MATCH
Communications in Mathematical and in Computer Chemistry

MATCH Commun. Math. Comput. Chem. **64** (2010) 513-550

ISSN 0340 - 6253

A General Model for Autocatalytic Zymogen Activation Inhibited by Two Different and Mutually Exclusive Inhibitors

J. Masia-Perez[a], E. Valero[b], J. Escribano[c], E. Arribas[d], J.M. Villalba[e], M. Garcia-Moreno[b], F. Garcia-Sevilla[f], M.L. Amo[b] and R. Varon[b*]

[a] *Servicio de Cardiología, Complejo Hospitalario Universitario de Albacete, Spain.*

[b] *Departamento de Química-Física, Universidad de Castilla-La Mancha, Albacete, Spain.*

[c] *Departamento de Producción Vegetal, Universidad de Castilla-La Mancha, Albacete, Spain.*

[d] *Departamento de Física Aplicada, Universidad de Castilla-La Mancha, Albacete, Spain.*

[e] *Departamento de Ciencias Médicas, Universidad de Castilla-La Mancha, Albacete, Spain.*

[f] *Departamento de Ingeniería Eléctrica, Electrónica, Automática y Comunicaciones, UCLM*

(Received March 1, 2010)

Abstract

In this paper, a general mechanism of autocatalytic zymogen activation and the simultaneous action of two different, mutually exclusive, two-step inhibitors acting on both the enzyme (which simultaneously is both the activating and the activated enzyme) and the complex enzyme-zymogen is suggested and kinetically analyzed. This generalization offers the advantages of being applicable to a high number of real cases since most mechanisms of autocatalytic zymogen activation involving reversible or irreversible, one or two step, equal or different inhibitors, with reversible steps in rapid equilibrium or not, are particular cases of the general model here studied. The number and type of the particular cases arising from the general model are obtained in a systematic way. Finally, as an example, the results obtained for the general model are applied to one of the thousands of its particular cases.

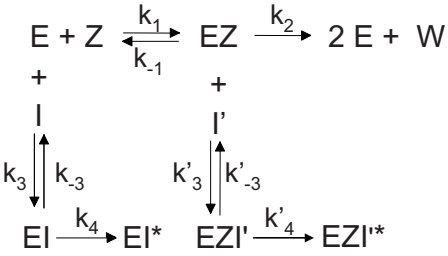
* Corresponding author, Departamento de Quimica-Fisica, Escuela de Ingenieros Industriales de Albacete, Avenida de España s/n, E-02071 Albacete, Spain. Tel.: +34 967599200, ext. 2480, Fax: +34 967599224, e-mail: ramon.varon@uclm.es

1. Introduction

An important number of proteases are normally synthesized and secreted as inactive precursors in a suitable time and place with the aim of protecting the cells that produce them. They are termed proenzymes or zymogens and must undergo an activation process, usually consisting of a limited proteolysis involving selective cleavage of a peptide bond, to attain their catalytic activity. This is a very important phenomenon in many fundamental physiological processes, such as digestion, metabolism, immunity, blood coagulation, fibrinolysis, cell apoptosis, tumor growth and metastasis [1-13].

In those cases in which the activating enzyme coincides with the activated one, the process is autocatalytic. Autocatalytic zymogen activation is a very important phenomenon to understand some fundamental physiological processes involved in regulation of enzymes in the gastrointestinal tract [14,15], on blood coagulation and fibrinolysis [16-18]. Specific examples of physiological processes of interest include the activation of trypsinogen to trypsin [9,19-21], the conversion of pepsinogen to pepsin [22-26] and prekallikrein to kallikrein [27-29], all of them controlled by natural inhibitors of proteases present in cells and body fluids. Therefore an important incentive is the research of inhibitors of proteolysis control because it has been shown that they are very effective in many human therapeutic processes [17,30,31]. A pathological increase in fibrinolysis, such as in leukemia or interventions involving organs with a high content of activator of fibrinolysis such as uterus, prostate or lungs, can be controlled by using inhibitors such as ε-aminocaproic acid, *p*-aminomethylbenzoic acid or aprotinin. These products inhibit plasmin, trypsin, chymotrypsin and kallikrein, the latter being the most important protein responsible for the release of bradykinin [17].

When working with protease inhibitors, the importance of reaction kinetics must be stressed. Analysis of the kinetics of autocatalytic activation of a zymogen overlapped with inhibition can indicate the most likely control point in complex biological media [32]. If one wishes to suppress the activity of a given protease and know the kinetic constants for the reaction of this protease with an inhibitor, it is possible to determine how much inhibitor to add and how much time to allow a wanted inhibition.



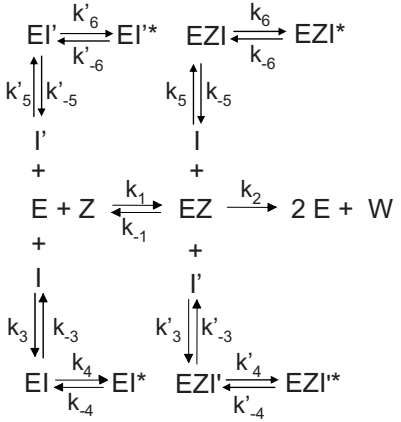
Scheme 1

Z is the zymogen, E is the activating (and activated) enzyme, W is the peptide(s) released during autocatalytic activation of Z, i.e. in the limited proteolysis of Z by E, I is a competitive inhibitor, I' is a uncompetitive inhibitor, EI and EZI' are the complex resulting of the binding of the inhibitors I and I' to E and EZ, respectively, EI^* is a isomeric form of EI and EZI'^* is a isomeric form of EZI'. The inclusion in the inhibition processes of steps in which the complexes containing I undergo an isomerisation to another complex is frequent in the literature to give a wider generality to the model under study [33-36].

Manjabacas et al. [37] obtained the kinetic equations for both the transient phase and the steady-state for a model of autocatalytic activation of zymogens. Furthermore, these authors established a new kinetic parameter that can predict if the route of activation or inhibition will prevail at steady state. They extended this analysis to different particular cases of the studied system. Subsequently, they carried out the analysis of this same scheme coupled to an enzymatic reaction of the activated enzyme on one substrate to yield a chromophore product [38]. In addition, Manjabacas et al. [20] conducted a study of an autocatalytic mechanism in the presence of a reversible inhibitor, illustrated with the experimental study of the inhibition by *p*-aminobenzamidine of trypsin activity in its action on trypsinogen. The results showed that the apparent activation rate constant decreased non-linearly with increasing the inhibitor concentration, according to the theoretical results. More recently Manjabacas et al. [39] extended the previous analysis so that the mixed inhibitor acted irreversibly in two steps on both the activating enzyme and the enzyme-zymogen complex in a general reaction scheme from which different particular cases could be obtained, to which they applied, as a limiting case, the overall results achieved. To our knowledge, the latest contribution in the scientific literature on the kinetics of inhibition of autocatalytic zymogen activation dates from 2006 [33] and we shall analyze the action of two different inhibitors, one competitive and the other one uncompetitive. Specifically these authors studied the reaction mechanism described in Scheme 1.

It remained to be done an analysis of a general model involving two, mixed or not, mutually exclusive inhibitors as shown in Scheme 2. This model is much more general and includes that shown in Scheme 1 as one of their thousands of particular cases. This generalization is reasonable if one considers the large number of inhibitors available in the cell, for a specific enzymatic process involved in autocatalytic activation of zymogens. Moreover, it has the advantage that it is applicable to a large number of real cases, because most of the autocatalytic activation mechanism of zymogens in the presence of inhibitors, competitive noncompetitive, uncompetitive or mixed, reversible or irreversible, in one or two steps, equal or different, can be considered particular cases of the general model proposed here (Scheme 2). Similarly, from the equations for the general mechanism or any of its particular cases the corresponding equations for those cases in which reversible steps are in rapid equilibrium can be obtained.

The aim of the present paper is to analyze the kinetic behaviour of an autocatalytic zymogen activation process overlapped with the action of two different inhibitors, in agreement with the general scheme reaction shown in Scheme 2. The specific objectives are: 1) To establish the initial and final conditions that allow linearizing the set of differential equations describing the kinetic behaviour of enzymatic systems whose reaction mechanism is indicated in Scheme 2. 2) To obtain the kinetic behaviour of the enzymatic species involved, valid for both the transient phase and the steady state by analytical integration of the differential equations system, once linearized, using the Laplace transform method. 3) To obtain, in a systematic way, the number of particular cases from Scheme 2. 4) To establish the method for applying the concentration-time equations obtained for Scheme 2, to the large number of processes whose reaction mechanisms are particular cases of this general scheme, using the example shown in Scheme 1, where the inhibitor I is competitive and I' uncompetitive, both of them irreversible in two steps, being all reversible steps in rapid equilibrium.



Scheme 2

Z, E, W, EI, EZI', EI* and EZI'* have the same meaning that in Scheme 1, I and I' are two non-competitive and mutually exclusive inhibitors, EI' and EZI are the complexes resulting of the binding of I' and I to E and EZ, respectively and EI'* and EZI* are isomeric forms of EI' and EZI, respectively.

2. Materials and methods

Approximated analytical solutions from the set of differential equations corresponding to Scheme 2, once linearized, were obtained by using the well-known mathematical method of Laplace transform, which turns linear systems of differential equations in algebraic systems of linear equations [40]. In Appendix B we show a flowchart to schematically summarise this method that we have applied it to the set of linear differential eqns. (27)-(36) below.

Numerical solutions of the set of differential equations corresponding to Scheme 2, without resort to its linearization, were obtained by means of the classical fourth-order Runge-Kutta formula, but applying an adaptive stepsize control originally invented by Fehlberg [41,42], using algorithms developed previously by our working group [43-47]. The programming language used was the Visual C++ 6.0 for Windows included in Microsoft Visual Studio 6.0 Professional Edition package. The above programs were run on a PC compatible computer based Pentium IV/2 GHz processor with 512 Mbytes of RAM.

Finally, data obtained from analytical and numerical solutions were plotted using the SigmaPlot Scientific Graphing System for Windows version 8.02.

3. Theory

3.1. Notation

The following notation will be used in the present paper:

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

$[\Sigma,E]$: Sum of the instantaneous concentrations of all enzymatic species involved in Scheme 2, i.e.:

$$[\Sigma,E]=[E]+[EI]+[EI^*]+[EI']+[EI'^*]+[EZ]+[EZI']+[EZI'^*]+[EZI]+[EZI^*] \quad (1)$$

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

rt: reaction time.

K_m: Michaelis-Menten constant for the zymogen in relation with the activating protease, i.e.:

$$K_m = (k_{-1} + k_2)/k_1 \quad (2)$$

K_j (j =1,3,4,5,6): Equilibrium constants defined as:

$$K_j = k_{-j}/k_j \quad (3)$$

$K_j^{'}$ (j =3,4,5,6): Equilibrium constants defined as:

$$K_j^{'} = k_{-j}^{'}/k_j^{'} \quad (4)$$

$D(\lambda)$:

the determinant:

$$D(\lambda)=\begin{vmatrix} K_{1,1}-\lambda & k_{-3} & 0 & k_{-5}^{'} & 0 & k_{-1}+2k_2 & 0 & 0 & 0 & 0 \\ k_3[I]_0 & K_{2,2}-\lambda & k_{-4} & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & k_4 & K_{3,3}-\lambda & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ k_5^{'}[I^{'}]_0 & 0 & 0 & K_{4,4}-\lambda & k_{-6}^{'} & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & k_6^{'} & K_{5,5}-\lambda & 0 & 0 & 0 & 0 & 0 \\ k_1[Z]_0 & 0 & 0 & 0 & 0 & K_{6,6}-\lambda & k_{-3}^{'} & 0 & k_{-5} & 0 \\ 0 & 0 & 0 & 0 & 0 & k_3[I^{'}]_0 & K_{7,7}-\lambda & k_{-4}^{'} & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & k_4^{'} & K_{8,8}-\lambda & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & k_5[I]_0 & 0 & 0 & K_{9,9}-\lambda & k_{-6} \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & k_6 & K_{10,10}-\lambda \end{vmatrix} \quad (5)$$

where:

$$K_{1,1}=-(k_3[I]_0+k_5^{'}[I^{'}]_0+k_1[Z]_0) \quad (6)$$

$$K_{2,2}=-(k_{-3}+k_4) \quad (7)$$

$$K_{3,3}=-k_{-4} \quad (8)$$

$$K_{4,4}=-(k_{-5}^{'}+k_6^{'}) \quad (9)$$

$$K_{5,5}=-k_{-6}^{'} \quad (10)$$

$$K_{6,6}=-(k_{-1}+k_2+k_3^{'}[I^{'}]_0+k_5[I]_0) \quad (11)$$

$$K_{7,7}=-(k_{-3}^{'}+k_4^{'}) \quad (12)$$

$$K_{8,8}=-k_{-4}^{'} \quad (13)$$

$$K_{8,7}=k_{-4}^{'} \quad (14)$$

$$K_{9,9}=-(k_{-5}+k_6) \quad (15)$$

$$K_{10,10}=-k_{-6} \quad (16)$$

G_q (q =1,2,...,10):

The coefficients of the polynomial obtained in the development of $D(\lambda)$, resulting in:

$$D(\lambda)=\lambda^{10}+G_1\lambda^9+G_2\lambda^8+G_3\lambda^7+G_4\lambda^6+G_5\lambda^5+G_6\lambda^4+G_7\lambda^3+G_8\lambda^2+G_9\lambda+G_{10} \quad (17)$$

These coefficients are the sum of terms each of them being products of rate constants and, where appropriate, of $[Z]_0$, $[I]_0$ and $[I']_0$ and, of course, are obtained simply by developing the determinant and putting it into the polynomial form (17). Just as an example of the complexity of these coefficients, the expression of G_{10} is given in the Appendix C, eqn. (C1).

$\lambda_1, \lambda_2, \ldots, \lambda_{10}$:

The roots of the polynomial $D(\lambda)$, none of them null because $G_{10} \neq 0$.

$D_{1,i}(\lambda)$:

Determinant obtained by deleting in the determinant $D(\lambda)$ the first row and the i-th column. Its development is a polynomial whose degree and coefficients depend on the value of i.

f_h (h =1,2,...,9):

The coefficients indicated in the development of $D_{1,1}(\lambda)$.

$$D_{1,1}(\lambda) = -\left(\lambda^9 + f_1\lambda^8 + f_2\lambda^7 + f_3\lambda^6 + f_4\lambda^5 + f_5\lambda^4 + f_6\lambda^3 + f_7\lambda^2 + f_8\lambda + f_9\right) \tag{18}$$

b_h (h=2,...,9):

The coefficients indicated in the development of $D_{1,3}(\lambda)$.

$$D_{1,3}(\lambda) = -\left(b_2\lambda^7 + b_3\lambda^6 + b_4\lambda^5 + b_5\lambda^4 + b_6\lambda^3 + b_7\lambda^2 + b_8\lambda + b_9\right) \tag{19}$$

a_h (h =2,...,9):

The coefficients indicated in the development of $D_{1,6}(\lambda)$.

$$D_{1,6}(\lambda) = a_1\lambda^8 + a_2\lambda^7 + a_3\lambda^6 + a_4\lambda^5 + a_5\lambda^4 + a_6\lambda^3 + a_7\lambda^2 + a_8\lambda + a_9 \tag{20}$$

c_h (h=3,...,9):

The coefficients indicated in the development of $D_{1,8}(\lambda)$.

$$D_{1,8}(\lambda) = c_3\lambda^6 + c_4\lambda^5 + c_5\lambda^4 + c_6\lambda^3 + c_7\lambda^2 + c_8\lambda + c_9\lambda + c_9 \tag{21}$$

These coefficients are expressions of the same characteristics as G_q (q=1,2 ,...,10) and they are sums of terms which in turn are products of rate constants and, where appropriate, of $[Z]_0$, $[I]_0$ and $[I']_0$ and, of course, are obtained simply by developing the corresponding determinant and putting it into polynomial form [eqns. (18)-(21)]. Just as an example of the

complexity of these coefficients, the expressions of f_8, b_8, a_8 and c_8 are shown in the Appendix C, eqns. (C2)-(C5).

3.2. Kinetic behaviour

The set of differential equations describing the kinetic behaviour corresponding to the mechanism shown in Scheme 2 is indicated in the Appendix A [eqns. (A1)-(A14)]. This set is nonlinear and so does not admit any analytical solution. However, under certain reasonable assumptions that are easy to implement experimentally, it is possible to linearize it to obtain approximate analytical solutions. To do this, we shall assume that the only species present at the onset of the reaction are E, Z, I and I'. In addition, we will assume the following initial conditions:

$$[Z]_0,\ [I]_0,\ [I']_0 \ >>\ [E]_0 \tag{22}$$

$$[I],\ [I']_0 \ >>\ [Z]_0 \tag{23}$$

We shall consider a reaction time, rt, during which $[E]$ is much smaller than $[Z]_0$, i.e.

$$[\Sigma, E] \ <<\ [Z]_0 \text{ during time } rt \tag{24}$$

This condition should be the next one, less restrictive:

$$[E] << [Z]_0 \text{ during the time } rt \tag{25}$$

but taking into account that the evolution of $[\Sigma, E]$ is easy to measure experimentally by a discontinuous method, we have chosen condition (24) which, in agreement with eqn. (1) includes the condition (25).

The above assumptions, which transform the set of differential equations (A1)-(A14) in linear, imply that during the reaction time considered, the instantaneous concentrations $[Z]$, $[I]$ and $[I']$ do not significantly differ (for example, no more than 10%) of $[Z]_0$, $[I]_0$ and $[I']_0$, respectively, i.e. at any reaction time less than or equal to rt, it is satisfied that:

$$\left.\begin{aligned} [Z] &\simeq [Z]_0 \\ [I] &\simeq [I]_0 \\ [I'] &\simeq [I']_0 \end{aligned}\right\} \tag{26}$$

In particular, eqns. (A1)-(A10) are now the following system of ordinary differential equations, linear, homogeneous and with constant coefficients:

$$\frac{d[E]}{dt} = -k_1[E][Z]_0 - k_3[I]_0[E] - k_5^{'}[I']_0[E] + (k_{-1} + 2k_2)[EZ] + k_{-3}[EI] + k_{-5}^{'}[EI'] \tag{27}$$

$$\frac{d[EI]}{dt} = k_3[E][I]_0 + k_{-4}[EI^*] - (k_{-3} + k_{-4})[EI] \tag{28}$$

$$\frac{d[EI^*]}{dt} = -k_{-4}[EI^*] + k_4[EI] \tag{29}$$

$$\frac{d[EI']}{dt} = k_5^{'}[E][I']_0 + k_{-6}^{'}[EI'^*] - (k_5^{'} + k_{-6}^{'})[EI'] \tag{30}$$

$$\frac{d[EI'^*]}{dt} = -k'_{-6}[EI'^*] + k'_6[EI'] \tag{31}$$

$$\frac{d[EZ]}{dt} = -k_5[EZ][I]_0 + k_{-5}[EZI] - (k_{-1} + k_2)[EZ] - k'_3[I']_0[EZ] + k_{-3}^{'}[EZI'] + k_1[E][Z]_0 \tag{32}$$

$$\frac{d[EZI']}{dt} = -(k'_4 + k'_{-3})[EZI'] + k'_3[EZ][I']_0 + k'_{-4}[EZI'^*] \tag{33}$$

$$\frac{d[EZI'^*]}{dt} = -k'_{-4}[EZI'^*] + k'_4[EZI'] \tag{34}$$

$$\frac{d[EZI]}{dt} = -(k_6 + k_{-5})[EZI] + k_5[EZ][I]_0 + k_{-6}[EZI^*] \tag{35}$$

$$\frac{d[EZI^*]}{dt} = -k_{-6}[EZI] + k_6[EZI] \tag{36}$$

This system of differential equations can be analytically solved by any method. We have used the Laplace transform method [40,42] (the details of the derivation are given in Appendix B).

3.3. Time course of the free enzyme concentration

The result obtained for the species *E* from the above set of differential equations is the following:

$$[E]=\sum_{h=1}^{10} A_{1,h}e^{\lambda_h t} \tag{37}$$

where:

$$A_{1,h}=\frac{\left(\lambda_h^9+f_1\lambda_h^8+f_2\lambda_h^7+f_3\lambda_h^6+f_4\lambda_h^5+f_5\lambda_h^4+f_6\lambda_h^3+f_7\lambda_h^2+f_8\lambda_h+f_9\right)[E]_0}{\prod_{\substack{p=1\\p\neq h}}^{10}\left(\lambda_p-\lambda_h\right)} \text{ (h=1,2,...,10)} \tag{38}$$

From polynomial theory, the following relations between the roots λ_1, λ_2, . . ., λ_{10} arise, that will be useful later:

$$\lambda_1+\lambda_2+\cdots+\lambda_{10}=-G_1 \tag{39}$$

$$\lambda_1\lambda_2+\lambda_1\lambda_3+\cdots+\lambda_9\lambda_{10}=G_2 \tag{40}$$

$$\lambda_1\lambda_2\lambda_3+\lambda_1\lambda_2\lambda_4+\cdots+\lambda_8\lambda_9\lambda_{10}=-G_3 \tag{41}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4+\lambda_1\lambda_2\lambda_3\lambda_5+\cdots+\lambda_7\lambda_8\lambda_9\lambda_{10}=G_4 \tag{42}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5+\lambda_1\lambda_2\lambda_3\lambda_4\lambda_6+\cdots+\lambda_6\lambda_7\lambda_8\lambda_9\lambda_{10}=-G_5 \tag{43}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6+\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_7+\cdots+\lambda_5\lambda_6\lambda_7\lambda_8\lambda_9\lambda_{10}=G_6 \tag{44}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7+\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_8+\cdots+\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8\lambda_9\lambda_{10}=-G_7 \tag{45}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8+\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_9+\quad\cdots+\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8\lambda_9\lambda_{10}=G_8 \tag{46}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8\lambda_9+\cdot\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8\lambda_{10}+\cdots+\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8\lambda_9\lambda_{10}=-G_9 \tag{47}$$

$$\lambda_1\lambda_2\lambda_3\lambda_4\lambda_5\lambda_6\lambda_7\lambda_8\lambda_9\lambda_{10}=G_{10} \tag{48}$$

3.4. Time course of other enzymatic forms

Analogously, the variation with time of the concentration of any other species from Scheme 2 can be obtained from the analytical solution of the set of differential eqns. (27)-(36). Just as examples, and because they will be useful later, the expressions for [*EZ*], [*EI**] and [*EZI'**] are given here:

$$[EZ] = \sum_{h=1}^{10} A_{6,h} e^{\lambda_h t} \tag{49}$$

where:

$$A_{6,h} = -\frac{\left(a_1\lambda_h^8 + a_2\lambda_h^7 + a_3\lambda_h^6 + a_4\lambda_h^5 + a_5\lambda_h^4 + a_6\lambda_h^3 + a_7\lambda_h^2 + a_8\lambda_h + a_9\right)[E]_0}{\prod_{\substack{p=1 \\ p\neq h}}^{10}\left(\lambda_p - \lambda_h\right)} \quad \text{(h=1,2,...,10)} \tag{50}$$

In turn:

$$[EI^*] = \sum_{h=1}^{10} A_{3,h} e^{\lambda_h t} \tag{51}$$

where:

$$A_{3,h} = -\frac{\left(b_2\lambda_h^7 + b_3\lambda_h^6 + b_4\lambda_h^5 + b_5\lambda_h^4 + b_6\lambda_h^3 + b_7\lambda_h^2 + b_8\lambda_h + b_9\right)[E]_0}{\prod_{\substack{p=1 \\ p\neq h}}^{10}\left(\lambda_p - \lambda_h\right)} \quad \text{(h=1,2,...,10)} \tag{52}$$

Finally:

$$[EZI^*] = \sum_{h=1}^{10} A_{8,h} e^{\lambda_h t} \tag{53}$$

where:

$$A_{8,h} = -\frac{\left(c_3\lambda_h^6 + c_4\lambda_h^5 + c_5\lambda_h^4 + c_6\lambda_h^3 + c_7\lambda_h^2 + c_8\lambda_h + c_9\right)[E]_0}{\prod_{\substack{p=1 \\ p\neq h}}^{10}\left(\lambda_p - \lambda_h\right)} \quad \text{(h=1,2,...,10)} \tag{54}$$

3.5. Time course equation of the enzyme activity, $[\Sigma, E]$

If eqns. (27)-(36) are added member to member, and the definition of $[\Sigma, E]$, given by eqn. (1), is taken into account, then it results:

$$\frac{d[\Sigma, E]}{dt} = k_2[EZ] \tag{55}$$

If eqn. (49) is taken into account into eqn. (55) and one integrates with the initial condition $[\Sigma, E]_0 = [E]_0$, it is obtained:

$$[\Sigma, E] = \sum_{h=1}^{10} \gamma_h e^{\lambda_h t} \tag{56}$$

where:

$$\gamma_h = -\frac{k_2 \left(a_1\lambda_h^8 + a_2\lambda_h^7 + a_3\lambda_h^6 + a_4\lambda_h^5 + a_5\lambda_h^4 + a_6\lambda_h^3 + a_7\lambda_h^2 + a_8\lambda_h + a_9\right)[E]_0}{\lambda_h \prod_{\substack{p=1 \\ p\neq h}}^{10} \left(\lambda_p - \lambda_h\right)} \text{ (h=1,2,...,10)} \tag{57}$$

4. Results and Discussion

In this contribution, a complete kinetic analysis of the general model shown in Scheme 2 has been performed. This model corresponds to an enzymatic system of autocatalytic zymogens activation in the presence of two different inhibitors for both the activating enzyme and the complex enzyme-zymogen. Approximated analytical solutions have been obtained for each enzymatic species involved in the mechanism (we have shown here those corresponding to E, EI^*, EZI'^* and $[\Sigma, E]$. These equations are valid during the transient phase and the steady-state, provided that the conditions assumed are fulfilled.

The instantaneous concentration of the free enzyme, [E], is described by eqn. (37), which consists of a sum of ten exponential terms. The same holds for all enzymatic species and for the total enzyme activity $[\Sigma, E]$.

Since the product of the ten roots, $\lambda_1\lambda_2\cdots\lambda_{10}$, is negative [see eqns. (48) and (A15)] there are an odd number, n (n =1,3,5,7 or 9) of roots which must be positive or complex with a positive real part and another odd number, 10-n, of roots negative or complex with a negative real part. This means that for high values of time, i.e., at steady state of reaction, the concentration of enzyme forms involved in the Scheme 2 is given by an equation n-

exponential because the 10-n exponential terms corresponding to the 10-n roots with negative values of the real part become negligible compared with the other ones.

The kinetic analysis we carried out here is based both on the numerical integration of the set of non-linear differential equations (A1)-(A14) as well as in the approached analytical solutions of the linear set of differential equations (27)-(36) obtained from the former one under the linear assumptions (22)-(24). The approached analytical solutions are fulfilled during a reaction time in which assumptions (22)-(24) remain valid.

The analysis carried out here is applicable not only to Scheme 2 but also to its numerous particular cases. Thus, this analysis offers a useful tool to characterize kinetically most of the enzyme reaction involving autocatalytic zymogen activation, at present known or not, overlapped with reversible or irreversible, competitive, non-competitive or uncompetitive inhibitions.

4.1. Comparison between data obtained and simulated progress curves

The goodness of the approximate analytical solutions obtained can be assessed by comparing them with the corresponding particular numerical solutions obtained from the system of differential eqns. (A1)-(A14) in Appendix A. This has been done for the species $[E]$ and $[\Sigma, E]$ for an arbitrary set of rate constants and initial conditions (Fig.1). As can be seen, there is good agreement between the analytical and numerical solutions until approximately the first 40 s. Obviously, the deviation of theoretical data from the analytical solutions respect to the numerical solutions will be greater as the time considered increases.

4.2. Particular cases of the general model

In this contribution we have made a kinetic analysis of a general model of proenzyme autocatalytic activation in the presence of two different, mutually exclusive, inhibitors (Scheme 2). Besides its physiological interest, this analysis will address, systematically, all the particular cases of the same Scheme 2. Indeed, one of the most important applications of the present paper is that the results obtained for the general model are applicable, without much mathematical effort, to any of its many particular cases (16724 as we shall see later). Therefore, for the first time, this study offers researchers on this topic a method based on

general solutions that only need to be particularized to their specific problem of zymogen activation.

Generally, from any reaction mechanism, other simpler reaction mechanisms can be obtained after setting some changes in the first one. The original reaction mechanism is also called primitive mechanism and the particular cases arising from it, derived mechanism [48]. The kinetic equations of any of the derived mechanism can be easily obtained setting in the kinetic equations of the primitive one the same changes that reduce it to the derived mechanism under study.

The most common changes to be made in the primitive mechanism to transform it into any of its particular cases, and which have been made in the present study, are the following: (A) make certain rate constants zero, (B) make one or more of the rate constants much greater than the others, i.e. let them tend to infinity and (C) match two different ligand species (in our case this change will be $I \equiv I'$ when they do not act on the same enzymatic species). Of course combinations of changes, AB, AC, BC and ABC, are also possible. For example, an AB change type means that certain constants are made zero in the primitive mechanism and, moreover, others tend to infinity.

4.2.1. Systematic retrieval of particular cases

In this section we shall systematically establish those mechanisms which can be considered particular cases of the mechanism shown in Scheme 2. So, we shall distinguish four inhibition routes (two for the activating enzyme and two for the enzyme-zymogen complex) and one route for zymogen activation (Fig. 2A). Fig. 2B shows the number of possibilities for each route. The following six situations indicated in Fig. 3 will be distinguished: (a) Particular cases where the inhibition routes of *I'* on *E* and of *I* on *EZ* are missing. (b) Particular cases where none of the inhibition routes is missing. (c) Particular cases where the inhibition route of *I* on *EZ* is missing. (d) Particular cases where the inhibition route of *I'* on *E* is missing. (e) Particular cases where both inhibition routes on *EZ* are missing. (f) Particular cases where both inhibition routes on *E* are missing.

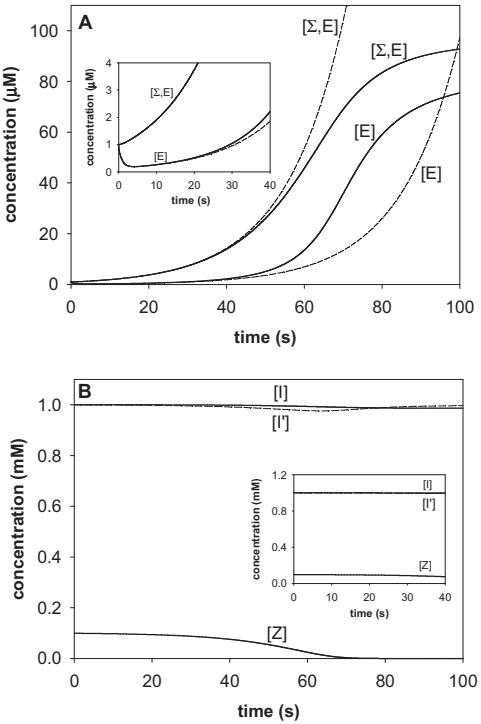


Figure 1. Time progress curve of [E] and $[\Sigma, E]$ obtained from plots of eqns. (37) and (56) derived by using the Laplace transform method (dashed lines) and from numerical integration of the set of differential equations (A1)-(A14) by using the numerical 4th-order Runge-Kutta method (solid lines) **(A)** and time progress curves corresponding to the consumption of Z, I and I' obtained by numerical integration of the set of differential equations (B1)-(B10) **(B)** for a same reaction time equal to 100 s and a same arbitrary set of values for the rate constants and initial concentrations: $k_1=10^4$ M^{-1} s^{-1}, $k_{-1}=0.1$ s^{-1}, $k_2=1$ s^{-1}, $k_3=10^5$ M^{-1} s^{-1}, $k_{-3}=10^3$ s^{-1}, $k_4=10^{-2}$ s^{-1}, $k_{-4}=100$ s^{-1}, $k'_3=10^5$ M^{-1} s^{-1}, $k'_{-3}=10$ s^{-1}, $k'_4=10^{-2}$ s^{-1}, $k'_{-4}=10$ s^{-1}, $k_5=10^3$ M^{-1} s^{-1}, $k_{-5}=1$ s^{-1}, $k_6=0.1$ s^{-1}, $k_{-6}=0.01$ s^{-1}, $k'_5=10^3$ M^{-1} s^{-1}, $k'_{-5}=50$ s^{-1}, $k'_6=0.01$ s^{-1}, $k'_{-6}=0.1$ s^{-1}, $[E]_0=1$ μM, $[I]_0=[I']_0=1$ mM and $[Z]_0=0.1$ mM. Note that the plots of the equations overlap practically with the simulated progress curves whenever the zymogen and inhibitors concentrations remain simultaneously and approximately constants, condition under which the equations have been derived. This approximated constancy is observed during the first 40 s approximately, so that the equations can be considered valid during this time. As the time increases, the deviation of the plots of the equations with respect to the simulated progress curves increases. Therefore, any experimental design and kinetic data analysis suggested for this enzyme system must be carried out in the time scale in which both methods approximately coincide.

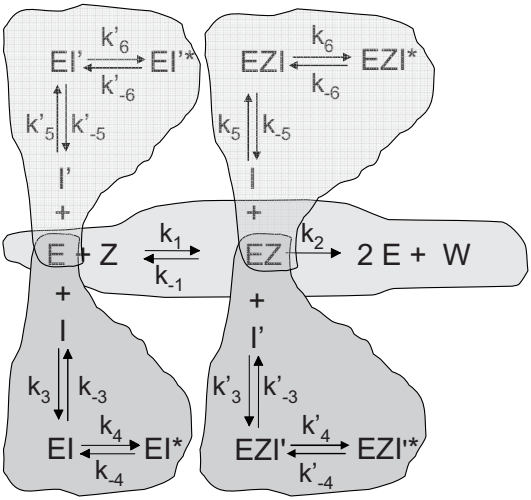


Figure 2A. Indication of the four inhibition routes (two routes for the activating enzyme and two for the activated one) and the activation route for the zymogen.

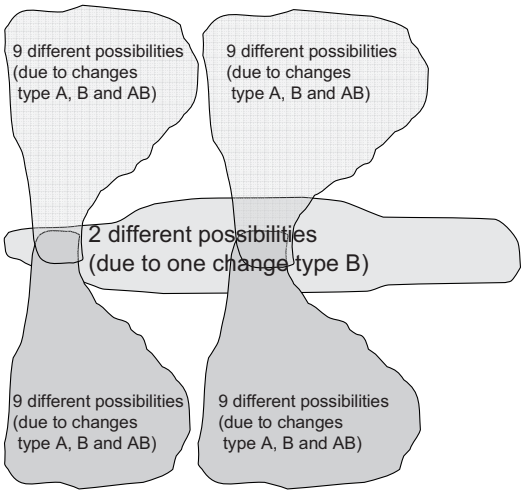


Figure 2B. Indication of the number of possibilities in each route.

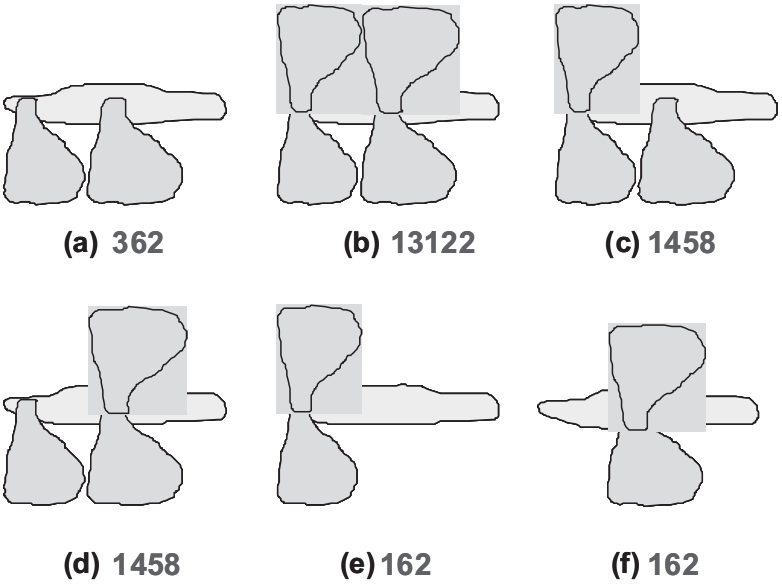
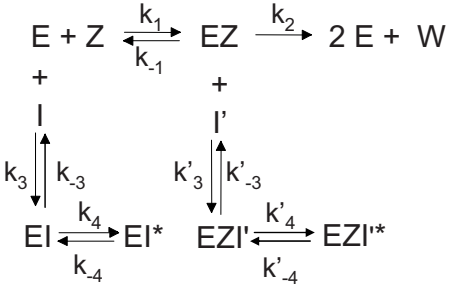


Figure 3. Different diagrams used to obtain the total number of cases of Scheme 2 can be obtained. Under each diagram the corresponding number of its particular cases, obtained as it is detailed in the main text, is shown. The algebraic operations giving them are: (a) 324+18+18+2=362; (b) 162x9x9=13122; (c) 162x9=1458; (d) 162x9=1458; (e) 18x9=162 and (f) 18x9=162.

(a) Particular cases where the inhibition routes of *I'* on *E* and of *I* on *EZ* are missing.

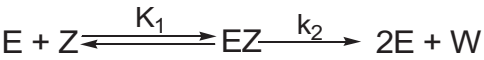
This case involves all the particular cases corresponding to the simplified mechanism shown in Scheme 3.



Scheme 3

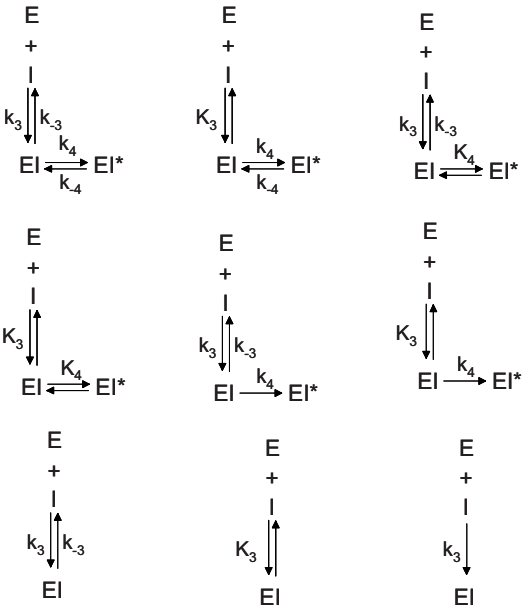
It is possible to distinguish three routes in this scheme. In the activation route, which is a particular type A case (k_3=k'_3=0), it is only possible a change type B, yielding the two following possibilities:

(a)



(b)

In turn, in each of the two inhibition routes there are nine possibilities corresponding to A, B and AB changes. Scheme 4 shows these in the case of inhibition of the activating enzyme. The nine possibilities for *EZ* can be established in a similar way.



Scheme 4

We could have included more possibilities in each of the inhibition routes, for example $E + I \rightarrow EI \rightarrow EI^*$, but these steps are non-viable. Nevertheless, the inclusion of more possibilities, whether they make sense or not does not change the analysis being carried out, although it does influence the number of particular cases, which would be greater. We have preferred to limit the possibilities to those which have an accepted meaning in the literature.

We shall now determine all the particular cases from Scheme 2. Then, four situations will be distinguished: a1) Particular cases where none of the inhibition routes is missing; a2) particular cases where the inhibition route on *E* is missing; a3) particular cases where the inhibition route on *EZ* is missing and a4) particular cases where both inhibition routes are missing.

a1) Particular cases where none of the inhibition routes is missing

The number of particular type A, B or AB cases, which will be denoted as $N_{I,I'}$, is the product of the number of possibilities of each one of the three routes (two inhibition and one activation), i.e. $N_{I,I'}$ =9x9x2=162, including the starting mechanism (Scheme 3). It is also possible that both inhibitors will match in each one of these mechanisms, so that the total number of particular cases with type C changes, which will be denoted as $N_{I,I}$, will also be 162, which indicates that the total number of cases is $N_{I,I'} + N_{I,I}$ =162+162 =324.

a2) Particular cases where the inhibition route on *E* is missing

The number of particular type A, B or AB cases, which will be denoted as $N_{I'}$, is the product of the different number of possibilities of both the inhibition and the activation routes, i.e. $N_{I'}$=9 x 2=18. In this case, it is not possible to make type C changes because there is only one inhibitor.

a3) Particular cases where the inhibition route on *EZ* is missing

The number of particular type A, B or AB cases, which will be denoted as N_I, is the product of the different number of possibilities of both the inhibition and the activation routes, i.e. N_I=9x2=18. In this case, it is not possible to do type C changes because there is only one inhibitor.

a4) Particular cases where both inhibition routes are missing

In this case, there are only two possibilities and so two particular cases, one type A and the other one type AB. This number will be denoted as N_0 (N_0 =2). No particular type C case is possible because there is no inhibitor.

Therefore, the total number of particular cases which can be derived from Scheme 2, including itself is: $N_{I,I'} + N_{I,I} + N_{I'} + N_I + N_0$ =324+18+18+2=362, of which $N_{I,I'} + N_{I'} + N_I + N_0$ =162+18+18+2=200 correspond to type A, B and AB changes, and the other 162 ($N_{I,I}$) correspond to particular cases involving a type C change, i.e. C, AC, BC and ABC changes.

b) Particular cases from Scheme 1 where none of the inhibition routes is missing

In agreement with the above, the number of particular cases in this situation will be, $N_{I,I'}$x9 x9=162x9x9=13122, a result obtained by combining the data obtained in point a1) with the 9 possibilities of each inhibition route.

(c) Particular cases where the inhibition route of *I* on *EZ* is missed

Analogously, the number of particular cases is $N_{I,I'}$x9=162x9=1458.

(d) Particular cases where the inhibition route of *I'* on *E* is missed

The number of particular cases is $N_{I,I'}$x9=162x9=1458.

(e) Particular cases where both inhibition routes on *EZ* are missed

The number of particular cases is N_Ix9 =8x9=162.

(f) Particular cases where both inhibition routes on *E* are missed

The number of particular cases is $N_{I'}$ x9=18x9=162.

Therefore, the number of particular cases derived from the general model shown in Scheme 2, including itself, is: 362+13122+1458+1458+162+162=16724. In this way, this study offers (for the first time) to the scientific community working on limited proteolysis regulation, a method based on general solutions which only needs to be particularized to the specific problem of zymogen activation.

4.2.2. Obtaining the kinetic equations for the particular cases

Each one of the particular cases derived from a primitive mechanism can be analyzed, in an individualized way, from its corresponding set of differential equations, taking into account the initial conditions and the reaction time allowing their linearization. However, this procedure would lose the power provided by having kinetic equations available for a model that includes the mechanism under study as a particular case, which constitutes a considerable saving of time and effort. In turn, usually, any of the particular cases of a primitive mechanism can be analyzed from the results of another particular case of the primitive mechanism if the mechanism under study is a particular case of the derived one.

When in the kinetic equations of a mechanism acting as primitive mechanism of another, one cancels the same rate constants allowing the primitive mechanism becomes the derived one under study, type 0/0 uncertainties can arise. Most of these possible uncertainties can be solved setting equal to a same quantity, ε, all of the rate constants to be annulled, cancelling with respect ε and then, if necessary making $\varepsilon \to 0$. Nevertheless, in some cases it can happen that, following this procedure, an unexpected, absurd result is obtained. In these cases one must proceed in the following two steps: 1) In the equation of the primitive mechanism, to cancel the minimum set of rate constants that, under the initial conditions used, convert it into an kinetically equivalent mechanism to the derived mechanism under study (whenever the initial conditions are the same) and 2) then to cancel, in the resulting equation, all other remaining rate constants that must be set equal to zero in the primitive mechanism. For more details about acquisition of the kinetic equations of a derived mechanism from any of its primitive mechanisms see reference [48].

4.2.2.1. Example. Mechanism shown in Scheme 1

The kinetic analysis of the enzyme system shown in Scheme 1 was carried out in an individualized way by Varon et al. [33]. Note that this enzyme system is one of the thousands particular cases of the general model shown in Scheme 2 and therefore, the kinetic behaviour of enzyme system in Scheme 1 can be obtained more easily from the kinetic equations corresponding to the general model by setting in the kinetic equations of the last one the same changes yielding Scheme 1 from Scheme 2. Analogously, the kinetic behaviour of the enzyme system shown in Scheme 1 could also have been obtained from those ones corresponding to

any other enzyme system from which that shown in Scheme 1 could been considered a particular case (e.g. that shown in Scheme 3).

In this section Scheme 1 is treated as a particular case of Scheme 2 to support the advantage of the kinetic analysis of a reaction mechanism of great complexity, such as that in Scheme 2 in order to derive from the results of this analysis, in easy and quick way, the kinetic behaviour of other enzyme systems which can be considered particular cases of the general complex model.

Kinetic equations for Scheme 1 can be obtained from those in Scheme 2 by setting in the latter:

$$k_{-4} = k'_{-4} = k_5 = k_{-5} = k'_5 = k'_{-5} = k_6 = k_{-6} = k'_6 = k'_{-6} = 0 \tag{58}$$

Moreover, the first or pseudofirst order reaction rate constants involved in the reversible steps, all of them assumed in rapid equilibrium, must be much higher than the other ones and mutually not very different [49]:

$$\left.\begin{array}{l} k_1[Z]_0, k_{-1}, k_3[I]_0, k_{-3}, k'_3[I']_0, k'_{-3} >> k_2, k_4, k'_4 \\ k_1[Z]_0, k_{-1}, k_3[I]_0, k_{-3}, k'_3[I']_0, k'_{-3} \text{ mutually not very different} \end{array}\right\} \tag{59}$$

The first of the above two conditions (59) is equivalent to state that from the point of view of the rate constants k_2, k_4 and k'_4, the remaining first or pseudofirst order reaction rate constants go to infinite. Therefore, condition (59) can also been written as:

$$\left.\begin{array}{l} k_1[Z]_0, k_{-1}, k_3[I]_0, k_{-3}, k'_3[I']_0, k'_{-3} \to \infty \\ k_1[Z]_0, k_{-1}, k_3[I]_0, k_{-3}, k'_3[I']_0, k'_{-3} \text{ mutually not very different} \end{array}\right\} \tag{60}$$

If conditions (59) or (60) are inserted in eqns. (37), (49), (51) and (53) corresponding to the general model (Scheme 2) the following eqns. (61)-(64) are obtained after some algebraic considerations (we omit the details in order to reduce the length of the paper, but a detailed derivation is available from the authors for the interested readers, on request):

$$[E] = \frac{K_1 K_3 K'_3 [E]_0}{K_1 K_3 K'_3 + K_1 K'_3 [I]_0 + K_3 K'_3 [Z]_0 + K_3 [Z]_0 [I']_0} e^{\lambda_1 t} \tag{61}$$

$$[\Sigma, E] = [E]_0 - \frac{k_2 K_3 K_3^{'} [Z]_0 [E]_0}{k_4 K_1 K_3^{'} [I]_0 + k_4^{'} K_3 [Z]_0 [I']_0 - k_2 K_3 K_3^{'} [Z]_0} \left(e^{\lambda_1 t} - 1 \right) \quad (62)$$

$$[EI^*] = - \frac{k_4 K_1 K_3^{'} [I]_0 [E]_0}{k_4 K_1 K_3^{'} [I]_0 + k_4^{'} K_3 [Z]_0 [I']_0 - k_2 K_3 K_3^{'} [Z]_0} \left(e^{\lambda_1 t} - 1 \right) \quad (63)$$

$$[EZI'^*] = - \frac{k_4^{'} K_3 [Z]_0 [I']_0 [E]_0}{k_4 K_1 K_3^{'} [I]_0 + k_4^{'} K_3 [Z]_0 [I']_0 - k_2 K_3 K_3^{'} [Z]_0} \left(e^{\lambda_1 t} - 1 \right) \quad (64)$$

where λ_1 is given by:

$$\lambda_1 = - \frac{k_4^{'} K_3 [Z]_0 [I']_0 + k_4 K_1 K_3^{'} [I]_0 - K_3 K_3^{'} k_2 [Z]_0}{K_1 K_3 K_3^{'} + K_1 K_3^{'} [I]_0 + K_3 K_3^{'} [Z]_0 + K_3 [Z]_0 [I']_0} \quad (65)$$

Note that for this particular case it is observed:

$$[\Sigma,E] = [E] + [EZ] + [EI] + [EI^*] + [EZI'] + [EZI'^*] \quad (66)$$

Residual enzyme activity

The instantaneous residual enzyme activity, $[E_T]$, in Scheme 1 is defined as the sum of the instantaneous concentrations of all of the enzyme forms involved in Scheme 1 minus the sum of the instantaneous concentrations of the inactive enzyme forms, i.e.:

$$[E_T] = [\Sigma, E] - ([EI^*] + [EZI'^*]) \quad (67)$$

If in eqn. (67), eqns. (62)-(64) are taken into account, we have:

$$[E_T] = [E]_0 e^{\lambda_1 t} \quad (68)$$

Note that, according to eqns. (66) and (67) it is also observed that:

$$[E_T] = [E] + [EZ] + [EI] + [EZI'] \quad (69)$$

From eqn. (68) it results $[E_T]_0 = [E]_0$, as expected.

Note that the approached analytical solution for the time course of the residual enzyme activity [eqn. (68)] has been obtained from the kinetic results for the general model in Scheme 2 as previously indicated, i.e., as a particular case of this Scheme.

4.3. Final Remarks

Eqns. (65) and (68) for the residual enzyme activity corresponding to Scheme 1 were obtained by our group [39] in an individualized analysis exclusively devoted to this Scheme. From the results obtained by these authors, the use of a dimensionless kinetic parameter giving the relative weight of both the activation and inhibition routes was suggested. In the present and in regard with Scheme 1 we want only point out that the kinetic results for a general model can serve as source to obtain the kinetic behaviour of any of the particular cases of the general model by only inserting in the time course equations for the general model the same changes reducing the last one to the particular cases under study.

Once obtained the equations corresponding to a determined mechanism, it is possible to make $[I]_0 = 0$ and/or $[I']_0 = 0$ to analyze the behaviour of the enzymatic system under study in the absence of one or the two inhibitors. The kinetic behaviour of any of the particular cases, allows to suggest experimental designs and analysis of kinetic data to estimate all or part of the kinetic parameters involved in the system, i.e., to characterize it.

The kinetic behaviour of any of the particular cases allows suggesting experimental design and kinetic data analysis to evaluate all or part of the kinetic parameters involved in the enzyme system, i.e. to characterize it and/or to suggest dimensionless kinetic parameters which provide the relative weight of the activation and inhibition routes and that of both inhibition routes.

The transient phase kinetic analysis of enzyme systems is a part of enzyme kinetics with increasingly importance because of it allows the determination of more kinetic parameters than in steady-state analysis. Nevertheless, transient phase kinetics requires to carry out kinetic measurements in a short reaction time because either the reaction is very rapid or due to, as in the present case, the obtained equations are valid during a short reaction time. Therefore, transient phase kinetic analysis requires instrumental allowing measurements at short reaction times such as a stopped flow coupled to the measurement instrument, e.g. spectrophotometer, spectrofluorimeter, etc. This instrumental allows to obtaining measurements from milliseconds. Thus, the experimental design and kinetic data analysis must be carried out at short time to be able to determine the involved parameters. But once these parameters have been obtained, the kinetic behaviour of the system for any reaction time

can be known from these parameters and the set of non-linear differential equations by numerical integration.

There are not yet too much reaction schemes of autocatalytic zymogen activation, overlapped or not with simultaneous inhibition, the kinetic analysis of which, experimentally, theoretically or both, has been carried out [9-14,31], some of them by ourselves [9-11,20,33,37-39]. Most of these contributions on prior work on enzyme-zymogen pairs fit any of the 16724 theoretically possible particular cases found from the present computation on Scheme 2.

Different researchers [9,12,13,20,22,27] have previously analysed experimental results corresponding to some schemes of autocatalytic zymogen activation, with or without the action of inhibitors, which are particular cases of Scheme 2. The experimental data were analysed by fitting them to the corresponding time course equations obtained from an individual kinetic analysis by analytically integrating the corresponding set of differential equations, once linearized. If these authors of the contributions above had provided the results of this paper, they could have particularized general model equations of Scheme 2 to their specific enzyme system under study, that is, in most cases, easier than obtaining individually. But the present theoretical contribution has not the need for previous experimental contributions on reaction schemes being particular cases of Scheme 2. When we began to develop this contribution, we did not think of applying the results to any real system as a part of the same contribution, but to carry out a kinetic analysis valid for any possible real system which fits the model as a particular case.

ACKNOWLEDGEMENTS

This work was supported by Projects No. PI-2007/53 from the Consejería de Sanidad (FISCAM) and PAI08-0175-8618 from the Consejería de Educación y Ciencia, both of them from Junta de Comunidades de Castilla-La Mancha (JCCM, Spain).

APPENDIX A

Set of differential equations describing the kinetic behaviour of those enzyme systems evolving according to reaction mechanism shown in Scheme 2.

$$\frac{d[E]}{dt} = -k_1[E][Z] - k_3[I][E] - k_5'[I'][E] + (k_{-1} + 2k_2)[EZ] + k_{-3}[EI] + k_{-5}'[EI'] \quad \text{(A1)}$$

$$\frac{d[EI]}{dt} = k_3[E][I] + k_{-4}[EI^*] - (k_{-3} + k_{-4})[EI] \quad \text{(A2)}$$

$$\frac{d[EI^*]}{dt} = -k_{-4}[EI^*] + k_4[EI] \quad \text{(A3)}$$

$$\frac{d[EI']}{dt} = k_5'[E][I'] + k_{-6}'[EI'^*] - (k_5' + k_{-6}')[EI'] \quad \text{(A4)}$$

$$\frac{d[EI'^*]}{dt} = -k'_{-6}[EI'^*] + k'_6[EI'] \quad \text{(A5)}$$

$$\frac{d[EZ]}{dt} = -k_5[EZ][I] + k_{-5}[EZI] - (k_{-1} + k_2)[EZ] - k'_3[I'][EZ] + k_{-3}'[EZI'] + k_1[E][Z] \quad \text{(A6)}$$

$$\frac{d[EZI']}{dt} = -(k'_4 + k'_{-3})[EZI'] + k'_3[EZ][I'] + k'_{-4}[EZI'^*] \quad \text{(A7)}$$

$$\frac{d[EZI'^*]}{dt} = -k'_{-4}[EZI'^*] + k'_4[EZI'] \quad \text{(A8)}$$

$$\frac{d[EZI]}{dt} = -(k_6 + k_{-5})[EZI] + k_5[EZ][I] + k_{-6}[EZI^*] \quad \text{(A9)}$$

$$\frac{d[EZI^*]}{dt} = -k_{-6}[EZI] + k_6[EZI] \quad \text{(A10)}$$

$$\frac{d[I]}{dt} = -k_3[E][I] + k_{-3}[EI] - k_5[EZ][I] + k_{-5}[EZI] \quad \text{(A11)}$$

$$\frac{d[I']}{dt} = -k'_5[E][I'] + k_{-5}[EI'] - k'_3[EZ][I'] + k'_{-3}[EZI'] \quad \text{(A12)}$$

$$\frac{d[Z]}{dt} = -k_1[E][Z] + k_{-1}[EZ] \quad \text{(A13)}$$

$$\frac{d[W]}{dt} = k_2[E] \quad \text{(A14)}$$

APPENDIX B

Analytical integration of the system of differential equations (27)-(36) in the main text, using the Laplace transform method. Obtaining the time course of [E], [EZ], [EI*] and [EZI*]

In Fig. B1 we summarise the steps in the application of the Laplace transform method that we detail step by step for the analytical solution of the set of linear differential equations (27)-(36)

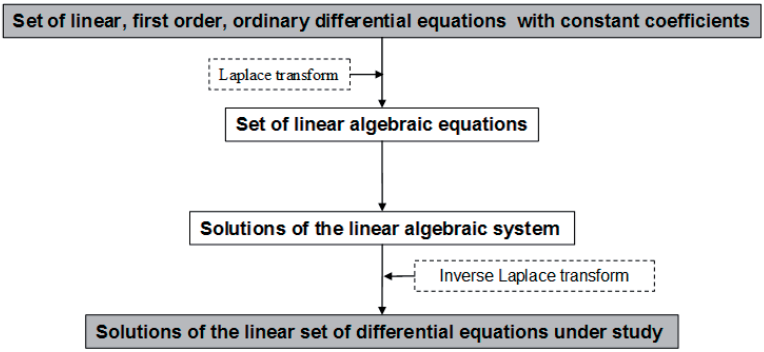


Figure B1. Simplified flowchart showing the steps in the Laplace transform method

Set of linear, first order, ordinary differential equations with constant coefficients

For convenience, we denote the species E, EI, EI*, EI', EI'*, EZ, EZI', EZI'*, EZI, EZI'* as X_1,X_2,....,X_{10}, respectively. Furthermore, $[X_1]$,$[X_2]$,....,$[X_{10}]$ denote the instantaneous concentrations of X_1,X_2,....,X_{10}, respectively, and $[X_1]_0$,$[X_2]_0$,....,$[X_{10}]_0$ denote the initial concentrations of X_1,X_2,....,X_{10}. With this notation, the system of differential equations (27)-(36) can be written as:

$$[X_1] = K_{1,1}[X_1] + K_{-3}[X_2] + K'_{-5}[X_4] + (K_{-1} + 2K_2)[X_6] \quad \text{(B1)}$$

$$[X_2] = K_3[I_0][X_1] + K_{2,2}[X_2] + K_{-4}[X_3] \quad \text{(B2)}$$

$$[X_3] = K_4[X_2] + K_{3,3}[X_3] \quad \text{(B3)}$$

$$[X_4] = K_5^{'}[I_0^{'}][X_1] + K_{4,4}[X_4] + K_{5,4}[X_5] \quad \text{(B4)}$$

$$[X_5] = K_6^{'}[X_4] + K_{5,5}[X_5] \quad \text{(B5)}$$

$$[X_6] = K_1[Z]_0[X_1] + K_{6,6}[X_6] + K_{-3}^{'}[X_7] + K_{-5}[X_9] \quad \text{(B6)}$$

$$[X_7] = K_3^{'}[I_0^{'}][X_6] + K_{7,7}[X_7] + K_{-4}[X_8] \quad \text{(B7)}$$

$$[X_8] = K_4^{'}[X_7] + K_{8,8}[X_8] \quad \text{(B8)}$$

$$[X_9] = K_5[I]_0[X_6] + K_{9,9}[X_9] + K_{-6}[X_{10}] \quad \text{(B9)}$$

$$[X_{10}] = K_6[X_9] + K_{10,10}[X_{10}] \quad \text{(B10)}$$

where $K_{i,i}$ (i=1,2,…,10) are given by eqns. (6)-(16) in the main text.

Laplace transform

If we apply the Laplace transform method to the linear system of differential equations (B1)-(B10) and assuming that the only enzyme species present at the start of the reaction is E, i.e. X_1 (which means that $[X_1]_0>0$), yields, after some rearrangement to the following equations:

Set of linear algebraic equations

$$-[X_1]_0 = (K_{1,1} - \lambda)L\{[X_1]\} + K_{-3}L\{[X_2]\} + K_{-5}^{'}L\{[X_4]\} + (K_{-1} + 2K_2)L\{[X_6]\} \quad \text{(B11)}$$

$$0 = K_3[I]_0 L\{[X_1]\} + (K_{2,2} - \lambda)L\{[X_2]\} + K_{-4}L\{[X_3]\} \quad \text{(B12)}$$

$$0 = K_4 L\{[X_2]\} + (K_{3,3} - \lambda)L\{[X_3]\} \quad \text{(B13)}$$

$$0 = K_5^{'}[I^{'}]_0 L\{[X_1]\} + (K_{4,4} - \lambda)L\{[X_4]\} + K_{-6}^{'}L\{[X_5]\} \quad \text{(B14)}$$

$$0 = K_1[Z]_0 L\{[X_4]\} + (K_{5,5} - \lambda)L\{[X_5]\} \quad \text{(B15)}$$

$$0 = K_3^{'}[I^{'}]_0 L\{[X_6]\} + (K_{7,7} - \lambda)L\{[X_7]\} + K_{-4}^{'}L\{[X_8]\} \quad \text{(B16)}$$

$$0 = K_4^{'}L\{[X_7]\} + (K_{8,8} - \lambda)L\{[X_8]\} \quad \text{(B17)}$$

$$0 = K_5[I]_0 L\{[X_6]\} + (K_{9,9} - \lambda)L\{[X_9]\} + K_{-6}L\{[X_{10}]\} \quad \text{(B18)}$$

$$0 = K_6 L\{[X_9]\} + (K_{10,10} - \lambda)L\{[X_{10}]\} \quad \text{(B19)}$$

where $L\{[X_i]\}$ is the Laplace transform of $[X_i]$ (i=1,2,...,10) and λ is the operator of Laplace transform.

Solutions of the linear algebraic system

The previous system of equations (B11)-(B19) is an algebraic system that can be solved by Cramer's rule. Thus $L\{[X_i]\}$ (i=1,2,...,10) can be expressed by:

$$L\{[X_i]\} = \frac{(-1)^i [X_1]_0 D_{1,i}(\lambda)}{D(\lambda)} \quad (i=1,2,...,10) \quad \text{(B20)}$$

In eqn. (B20) above $D(\lambda)$ is the determinant given by eqn. (5) and $D_{1,i}(\lambda)$ is the minor that results from deleting the first row and i-th column. The development of the determinant $D(\lambda)$ is given by eqn. (17) in the main text. The polynomial $D(\lambda)$ has 10 roots, $\lambda_1, \lambda_2, \ldots, \lambda_{10}$, none of them as null because $G_{10} \neq 0$. Furthermore it is assumed that these 10 roots are simple, i.e., there is no one repeated, which in the practice is most probably. Therefore, the polynomial $D(\lambda)$ can be written as:

$$D(\lambda) = (\lambda - \lambda_1)(\lambda - \lambda_2)\cdots(\lambda - \lambda_{10}) \quad \text{(B21)}$$

At the same time, the expansion of $D_{1,i}(\lambda)$ is a polynomial whose degree and coefficients depend on what the value of i be. Specifically, for i=1,3,6 and 8, these expansions are given by eqns. (18)-(21) of the main text. $D(\lambda)$ is a polynomial of degree 10, while $D_{1,i}(\lambda)$ is a polynomial of degree 9 at the most, therefore, the second member of the eqn. (B20) can be decomposed into simple fractions. Moreover, taking into account the expression of $D(\lambda)$ given by eqn. (B21), can be written:

$$\frac{(-1)^i [X_1]_0 D_{1,i}(\lambda)}{(\lambda - \lambda_1)(\lambda - \lambda_2)\cdots(\lambda - \lambda_{10})} = \frac{A_{1,i}}{\lambda - \lambda_1} + \frac{A_{2,i}}{\lambda - \lambda_2} + \cdots + \frac{A_{10,i}}{\lambda - \lambda_{10}} \quad \text{(B22)}$$

If the sum of the terms of the second member is made:

$$\frac{(-1)^i[X_1]_0 D_{1,i}(\lambda)}{(\lambda-\lambda_1)(\lambda-\lambda_2)\cdots(\lambda-\lambda_{10})} = \frac{\sum_{h=1}^{10}\left\{A_{i,h}\prod_{\substack{p=1\\p\neq h}}^{10}(\lambda-\lambda_p)\right\}}{(\lambda-\lambda_1)(\lambda-\lambda_2)\cdots(\lambda-\lambda_{10})} \qquad \text{(B23)}$$

As the denominators of both sides of the equation above are equal, the numerators must be equal too, i.e.,

$$(-1)^i[X_1]_0 D_{1,i}(\lambda) = \sum_{h=1}^{10}\left\{A_{i,h}\prod_{\substack{p=1\\p\neq h}}^{10}(\lambda-\lambda_p)\right\} \qquad \text{(B24)}$$

If in eqn. (B24) we set $\lambda = \lambda_h$ (h =1,2,...,10) all summands of the second member become null, except the summand $A_{i,h}\prod_{\substack{p=1\\p\neq h}}^{10}(\lambda_h-\lambda_p)$ that will be written for convenience as $-A_{i,h}\prod_{\substack{p=1\\p\neq h}}^{10}(\lambda_p-\lambda_h)$. So, from eqn. (B24) we have:

$$(-1)^i[X_1]_0 D_{1,i}(\lambda_h) = -A_{i,h}\prod_{\substack{p=1\\p\neq h}}^{10}(\lambda_p-\lambda_h) \qquad \text{(h=1,2,…,10)} \qquad \text{(B25)}$$

from which we obtain:

$$A_{i,h} = \frac{(-1)^{i-1}[X_1]_0 D_{1,i}(\lambda_h)}{\prod_{\substack{p=1\\p\neq h}}^{10}(\lambda_p-\lambda_h)} \qquad \text{(h=1,2,…,10)} \qquad \text{(B26)}$$

If in the last eqn. (B26) $[X_1]_0$ is replaced for $[E]_0$ and $D_{1,i}(\lambda_h)$ for the expression corresponding to each of the different i-values, we obtain the expressions of the ten

coefficients $A_{i,h}$, for each i. In particular, for i=1,3,6 and 8 are obtained eqns. (38), (50), (52) and (54) in the main text. From eqns. (B20) and (B22) we get:

$$L\{[X_i]\} = \frac{A_{1,i}}{\lambda - \lambda_1} + \frac{A_{2,i}}{\lambda - \lambda_2} + \cdots + \frac{A_{10,i}}{\lambda - \lambda_{10}} \tag{B27}$$

Inverse Laplace transform

Taking inverse Laplace transform in the above equation we obtain, finally:

Solutions of the linear set of differential equations under study

$$[X_i] = \sum_{h=1}^{10} A_{i,h} e^{\lambda_h t} \tag{B28}$$

where $A_{i,h}$ (i=1,2,...,10; h=1,2,...,10) are given by eqn. (B26). Eqn. (B28) provides the instantaneous concentration of any of the ten species involved in the enzymatic reaction mechanism of Scheme 2.

APPENDIX C

Expressions of the coefficients G_{10}, f_8, b_8, a_8 and c_8 involved in eqns. (17)-(21) in the main text. The expressions which appear in eqns. (C1)-(C5) as k1, k2, k3, k'3, k4, k'4, k5, k'5, k6, k'6, (k-1), (k-3), (k'-3), (k-4), (k'-4), (k-5), (k'-5), (k-6) and (k'-6) correspond to k_1, k_2, k_3, k'_3, k_4, k'_4, k_5, k'_5, k_6, k'_6, k_{-1}, k_{-3}, k'_{-3}, k_{-4}, k'_{-4}, k_{-5}, k'_{-5}, k_{-6} and k'_{-6}. Likewise, the expressions [Z]0, [I]0 and [I']0 correspond to the initial concentrations $[Z]_0$, $[I]_0$ and $[I']_0$

$$G_{10} = - k1k2(k-3)(k-4)(k'-5)(k'-6)(k'-3)(k'-4)(k-5)(k-6)[Z]0 \quad \text{(C1)}$$

$f_8 =$

(k-3)(k-4)(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5) + (k-3)(k-4)(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)k6 +
+ (k-3)(k-4)(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-6) + (k-3)(k-4)(k'-5)(k'-6)(k-1+k2)(k'-3)(k-5)(k-6) + (k-3)(k-4)(k'-5)(k'-6)(k-1+k2)k'4(k-5)(k-6) + (k-3)(k-4)(k'-5)(k'-6)(k-1+k2)(k'-4)(k-5)(k-6) + (k-3)(k-4)(k'-5)(k'-6)k'3[I']0k'4(k-5)(k-6) + (k-3)(k-4)(k'-5)(k'-6)k'3[I']0(k'-4)(k-5)(k-6) +
+ (k-3)(k-4)(k'-5)(k'-6)k5[I]0(k'-3)(k'-4)k6 + (k-3)(k-4)(k'-5)(k'-6)k5[I]0(k'-3)(k'-4)(k-6) +
+ (k-3)(k-4)(k'-5)(k'-6)(k'-3)(k'-4)(k-5)(k-6) + (k-3)(k-4)(k'-5)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6) +
+ (k-3)(k-4)k'6(k-1+k2)(k'-3)(k'-4)(k-5)(k-6) + (k-3)(k-4)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6)
+ (k-3)(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6) + k4(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6)
+ (k-4)(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6) (C2)

$b_8 =$

k3[I]0k4(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5) + k3[I]0k4(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)k6 +
+ k3[I]0k4(k'-5)(k'-6)(k-1+k2)(k'-3)(k'-4)(k-6) + k3[I]0k4(k'-5)(k'-6)(k-1+k2)(k'-3)(k-5)(k-6)
+ k3[I]0k4(k'-5)(k'-6)(k-1+k2)k'4(k-5)(k-6) + k3[I]0k4(k'-5)(k'-6)(k-1+k2)(k'-4)(k-5)(k-6) +
+ k3[I]0k4(k'-5)(k'-6)k'3[I']0k'4(k-5)(k-6) + k3[I]0k4(k'-5)(k'-6)k'3[I']0(k'-4)(k-5)(k-6) +
+ k3[I]0k4(k'-5)(k'-6)k5[I]0(k'-3)(k'-4)k6 + k3[I]0k4(k'-5)(k'-6)k5[I]0(k'-3)(k'-4)(k-6) +
+ k3[I]0k4(k'-5)(k'-6)(k'-3)(k'-4)(k-5)(k-6) + k3[I]0k4(k'-5)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6) +
+ k3[I]0k4k'6(k-1+k2)(k'-3)(k'-4)(k-5)(k-6) + k3[I]0k4(k'-6)(k-1+k2)(k'-3)(k'-4)(k-5)(k-6)
(C3)

a_8 =

k1[Z]0(k-3)(k-4)(k'-5)(k'-6)(k'-3)(k'-4)(k-5) + k1[Z]0(k-3)(k-4)(k'-5)(k'-6)(k'-3)(k'-4)k6 +
+ k1[Z]0(k-3)(k-4)(k'-5)(k'-6)(k'-3)(k'-4)(k-6) + k1[Z]0(k-3)(k-4)(k'-5)(k'-6)(k'-3)(k-5)(k-6) +
+ k1[Z]0(k-3)(k-4)(k'-5)(k'-6)k'4(k-5)(k-6) + k1[Z]0(k-3)(k-4)(k'-5)(k'-6)(k'-4)(k-5)(k-6) +
+ k1[Z]0(k-3)(k-4)(k'-5)(k'-3)(k'-4)(k-5)(k-6) + k1[Z]0(k-3)(k-4)k'6(k'-3)(k'-4)(k-5)(k-6) +
+ k1[Z]0(k-3)(k-4)(k'-6)(k'-3)(k'-4)(k-5)(k-6) + k1[Z]0(k-3)(k'-5)(k'-6)(k'-3)(k'-4)(k-5)(k-6) +
+ k1[Z]0k4(k'-5)(k'-6)(k'-3)(k'-4)(k-5)(k-6) + k1[Z]0(k-4)(k'-5)(k'-6)(k'-3)(k'-4)(k-5)(k-6)

(C4)

c_8 =

k1[Z]0(k-3)(k-4)(k'-5)(k'-6)k'3[I']0k'4(k-5) + k1[Z]0(k-3)(k-4)(k'-5)(k'-6)k'3[I']0k'4k6 +
+ k1[Z]0(k-3)(k-4)(k'-5)(k'-6)k'3[I']0k'4(k-6) + k1[Z]0(k-3)(k-4)(k'-5)k'3[I']0k'4(k-5)(k-6) +
+ k1[Z]0(k-3)(k-4)k'6k'3[I']0k'4(k-5)(k-6) + k1[Z]0(k-3)(k-4)(k'-6)k'3[I']0k'4(k-5)(k-6) +
+ k1[Z]0(k-3)(k'-5)(k'-6)k'3[I']0k'4(k-5)(k-6) + k1[Z]0k4(k'-5)(k'-6)k'3[I']0k'4(k-5)(k-6) +
+ k1[Z]0(k-4)(k'-5)(k'-6)k'3[I']0k'4(k-5)(k-6) (C5)

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