we resume the results obtained [75] for sums, such as those indicated in Eq. 41 in the main text which appear in our analysis of dynamic linear time-invariant systems.

In the sum types indicated in Eq. 41, $n$ is an integer number higher than unity (i.e. $n=2,3 \ldots$ ), $r$ is any positive, negative or null integer number and $\lambda_{h}$ and $\lambda_{p}$ ( $h, p=1,2,3, \ldots, n$ ) are different, non-null, complex numbers. For ease, we will denote expressions like those in Eq. 41 as $Q(n, r)$, i.e.:

$$
\begin{equation*}
Q(n, r) \equiv \sum_{h=1}^{n} \frac{1}{s_{h}^{r} \prod_{\substack{p=1 \\ p \neq h}}^{n}\left(\lambda_{p}-\lambda_{h}\right)} \tag{A.1}
\end{equation*}
$$

We have shown (derivation not given) the following summarized results:

$$
Q(n, r)= \begin{cases}\frac{R(n, r)}{P_{n}^{r}} & \text { if } r \geq 0  \tag{A.2}\\ (-1)^{n-1} R^{\prime}(n, r) & \text { if } r<0\end{cases}
$$

where:

$$
\begin{equation*}
R(n, 0)=0 \tag{A.3}
\end{equation*}
$$

$$
\begin{equation*}
R(n, 1)=1 \tag{A.4}
\end{equation*}
$$

$$
R(n, r)=\left|\begin{array}{cccc}
P_{n-1} & P_{n-2} & \cdots & P_{n-r+1}  \tag{A.5}\\
P_{n} & P_{n-1} & \cdots & P_{n-r+2} \\
0 & P_{n} & \cdots & P_{n-r+3} \\
\cdot & \cdot & \cdots & \cdot \\
\cdot & \cdot & \cdots & \cdot \\
\cdot & \cdot & \cdots & \cdot \\
0 & 0 & \cdots & P_{n-1}
\end{array}\right| \quad \text { if } r>1
$$

$$
\begin{equation*}
R^{\prime}(n, r)=0 \text { if }-r<n-1 \tag{A.6}
\end{equation*}
$$

$$
\begin{equation*}
R^{\prime}(n, r)=1 \quad \text { if }-r=n-1 \tag{A.7}
\end{equation*}
$$

$$
R^{\prime}(n, r)=\left|\begin{array}{ccccc}
P_{1} & 1 & 0 & \cdots & 0  \tag{A.8}\\
P_{2} & P_{1} & 1 & \cdots & 0 \\
P_{3} & P_{2} & P_{1} & \cdots & 0 \\
\cdot & \cdot & \cdot & \cdots & \cdot \\
\cdot & \cdot & \cdot & \cdots & \cdot \\
\cdot & \cdot & \cdot & \cdots & \cdot \\
P_{-(n+r-1)} & P_{-(n+r)} & P_{-(n+r-1)} & \cdots & P_{1}
\end{array}\right| \text { if }-r>n-1
$$

In the Eqs. (A5) and (A8), $P_{v}(v=1,2, \ldots, n)$ is equal to the sum of all the $v$ nary ( $v \leq n$ ) products distinct from $\lambda_{1}, \lambda_{2}, \ldots, \lambda_{n}$. Always $P_{0}$ appears it must be set $P_{0}=1$.

From the definition of $P_{v}(v=1,2, \ldots, n)$ and Eq. 4, we obtain:

$$
\begin{equation*}
P_{v}=(-1)^{v} F_{v} \tag{A.9}
\end{equation*}
$$

Therefore, $R(n, r)$ (for $r>1$ ) and $R^{\prime}(n, r)$ (for $-r>n-1$ ) can be expressed in terms of the coefficients $F_{1}, F_{2}, \ldots, F_{\mathrm{n}}$ of Eq. 2, rather than in terms of its roots $\lambda_{1}, \lambda_{2}, \ldots, \lambda_{\mathrm{n}}$.

## Appendix B

Set of differential equations describing the kinetic behaviour of the unstable enzyme system evolving according to the reaction mechanism in Scheme 1:

$$
\begin{equation*}
\frac{\mathrm{d}[\mathrm{E}]}{\mathrm{d} t}=-k_{1}[\mathrm{~S}][\mathrm{E}]-k_{3}[\mathrm{E}]+\left(k_{-1}+k_{2}\right)[\mathrm{ES}] \tag{B.1}
\end{equation*}
$$

$$
\begin{gather*}
\frac{\mathrm{d}[\mathrm{ES}]}{\mathrm{d} t}=k_{1}[\mathrm{~S}][\mathrm{E}]-\left(k_{-1}+k_{2}+k_{4}\right)[\mathrm{ES}]  \tag{B.2}\\
\frac{\mathrm{d}\left[\mathrm{E}_{\mathrm{i}}\right]}{\mathrm{d} t}=k_{3}[\mathrm{E}] \tag{B.3}
\end{gather*}
$$

$$
\begin{equation*}
\frac{\mathrm{d}\left[\mathrm{ES}_{\mathrm{i}}\right]}{\mathrm{d} t}=k_{4}[\mathrm{ES}] \tag{B.4}
\end{equation*}
$$

$$
\begin{align*}
& \frac{\mathrm{d}[\mathrm{~S}]}{\mathrm{d} t}=-k_{1}[\mathrm{~S}][\mathrm{E}]+k_{-1}[\mathrm{ES}]  \tag{B.5}\\
& \frac{\mathrm{d}[\mathrm{P}]}{\mathrm{d} t}=k_{2}[\mathrm{ES}] \tag{B.6}
\end{align*}
$$

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