Journal of Enzyme Inhibition and Medicinal Chemistry, December 2006; 21(6): 635–645

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Competitive and uncompetitive inhibitors simultaneously acting on an autocatalytic zymogen activation reaction

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(Received 20 October 2005; accepted 22 April 2006)

Abstract

The time course of the residual enzyme activity of a general model consisting of an autocatalytic zymogen activation process inhibited by an irreversible competitive inhibitor and an irreversible uncompetitive inhibitor has been studied. Approached analytical expressions which furnish the time course of the residual enzyme activity from the onset of the reaction depending on the rate constants and initial concentration have been obtained. The goodness and limitations of the analytical equations were checked by comparing with the results obtained from the numerical integration, i.e. with the simulated progress curves. A dimensionless parameter giving the relative contributions of both the activation and the inhibitions routes is suggested, so that the value of this parameter determines whether the activation or the inhibitions routes prevail or if both processes are balanced during the time for which the analytical expressions are valid. The effects of the initial zymogen, free enzyme and inhibitors concentrations are analysed. Finally an experimental design and kinetic data analysis is proposed to evaluate simultaneously the kinetic parameters involved and to discriminate between different zymogen activation processes which can be considered particular cases of the general model.

Keywords: *Enzyme inhibition, autocatalysis, proenzyme activation, competitive, uncompetitive, kinetics*

Introduction

Autocatalytic zymogen activation is a phenomenon of great importance for understanding some fundamental physiological processes involved in the enzyme regulation of gastrointestinal-track enzymes [1,2], blood coagulation and fibrinolysis [3–5]. Examples of such processes are the activation of prekallikrein, trypsinogen and pepsinogen, all of them being controlled by natural proteinase inhibitors [6].

A major incentive in inhibitor research is that control of proteolysis is a valid pharmacological principle. Inhibitors have indeed proved useful in controlling pathogenesis in many animal models of proteolysis. The proteinase inhibitors are effective in human therapy [3,6,7]. A pathological increase in fibrinolysis, e.g., in leukaemia or in operations involving organs with a high fibrinolysis activator content such as the uterus, prostate or lungs, can be controlled by the use of inhibitors such as ε-aminocaproic acid, *p*-aminomethylbenzoic acid or aprotinin. These products inhibit plasmin, trypsin, chymotrypsin and kallikrein, the last one being the most important protein responsible for the release of bradykinin from kininogen [3].

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ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360600858562

The transient phase kinetic analysis of a global model of an autocatalytic zymogen activation process overlapped with irreversible competitive inhibition was carried out by Manjabacas et al. [8]. More recently Manjabacas et al. [9] extended their analysis to the case in which the irreversible inhibition is mixed, therefore including as particular cases those in which the inhibitor is competitive, noncompetitive, mixed or uncompetitive, having in this way a more general vision of the kinetic behaviour of zymogen activation reactions in the presence of one irreversible inhibitor.

In this contribution we treat the case in which two different two-steps irreversible inhibitors act on an autocatalytic zymogen activation system, one of them being competitive and the other uncompetitive. The reaction mechanism proposed is shown in Scheme 1 where Z, E, W, I and I′ are the zymogen, the activating (and activated) enzyme, one or more peptides released from Z, and the two different inhibitors, respectively. We will assume that the three reversible steps are in rapid equilibrium.

Notation and definitions

[E], [Z], [I], [I′], [EZ], [EI], [EZI′]: Instantaneous concentrations of the species indicated.

$[E]_0$, $[Z]_0$, $[I]_0$, $[I']_0$: Initial concentrations of E, Z, I and I′, respectively.

$[E_T]$: Instantaneous residual enzyme activity, i.e. the total concentration, at time t, of the active enzyme forms:

$$[E_T] = [E] + [EZ] + [EI] + [EZI'] \tag{1}$$

$[E_T]_0$: Value of $[E_T]$ at $t = 0$, i.e.:

$$[E_T]_0 = [E]_0 \tag{2}$$

K_1, K_3, K_3': Equilibrium dissociation constants of the complexes EZ, EI and EZI′, respectively:

$$K_1 = \frac{k_{-1}}{k_1} \tag{3}$$

$$K_3 = \frac{k_{-3}}{k_3} \tag{4}$$

$$K_3' = \frac{k_{-3}'}{k_3'} \tag{5}$$

Q_1, Q_3, Q_3': The expressions:

$$Q_1 = \frac{[E][Z]}{[EZ]} \tag{6}$$

$$Q_3 = \frac{[E][I]}{[EI]} \tag{7}$$

$$Q_3' = \frac{[EZ][I']}{[EZI']} \tag{8}$$

η_∞:

Near the end of the reaction either $[E]/[E]_0$ or $[Z]/[Z]_0$ must necessarily be less than unity according to the inhibition or the activation which prevails at this reaction time. We must arbitrarily fix a value to that of the above quotients being less than the unity for which we can consider the reaction is practically finished. We denote this quotient as η_∞ and give it the value 10^{-4}, what means that we consider a reaction time at which either $[E]/[E]_0 = 10^{-4}$ or $[Z]/[Z]_0 = 10^{-4}$.

t_∞:

The time the system takes to either $[E]/[E]_0$ or $[Z]/[Z]_0$ is equal to η_∞

θ:

If there is a reaction time different to zero at which $[E_T] = [E_0]$, we name it θ. This parameter, θ, means the time at which the prevalence of the activation changes to the prevalence of the inhibition or *vice versa* in Scheme 1 [see Figures 2(A) and 4(A)].

Kinetic behaviour from numerical integration

The kinetic behaviour of enzyme systems evolving according to Scheme 1 is described by the set of differential equations (A1)–(A10) in the Appendix. This system is not linear and, therefore, it does not admit any exact analytical solution. Thus, a complete vision of the time course of the systems is only reachable by numerical integration from arbitrary sets of values of the rate constants and initial concentrations. We have selected three cases giving considerably different kinetic behaviours. The values of the rate constants and $[E]_0$, $[Z]_0$, $[I]_0$ and $[I']_0$ for each of the cases are summarised in Table I.

By numerical integration for the above initial conditions and kinetic parameter values we obtained, for each case (1–3) in Table I, the time course of [E], [EZ], [EI], [EZI′], [Z], [I] and [I′] and, using Equation (1), the simulated progress curve of $[E_T]$ [Figures 1(A)–3(A)], using Equations (6)–(8) the time progress of Q_1/K_1, Q_3/K_3, Q_3'/K_3' [Figures 1(B)–3(B)] and using the $[Z]_0$–, $[I]_0$– and $[I']_0$-values the time progress curves of $[Z]/[Z]_0$, $[I]/[I]_0$ and $[I']/[I']_0$ [Figures 1(C)–3(C)]. The values of K_1, K_3 and K_3' needed for plots in Figures 1(B)–(3(B) are obtained from Equations (3)–(5) and the corresponding values

Table I. Arbitrary set of values of the rate constants and initial concentrations corresponding to the three cases (1–3). For all of these cases $[E]_0 = 10\,nM$ and $k_{-1} = 900\,s^{-1}$, $k_3 = 10^7\,M^{-1}\,s^{-1}$, $k'_{-3} = 500\,s^{-1}$ and $k'_4 = 0.015\,s^{-1}$. The values of k_1, k_2, k_{-3}, k_4, $[Z]_0$, $[I]_0$ and $[I']_0$ are indicated in the corresponding columns.

Case	k_1 ($M^{-1}s^{-1}$)	k_2 (s^{-1})	k_{-3} (s^{-1})	k_4 (s^{-1})	k'_3 ($M^{-1}\,s^{-1}$)	$[Z]_0$ (mM)	$[I]_0$ (mM)	$[I']_0$ (mM)
1	1.0×10^7	0.5	800	0.1	5×10^5	0.10	0.001	0.01
2	1.0×10^7	5	800	0.1	5×10^5	0.10	1.000	1.00
3	1.0×10^7	5	800	0.1	5×10^5	0.01	1.00	1.00

of k_1, k_{-1}, k_3, k_{-3}, k'_3 and k'_{-3} in Table I for the corresponding cases. For all cases (1–3) the values of the equilibrium constants are: $K_1 = 9 \times 10^{-5}\,M$, $K_3 = 8 \times 10^{-5}\,M$ and $K'_3 = 10^{-3}\,M$.

In Figures 1–3 we have arbitrarily chosen $\eta_\infty = 0.0001$ and, therefore, the simulated progress curves in Figures 1–3 were plotted up to the corresponding t_∞ defined above. In Table II we summarise the t_∞-values for each case. Note that only the numerical integration of the differential Equations (A1)–(A7) is needed to have [E], [EZ], [EI], [EZI′], [Z], [I] and [I′].

Approached analytical solution giving the time course of $[E_T]$

Assumptions

In order to obtain approached analytical solutions we have to make some assumptions for the set of differential equations (A1)–(A10) approximately to become linear. For this task we will consider a reaction time for which [Z], [I] and [I′] do not considerably differ (e.g. not more than 10%) from $[Z]_0$, $[I]_0$ and $[I']_0$, respectively.

$$\left.\begin{aligned} [Z]/[Z]_0 &\backsimeq 1 \\ [I]/[I]_0 &\backsimeq 1 \\ [I']/[I']_0 &\backsimeq 1 \end{aligned}\right\} \qquad (9)$$

In order to assumptions (9) are observed, the following conditions, easy to be reach experimentally, are necessary:

$$\left.\begin{aligned} &[Z]_0 >> [E]_0 \\ &[I]_0 >> [E]_0 + [Z]_0 \\ &[I']_0 >> [Z]_0 \\ &\text{Reaction time assayed so that } [E_T] \ll [Z]_0 \end{aligned}\right\} \qquad (10)$$

The last of conditions (10) should really be the less restrictive one:

$$\text{Reaction time assayed so that } [E] \ll [Z]_0 \qquad (11)$$

but since $[E_T]$ is easy to measure experimentally by a discontinuous method, we have chosen this last more restrictive condition which, according to Equation (1), includes condition (11).

If in Equations (A1)–(A4) we insert conditions (9), i.e. we replace [Z], [I] and [I′] by $[Z]_0$, $[I]_0$ and $[I']_0$, we have a linear set of four differential equations which already admits analytical solution. Nevertheless, these analytical solutions giving [E], [EZ], [EI] and [EZI] and, therefore, $[E_T]$ would result in tetraexponential equations which are not suitable for any easy treatment. Thus, we make the additional assumption that the three reversible steps in Scheme 1 reach the equilibrium from practically the onset of the reaction, i.e. that:

$$\left.\begin{aligned} Q_1/K_1 &\backsimeq 1 \\ Q_3/K_3 &\backsimeq 1 \\ Q'_3/K'_3 &\backsimeq 1 \end{aligned}\right\} \qquad (12)$$

From Equations (6)–(8) assumptions (12) can also be expressed as:

$$\left.\begin{aligned} [E][Z]/[EZ] &\backsimeq K_1 \\ [E][I]/[EI] &\backsimeq K_3 \\ [EZ][EZI']/[EZI'] &\backsimeq K'_3 \end{aligned}\right\} \qquad (13)$$

Kinetic analysis

From assumptions (12) and Equation (1) we obtain:

$$[EZ] = \frac{[E_T][Z]}{K_1 D} \qquad (14)$$

$$[EI] = \frac{[E_T][I]}{K_3 D} \qquad (15)$$

$$[EZI'] = \frac{[E_T][Z][I']}{K_1 K'_3 D} \qquad (16)$$

Table II. Values of t_∞, θ, t_{max} and $[E_T]_{max}$ for each of the cases (1–3) of Table 1 obtained from the numerical integration.

Case	t_∞ (s)	θ (s)	t_{max} (s)	$[E_T]_{max}$ (nM)
1	60.804	—	—	—
2	218.56	141.27	38.320	43289
3	129.30	—	—	—

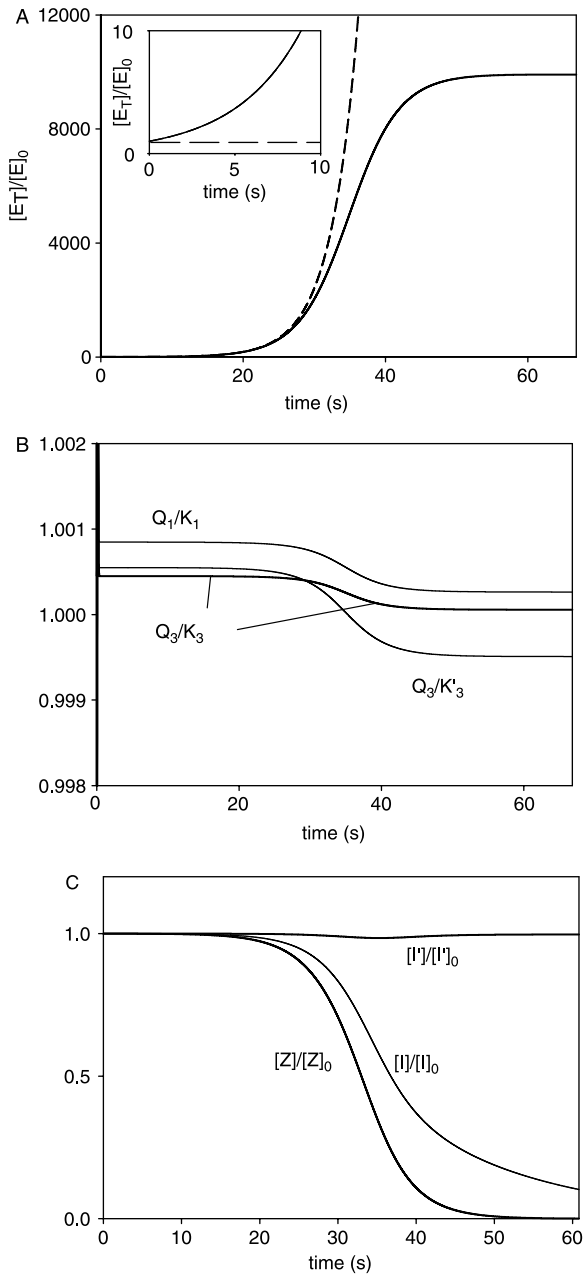


Figure 1. Time progress curves for case (1) in Table I. (A) Simulated progress curve of $[E_T]$ (———) and plot of Equation (20) (– – – –). Insert: the details in the 10 first seconds of the reaction. The simulated progress curves of $[E_T]$ has been obtained from the simulated progress curves of [E], [EZ], [EI] and [EZI′] and Equation (1). For ease we plotted the dimensionless quotient $[E_T]/[E]_0$. (B) Time progress curves of the dimensionless quotients Q_1/K_1, Q_3/K_3 and Q'_3/K'_3. The time courses of Q_1, Q_3 and Q'_3 has been obtained from Equations (6)–(8) and from simulated progress curves of [E], [EZ], [EI] and [EZI′]. The values of K_1; K_3 and K'_3 are given in Table IV. (C) Simulated progress curves of the dimensionless quotients $[Z]/[Z]_0$, $[I]/[I]_0$ and $[I']/[I']_0$. In (A), (B) and (C) the up reaction time value is the corresponding t_∞-value in Table II.

where

$$D = 1 + \frac{[I]}{K_3} + \frac{[Z]}{K_1} + \frac{[Z][I']}{K_1 K'_3} \tag{17}$$

To derive the time course of $[E_T]$ under the above assumptions we add, side by side differential Equations (A.1)–(A.4) in the Appendix, resulting:

$$\frac{d[E_T]}{dt} = k_2[EZ] - k_4[EI] - k'_4[EZI'] \tag{18}$$

where Equation (1) has been taken into account. Then, if we replace in Equation (18) [E], [EZ], [EI] and [EZI′] by their expressions in Equations (14)–(16) [i.e. we use assumptions (12)], we have:

$$\frac{d[E_T]}{dt} \approx -\frac{k'_4 K_3[Z][I'] + k_4 K_1 K'_3[I] - K_3 K'_3 k_2[Z]}{K_1 K_3 K'_3 + K_1 K'_3 + K_3 K'_3[Z] + K_3[Z][I']} \cdot [E_T] \tag{19}$$

If in Equation (19) we replace [Z], [I] and [I′] by $[Z]_0$, $[I]_0$ and $[I']_0$ and we integrate with the initial condition given by Equation (2) one obtains:

$$[E_T] \backsimeq [E]_0 . \mathbf{e}^{-\lambda t} \tag{20}$$

where

$$\lambda = \frac{k'_4 K_3[Z]_0[I']_0 + k_4 K_1 K'_3[I]_0 - K_3 K'_3 k_2[Z]_0}{K_1 K_3 K'_3 + K_1 K'_3[I]_0 + K_3 K'_3[Z]_0 + K_3[Z]_0[I']_0} \tag{21}$$

Equation (21) can also be rewritten as:

$$\lambda = \frac{(k'_4 K_3[Z]_0[I']_0 + k_4 K_1 K'_3[I]_0) \cdot (1 - r)}{K_1 K_3 K'_3 + K_1 K'_3[I]_0 + K_3 K'_3[Z]_0 + K_3[Z]_0[I']_0} \tag{22}$$

with

$$r = \frac{K_3 K'_3 k_2[Z]_0}{k'_4 K_3[Z]_0[I']_0 + k_4 K_1 K'_3[I]_0} \tag{23}$$

In Figures 1(A)–3(A) we have plotted Equation (20) for cases [1–3] in Table I, respectively.

Material and methods

The simulated progress curves were obtained by numerical integration (A1)–(A7), using a set of arbitrary, but realistic values of the initial concentrations and the rate constants. This numerical solution was found by using the classical fourth-order Runge-Kutta formula, but applying an adaptative stepsize control originally invented by Fehlberg [10,11] using the computer program WES implemented in MicroSoft Visual C++6.0 [12].

The plots of the data obtained from the numerical integration as well as the plot of Equations (20) and

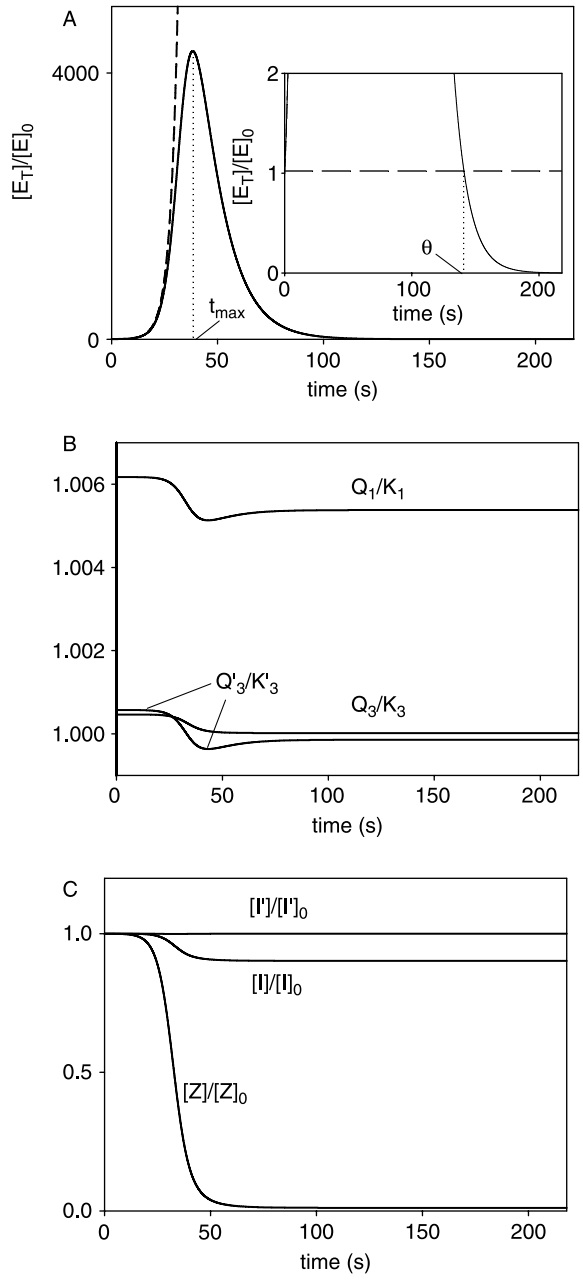


Figure 2. Time progress curves for case (2) in Table I. (A) Simulated progress curve of $[E_T]$ (———) and plot of Equation (20) (– – – –). Insert: the details showing the θ-value. The simulated progress curves of $[E_T]$ have been obtained as explained in Figure 1. For ease we plotted the dimensionless quotient $[E_T]/[E]_0$. (B) Time progress curves of the dimensionless quotients Q_1/K_1, Q_3/K_3 and Q'_3/K'_3 obtained as explained in Figure 1. (C) Simulated progress curves of the dimensionless quotients $[Z]/[Z]_0$, $[I]/[I]_0$ and $[I']/[I']_0$. In (A), (B) and (C) the up reaction time value is the corresponding t_∞-value in Table II.

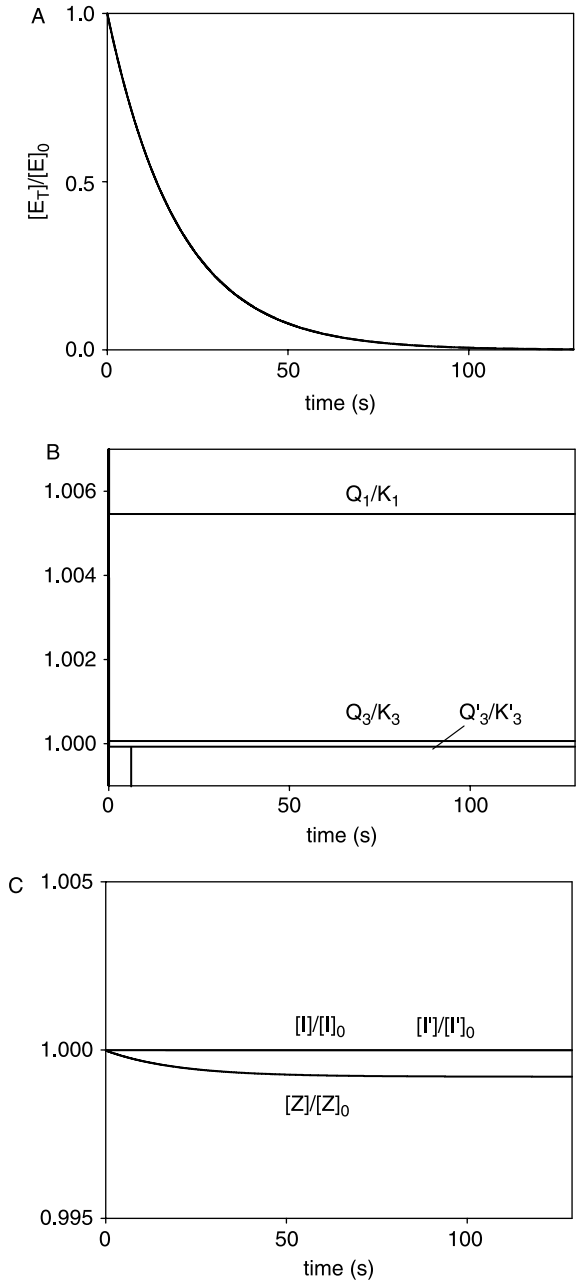


Figure 3. Time progress curves for case (3) in Table I. (A) Simulated progress curve of $[E_T]$ (———) and plot of Equation (20) (– – – –). The simulated progress curves of $[E_T]$ have been obtained as explained in Figure 1. For ease we plotted the dimensionless quotient $[E_T]/[E]_0$. (B) Time progress curves of the dimensionless quotients Q_1/K_1, Q_3/K_3 and Q'_3/K'_3 obtained as explained in Figure 1. (C) Simulated progress curves of the dimensionless quotients $[Z]/[Z]_0$, $[I]/[I]_0$ and $[I']/[I']_0$. In (A), (B) and (C) the up reaction time value is the corresponding t_∞-value in Table II.

(25) were performed using the software SigmaPlot Scientific Graphing System, version 8.02 (2002, SPSS Inc) for Windows.

The above program was run on a PC compatible computer based on a Pentium IV processor, running at 2 GHz and with 512 Mbytes of RAM.

Results and discussion

We have analysed the kinetic behaviour of enzyme systems evolving according to Scheme 1 both from numerical integration of the set of Equations (A1)–(A7) in the Appendix and from approached analytical integration of the set of Equations (A1)–(A4) in the Appendix.

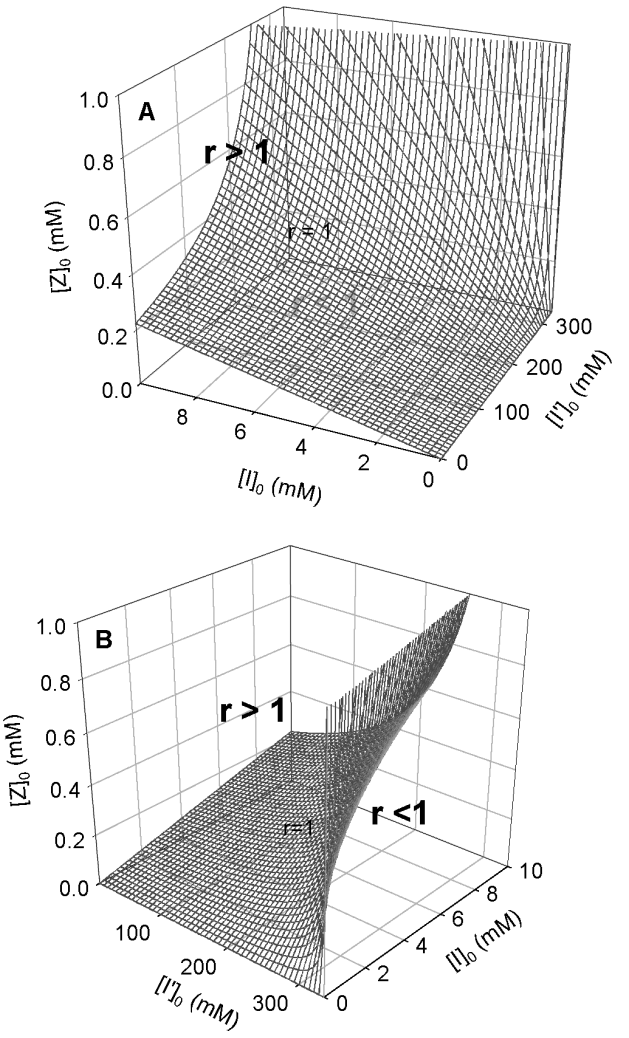


Figure 4. Spatial regions, according to Equation (23), corresponding to the rate constants of cases (2) and (3) in Table I which prevail, according to the values of $[Z]_0$, $[I]_0$ and $[I']_0$, the activation ($r > 1$) or the inhibition ($r < 1$). The surface separating both special regions, i.e. the *$r = 1$-surface,* corresponds to those $[Z]_0$-, $[I]_0$- and $[I']_0$-values for which the activation and the inhibition routes are balanced ($r = 1$). (A) A perspective. (B) Another perspective.

Numerical solution

For the kinetic behaviour from numerical integration we have chosen, as examples, the three cases (1–3) in Table I. Note that both activation and inhibition routes compete and that, according to the rate and initial concentrations values, the time course of $[E_T]$ can be very different; thus, in case (1) the activation of E prevails from the onset of the reaction [Figure 1(A)]; in case (2) the activation prevails until $t = \theta$ and then the inhibition prevails until the end of the reaction; before the reaction time reaches the θ-value, $[E_T]$ reaches its maximum $[E_T]_{max}$ at $t = t_{max}$ [Figure 2(A), Table II]; in case (3) the inhibition route prevails from the onset of the reaction and $[E_T]$ continuously decreases from $t = 0$ until it vanishes at the end of the reaction [Figure 3(A)]

Moreover, for each of these cases we have plotted in Figures 1(B)–3(B) the quotients Q_1/K_1, Q_3/K_3 and Q'_3/K'_3. Note that for cases 1–3 the quotients Q_1/K_1, Q_3/K_3 and Q'_3/K'_3 remain near unity, i.e. the reversible steps in these cases can be considered, in the three cases, in a rapid equilibrium from practically the onset of the reaction.

In Figures 1(C)– 3(C) we have plotted the time courses of the quotients $[Z]/[Z]_0$, $[I]/[I]_0$ and $[I']/[I']_0$ where the time course of [Z], [I] and [I′] were obtained from the corresponding numerical integration. Note that, except in case (3), at least one of these quotients decreases greatly from a certain reaction time. In case (3) these three quotients remain near unity during whole course of the reaction.

Analytical approached solution

Apart from the results obtained from the numerical integration, we obtained approached analytical Equation (20) valid for that reaction time from the onset of the reaction for which assumptions (9) and (12) are observed [Figures 1(A)–3(A)]. Since, according to the numerical integration, in cases (1), (2) and (3) a rapid equilibrium exists in each of the reversible steps [Figures 1(B)–3(B)], the time range of fulfilment of Equation (20) is only determined by the time range for which assumptions (9) prevail as is easy to see by comparing Figures 1(C), 2(C) and 3(C) with Figures 1(A), 2(A) and 3(A), respectively. Note that, in case 3, Equation (20) is valid during the whole course of the reaction because both assumptions (9) and (12) are also observed during the whole reaction.

Dimensionless parameter r

According to Equation (23) r can be higher than, equal to or less than unity. Depending on whether $r > 1$ [or according to Equation (7) $\lambda < 1$], $r = 1$ [or according to Equation (7) $\lambda = 0$], or $r < 1$ [or according to Equation (7) $\lambda > 1$], the residual activity exponentially increases (the activation prevails on the inhibition), remains constant and equal to the unity (the activation and the inhibition are balanced) or exponentially decreases (the inhibition prevails on the activation). In short, the parameter r determines the relative contribution of both routes. In Table III we show the λ- and r-values for each of cases (1–3).

Parameter r is $[E]_0$-independent and $[Z]_0$–, $[I]_0$– and $[I']_0$–dependent. Therefore, for any set of values of the rate constants, the $[Z]_0$–, $[I]_{0-}$ and $[I']_0$-values determine whether $r > 1$, $r = 1$ and $r < 1$. The set of points representing all different possible combinations of $[Z]_0$–, $[I]_0$ and $[I']_0$-values for which $r = 1$ forms a three dimensional surface that we will name as *$r = 1$-surface*. On one side of these surfaces $r > 1$ and on the other side $r < 1$. For any *$r = 1$–surface* $[Z]_0$ is related

Table III. Changes to be made in Scheme 1, so that it becomes Schemes 2–9.

Scheme	Changes in Scheme 1
2	$I' \eta I$ (i.e. both inhibitors coincide)
3	$k'_4 = 0$
4	$k_4 = 0$
5	$k_4 = k'_4 = 0$
6	$[I']_0 = 0$ (or $k'_3 = 0$)
7	$[I']_0 = 0$ (or $k'_3 = 0$)
8	$[I]_0 = 0$ (or $k_3 = 0$) and $[I']_0 = 0$ (or $k'_3 = 0$)
9	$k_4 = 0$ and $[I']_0 = 0$ (or $k'_3 = 0$)

with $[I]_0$ and $[I']_0$ through:

$$[Z]_0 = \frac{k_4 K_1 K'_3 [I]_0}{k_2 K_3 K'_3 - k'_4 K_3 [I']_0} \tag{24}$$

Note that, because of $[Z]_0$ being a non-negative quantity, the possible upper $[I']_0$–value in the *r = 1-surfaces* is given by $k_2K'_3/k'_4$ which is $[Z]_0$– and $[I]_0$-independent. In Figure 4 we represent the *r = 1-surface* for the arbitrary common set of values of the rate constants of cases (2) and (3).

Particular cases of Scheme 1

From Scheme 1 arise many other schemes which can be regarded as real or formal particular cases of Scheme 1, such as Schemes 2–9. In Table IV are shown the changes reducing Scheme 1 to its corresponding particular case.

Kinetic equations of the particular cases of Scheme 1

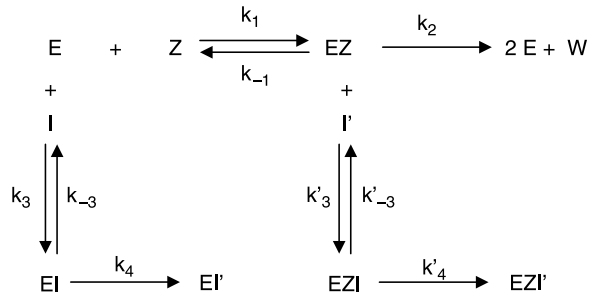
$[E_T]$ for any of the several particular cases of Scheme 1 can be obtained in an individualised way from its corresponding set of differential equations or, what is easier, setting in Equation (20) for Scheme 1 the same changes allowing Scheme 1 to become the scheme under study. As an example, for Scheme 7 the time course of $[E_T]$ is given by:

$$[ET] = \lim_{[I']_0 \to 0} [E]_0 e^{\lambda t} \quad \text{(Scheme 7)} \tag{25}$$

where λ is given by Equation (21). The result is, after insertion into Equation (21) of $[I']_0 = 0$, an equation

Table IV. Values of *r* and λ for cases 1–3 in Table I.

Case	*r*	(s^{-1})
1	392.1568	−0.2595836
2	4.385964	−0.2727915
3	0.443853	0.0507288



Scheme 1.

as Equation (20), but where now λ is given by:

$$\lambda = \frac{k_4 K_1 [I]_0 - K_3 [Z]_0}{K_1 K_3 + K_1 [I]_0 + K_3 [Z]_0} \quad \text{(Scheme 7)} \tag{26}$$

Hence, the corresponding expressions of *r* is obtained as:

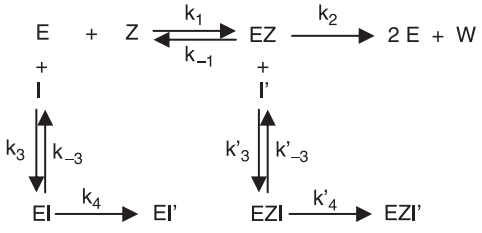
$$r = \lim_{[I']0 \to 0} \frac{K_3 K'_3 k_2 [Z]_0}{k'_4 K_3 [Z]_0 [I']_0 + k_4 K_1 K'_3 [I]_0} \tag{27}$$

(Scheme 7)

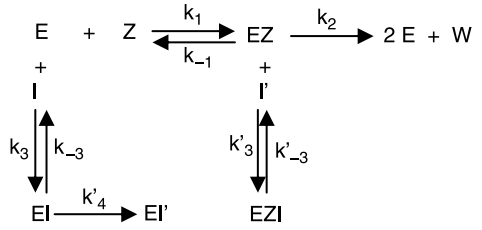
i.e.,

$$r = \frac{K_3 k_2 [Z]_0}{k_4 K_1 [I]_0} \quad \text{(Scheme7)} \tag{28}$$

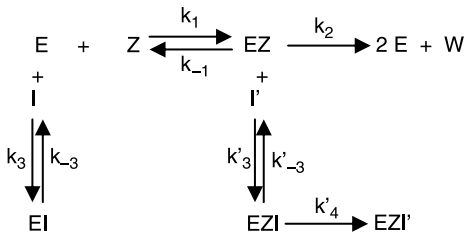
Analogously one can proceed for all of the other particular schemes.



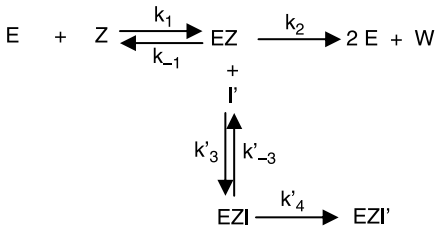
Scheme 2.



Scheme 3.



Scheme 4.



Scheme 6.

Experimental design, kinetic data analysis and discrimination between the particular cases of Scheme 1

Equation (21) can be rewritten as:

$$\lambda = \frac{a + b[I]_0 + b'[I']_0}{c + d[I]_0 + d'[I']_0} \tag{29}$$

where for Scheme 1 the coefficients a,b,b′,c, d and d′ are:

$$a = -k_2 K_3 K_3' [Z]_0 \tag{30}$$

$$b = K_1 K_3' k_4 \tag{31}$$

$$b' = k_4' K_3 [Z]_0 \tag{32}$$

$$c = K_3 K_3' ([Z]_0 + K_1) \tag{33}$$

$$d = K_1 K_3' \tag{34}$$

$$d' = K_3 [Z]_0 \tag{35}$$

Note that it is observed that:

$$b/d = k_4 \tag{36}$$

$$b'/d' = k_4' \tag{37}$$

Equations (29)–(35) for any particular case of Scheme 1 become simplified after inserting the same changes which allowed Scheme 1 to become the scheme under study, as explained above. For the purpose of this section, it is interesting to dispose of the expressions of λ for Schemes 8, 7 and 6 which are:

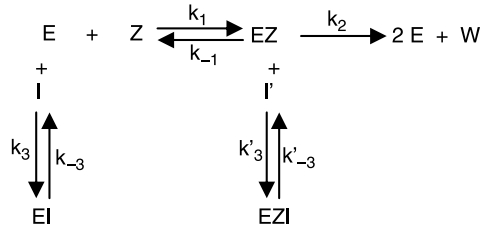
$$\lambda = \frac{-k_2 [Z]_0}{K_1 + [Z]_0} \quad \text{(Scheme 8)} \tag{38}$$

$$\lambda = \frac{a + b[I]_0}{c + d[I]_0} \text{(Scheme 7)} \tag{39}$$

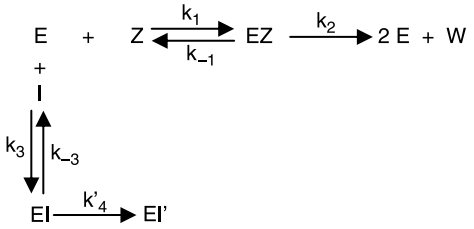
$$\lambda = \frac{a + b'[I']_0}{c + d'[I']_0} \quad \text{(Scheme 6)} \tag{40}$$

If the inhibitors I and I′ are different, we suggest the following steps in the evaluation of the kinetic parameters additionally yielding the compatible scheme of activation:

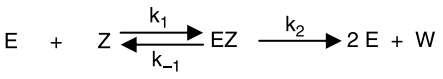
(1) Carrying out different assays in the absence of inhibitors (so that Scheme 1 results reduced to Scheme 8) in order to obtain different time progress curves of $[E_T]$ at different $[Z]_0$-values.
(2) Fitting each of the progress curves of $[E_T]$ obtained in step (1) to Equation (20) in order to obtain the corresponding λ-value for each of the progress curves.
(3) According to Equation (38), a plot of the $(-1/\lambda)$-values *vs* $1/[Z]_0$ gives a straight line with the slope K_1/k_2 and the ordinate intercept $1/k_2$ from where the evaluation of k_2 and K_1 is immediate.
(4) In different assays in absence of inhibitor I′ but in presence of inhibitor I (so that Scheme 1 results reduced to Scheme 7) one obtains different time progress curves, all of them at a fixed $[Z]_0$–value, but each of them at a different $[I]_0$–value.
(5) Proceeding as in step (2).



Scheme 5.



Scheme 7.



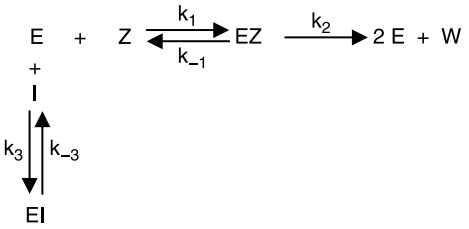
Scheme 8.

(6) Fitting the different λ-values to Equation (39) in order to obtain the coefficients *b* and d.
(7) According to Equation (36) the quotient b/d directly gives k_4.
(8) From the *d*- and K_1-values we have, according to Equation (34), the K_3'-value.
(9) Proceeding as in steps (4)–(7), but in absence of I and in presence of I′ (so that Scheme 1 results reduced to Scheme 6) and fitting the λ-values to Equation (40) in order to obtain the coefficients b′ and d′ from which, according to Equation (37), we yield directly k_4'.
(10) According to Equation (35), from the d′- and the fixed $[Z]_0$– values one obtains K_3.

If the inhibitors I and I′ coincide, then we are in the case already studied by Manjabacas et al. [9].

Additional remarks

It is known that the action of only one inhibitor (competitive, non-competitive, uncompetitive or mixed) on enzyme systems not involving zymogen activation is a aspect of the enzyme kinetics widely studied and they are already part of all text books on enzyme kinetics. Nevertheless, some years ago, there were no analyses yet on the kinetic behaviour of enzyme systems involving zymogen activation under the action of one only inhibitor (competitive, non-competitive, uncompetitive or mixed). Thus, to fill this void, not very long ago our research team began to analyse the kinetics of these types of inhibitory processes for completeness [8,9,13–15]. More recently, other groups have also provided some contributions in this field [16]. With regard to the kinetic analyses of the action of not only one inhibitor but of a mixture of different inhibitors on an enzyme system, there are also analyses handling enzyme systems not involving zymogen activation [17–21], but no similar contributions concerned with zymogen activation processes have yet been reported. Thus, for analogy with the case of the action of one only



Scheme 9.

inhibitor, with the present contribution our aim is to begin to extend kinetic analyses involving a mixture of inhibitors to zymogen activation processes.

Pepsinogen, trypsinogen, kallikrein, etc. are some examples of the activating enzymes of their own zymogens and, at the present, many competitive and uncompetitive inhibitors, reversible or irreversible, of these activating enzymes are known and continuously more and more natural or synthetic inhibitors are being reported [3,6,7,22–24]. Moreover, cells have different types of proteases and mixtures of different inhibitors. So it is very probable that an experimentalist worker will need to handle an autocatalytic zymogen activation process inhibited by a mixture of two different inhibitors whose reaction mechanism fits Scheme 1 or to any of its particular cases described below.

The kinetic study made here is based both in the numerical integration of the set of non linear differential equations (A1)–(A7) as well as in the approached analytical equations obtained from the above system under linearising [assumptions (9)] and simplifying ones [assumptions (12)]. The approached analytical solutions have validity during the reaction time for which both assumptions (9) and (12) are also observed. The values of the initial concentrations used are similar to those in different experimental [14,20–25] as well as theoretical [15,31] previous contributions on zymogen activation.

The parameters η_∞ and t_∞ have been defined to have a criterion to know when we can consider the reaction to be finished, because in the simulation the concentrations approach asymptotically to their limit values and thus, we would need an infinite simulation time. On the other hand, the parameter θ is inserted to quantify the time, if any, at which the predominance of the activation changes to the inhibition or *vice versa*.

Figures 1–3 show different degrees of goodness of the analysis according to the higher or less degree of observation of assumptions (10). Figure 1 corresponds to a case where the 2nd and 3rd of assumptions (10) are not observed and Figures 2 and 3 correspond to cases were assumptions (10) are observed (better in case (3) than in case (2)). The better the observation of the first three of the assumptions (10) is, the higher is the reaction time in which the simulated (experimental) progress curve would overlap with the plot of the corresponding analytical equation. The uniexponential Equation (20) has been obtained under assumptions (10) and, therefore, under the assumption that the reaction time assayed is such that $[E_T] \ll [Z]_0$ [last of assumptions (10)]. Therefore only that part of the progress curve from $t = 0$ where it is observed $[E_T] \ll [Z]_0$ must be fitted to the uniexponential Equation (20).

The assumption of rapid equilibrium of the reversible steps made in this contribution to obtain Equation (20), apart from being the most frequently used in enzyme kinetics [17,32,34], is specially justified. Effectively, the step EZ→2E + W requires

the cleavage of the propeptide, W, from *Z* in what is a slow process, since a peptide bond must be cleaved [28–32]. Moreover, in the two-steps irreversible inhibitors, the first, reversible step is usually assumed to be in rapid equilibrium [32–34]. The obtaining of the time course of $[E_T]$ corresponding to the Briggs-Haldane assumptions (no rapid equilibrium in the reversible steps) has no difficulty, but once obtained it is necessary, through reasonable assumptions, to reduce it so that it becomes manageable. In our case, as commented above, the time course equation for $[E_T]$ derived under Briggs-Haldane assumptions would result tetraexponential. If then it is assumed that the reversible steps are in rapid equilibrium, the equation for $[E_T]$ reduces to the uniexponential Equation (20). In the present contribution we have directly obtained Equation (20).

The analysis made here is applicable not only to Scheme 1, but also to all the several different reaction schemes which can be considered particular cases of it, e.g. schemes 2–9. Thus, this analysis offers a useful tool to kinetically characterise most of the autocatalytic zymogen activation reactions involving reversible or irreversible, competitive, noncompetitive or uncompetitive inhibitions, known or not at the present.

Acknowledgements

This work was partially supported by grants from the comisión interministerial de ciencia y tecnología (mcyt, spain), project no. Bqu2002-01960 and from junta de comunidades de castilla-la mancha, Project no. PAI-05-036.

References

[1] Hadorn B. Pancreatic proteinases. Med Clin North Am 1974;58:1319–1331.
[2] Rovery M. Limited proteolisis in pancreatic chymotrypsinogens and trypsinogens. Biochimie 1988;70:1131–1135.
[3] Löffler G, Petrides PE. Physiologische Chemie. Berlin Heidelberg and New York: Springer; 1988.
[4] Neurath H, Walsh KA In: Ribbons EW, Brew K, editors. In Proteolysis and physiological regulation. New York: Academic Press; 1976. p 29–40.
[5] Angles-Cano E. Overview on fibrinolisis: Plasminogen activation pathways on fibrin and cell surfaces. Chem Phys Lipids 1994;67–68:353–362.
[6] Rappay G. Proteinases and their inhibitors in cells and tissues. Stuttgart and New York: Gustav Fischer; 1989.
[7] Scharpe S, De Meester I, Hendriks A, Vanhoof G, Van Sande M, Vriend G. Biochimie 1991;73:121–126.
[8] Manjabacas MC, Valero E, García-Moreno M, García-Cánovas F, Rodríguez JN, Varón R. Kinetic análisis of the control through inhibition of autocatalytic zymogen activation. Biochem J. 1992;282:583–587.
[9] Manjabacas MC, Valero E, Moreno-Conesa M, García-Moreno M, Molina-Alarcón M, Varón R. Linear mixed irreversible inhibition of the autocatalytic activation of zymogens. Kinetic analysis checked by simulated progress curves. Int J Biochem & Cell Biol 2002;34:358–369.
[10] Fehlberg E, Runge- Kutta K. Formeln vierter und niedregerer ordnung mit schrittweiten-kontrole und ihre anwendung auf wärmeleitungsprobleme. Computing 1970;6:61–71.
[11] Burden R, Faires J. Numerical Análysis. Boston: PWS; 1985.
[12] García-Sevilla F, Garrido del Solo C, Duggleby R,G, García-Cánovas F, Peyró García R, Varón R. Use of a windows program for simulation of the progress curves of reactants and intermediates involved in enzyme-catalyzed reactions. Biosystems 2000;54:151–164.
[13] Varón R, Manjabacas MC, García Moreno M, Valero E, García Cánovas F. Kinetic behaviour of zymogen activation processes in the presence of an inhibitor. Biochem J 1993;290:463–470.
[14] Manjabacas MC, Valero E, García Moreno M, Varón R. Kinetic análisis of an autocatalytic process coupled to a reversible inhibition. The inhibition of system trypsinogen-trypsin by *p*-aminobenzamidine. Biological Chemistry Hoppe-Seyler 1995;376:577–580.
[15] Manjabacas-Tendero MC, Valero-Ruiz E, García Moreno M, Garrido-del Solo C, Varón-Castellanos R. Kinetics of an autocatalytic zymogen reaction in the presence of an inhibitor coupled to a monitoring reaction. Bull Math Biol 1996;58:19–41.
[16] Wang W-N, Pan X-M, Wang Z-X. Kinetic analysis of zymogen autoactivation in the presence of a reversible inhibitor. Eur J Biochem 2004;271:4638–4645.
[17] Segel IH. Enzyme Kinetics. New York: John Wiley & Sons; 1975.
[18] Lucas JJ, Burchiel SW, Segel IH. Choline sulfatase of *Pseudomonas aeruginosa*. Arch Biochem Biophys 1972;153: 664–672.
[19] Semenza G, von Balthazar AK. Steady-state kinetics of rabbit-intestinal sucrase. Kinetic mechanism, Na^+ activation, inhibition by tris(hydroxymethyl) aminomethane at the glucose subsite. Eur J Biochem 1974;:149–162.
[20] Kishioka S, Miyamoto Y, Fukunaga Y, Nishida S, Yamamoto H. Effects of a mixture of peptidase inhibitors (amastatin, captopril and phosphoramidon) on met-enkephalin-, beta-endorphin-, dynorphin-(1–13)- and electroacupuncture-induced antinociception in rats. Jpn J Pharmacol 1994;66:337–345.
[21] Cárdenas ML, Ortega F, Cascante M, Cornish-Bowden A. Modulation of metabolite concentrations with no net effect on fluxes. Mol Biol Rep 2002;29:17–20.
[22] Zollner H. Handbook of enzyme inhibitors. Weinheim: VCH; 1990.
[23] Venkatesan N, Hyean KB. Synthesis and enzyme inhibitory activities of novel peptide isosteres. Curr Med Chem 2002;9:2243–2270.
[24] Barret AJ, Rawlings ND, Woessner JF. Handbook of proteolytic enzymes. 2nd ed. London: Elsevier; 2004.
[25] Al-Janabi J, Hartsuck JA, Tang J. Kinetics and mechanism of pepsinogen activation. J Biol Chem 1972;247:4628–4632.
[26] Bajaj SP, Castellino FJ. Activation of human plasminogen by equimolecular levels of streptokinase. J Biol Chem 1977;252:492–498.
[27] Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. J Biol Chem 1982;257:2912–2919.
[28] García-Moreno M, Havsteen BH, Varón R, Rix-Matzen H. Evaluation of the kinetic parameters of the activation of trypsinogen by trypsin. Biochim Biophys Acta 1991;1080: 143–147.
[29] Matilde Esther Fuentes, Ramón Varón, Manuela García-Moreno, Edelmira Valero. Kinetics of intra- and intermolecular zymogen activation with formation of an enzyme complex. FEBS Journal 2005;272:85–96.
[30] Fuentes ME, Varón R, García-Moreno M, Valero E. Kinetics of autocatalytic zymogen activation measured by a coupled reaction: Pepsinogen autoactivation. Biol Chem 2005;386: 689–698.

[31] Vázquez A, Varón R, Tudela J, García-Cánovas F. Kinetic characterization of a model for zymogen activation: An experimental design and kinetic data análisis. J Mol Cat 1993;79:347–363.
[32] Dixon M, Webb EC, Thorne CJR, Trypton KF. Enzymes. 3rd ed. London: Longman; 1979.
[33] Cornish-Bowden A. Fundamentals of Enzyme Kinetics. London: Portland Press; 1995.
[34] Leytus SP, Toledo DL, Mangel WF. Theory and experimental method for determining individual kinetic constants of fast-acting, irreversible proteinase inhibitors Biochim Biophys Acta, 788: 74–86

Appendix

Set of differential equations describing the time course of all of the species involved in Scheme 1

$$\frac{d[E]}{dt} = -k_1[E][Z] - k_3[E][I] + (k_{-1} + 2k_2) \times [EZ] + k_{-3}[EI] \quad \text{(A.1)}$$

$$\frac{d[EZ]}{dt} = -(k_{-1} + k_2)[EZ] - k_3'[EZ][I'] + k_1[E][Z] + k_{-3}'[EZI'] \quad \text{(A.2)}$$

$$\frac{d[EI]}{dt} = -(k_{-3} + k_4)[EI] + k_3[E][I] \quad \text{(A.3)}$$

$$\frac{d[EZI']}{dt} = -(k_{-3}' + k_4')[EZI'] + k_3'[EZ][I'] \quad \text{(A.4)}$$

$$\frac{d[Z]}{dt} = k_{-1}[EZ] - k_1[E][Z] \quad \text{(A.5)}$$

$$\frac{d[I]}{dt} = -k_3[E][I] + k_{-3}[EI] \quad \text{(A.6)}$$

$$\frac{d[I']}{dt} = -k_3'[EZ][I'] + k_{-3}'[EZI'] \quad \text{(A.7)}$$

$$\frac{d[EI^\star]}{dt} = k_4[EI] \quad \text{(A.8)}$$

$$\frac{d[EZI'^\star]}{dt} = k_4'[EZI'] \quad \text{(A.9)}$$

$$\frac{d[W]}{dt} = k_2[EZ] \quad \text{(A.10)}$$