J Math Chem (2010) 48:635–652
DOI 10.1007/s10910-010-9697-z

ORIGINAL PAPER

A general model for non-autocatalytic zymogen activation in the presence of two different and mutually exclusive inhibitors. II. Relative weight of activation and inhibition processes

J. Masiá-Pérez · J. Escribano · E. Valero · E. Arribas · M. García-Moreno · J. L. Muñoz-Muñoz · R. Gómez-Ladrón de Guevara · R. Varón

Received: 15 January 2010 / Accepted: 4 May 2010 / Published online: 26 May 2010
© Springer Science+Business Media, LLC 2010

Abstract The kinetic study carried out in paper I of this series (preceding article) on enzyme systems involving zymogen activation and the simultaneous action of two different, mutually exclusive inhibitors (Scheme 1) allows, new dimensionless kinetic parameters to be suggested. These parameters furnish quantitative information about the relative weight of the activation and inhibition routes, i.e. the conditions under which the activation prevails over the inhibition and vice versa, as well as the absolute and relative contributions to overall inhibition of each of the inhibition routes and their synergistic effect. These results can be easily and directly applied to any of the thousands of particular cases of the model. Examples are given for different particular cases.

Keywords Enzyme kinetics · Zymogen activation · Enzymatic inhibition · Relative weight · Exclusive

J. Masiá-Pérez
Servicio de Cardiología, Complejo Hospitalario Universitario de Albacete, Albacete, Spain

J. Escribano · R. Gómez-Ladrón de Guevara
Departamento de Ciencia y Tecnología Agroforestal y Genética, Universidad de Castilla-La Mancha, Albacete, Spain

E. Valero · M. García-Moreno · R. Varón (✉)
Departamento de Química-Física, Universidad de Castilla-La Mancha, Albacete, Spain
e-mail: ramon.varon@uclm.es

E. Arribas
Departamento de Física Aplicada, Universidad de Castilla-La Mancha, Albacete, Spain

J. L. Muñoz-Muñoz
Departamento de Bioquímica y Biología Molecular A, Universidad de Murcia, Murcia, Spain

1 Introduction

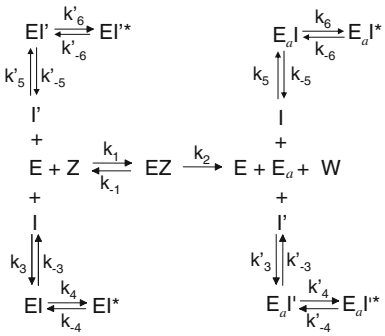
The ability of metabolic processes to regulate different processes is a consequence of the evolution of an extremely complex system of regulatory mechanisms. Thus, living organisms possess several biological amplification systems which help them achieve a fast response to a given stimulus, such as enzyme cascades [1,2], substrate cycling [3,4] and limited proteolysis reactions [5–7]. These mechanisms have special characteristics which make them well-suited to playing a central role in metabolism, so they are tightly buffered and controlled more. Particularly, proteolytic enzymes are of widespread interest to the scientific community because, besides the fact that they can be used as tools, they play very critical roles in biological systems, where they are involved in a multitude of important physiological processes that range from the functional activation or inactivation of proteins by single proteolytic events, to the complete dissolution of proteins into their constituent amino acids. For this reason they have become the focus of a wide range of basic and applied research, and are targets for intervention, both experimentally and therapeutically [8,9].

Limited proteolysis reactions are controlled in the metabolism by the natural protease inhibitors present in cells and body fluids. When working with protease inhibitors, whether discovering new ones and characterizing them, using them as practical tools, or for therapeutic intervention, the importance of reaction kinetics cannot be overstressed. Analysis of the kinetics of a reaction between a protease and its potential inhibitors delineates the likely control point in complex biological media [10]. If one wishes to suppress the activity of a given protease and knows the kinetic constants for the reaction of this protease with an inhibitor, one can determine how much inhibitor to add and how much time to allow for the inhibition.

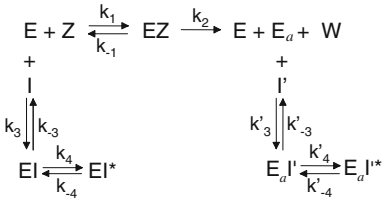
In a previous contribution (paper I of this series; [11]) we performed a kinetic analysis of a general model of a zymogen activation process involving two different, mutually exclusive inhibitors, which can act simultaneously on both the activating and the active enzyme (Scheme 1). In this respect it would be very interesting to establish the relative weight of the activation and inhibition routes, i.e. the conditions in which the activation prevails over the inhibition and vice versa. Moreover, it could also be interesting to know the relative contribution of both inhibitors to the total inhibition. Such knowledge would be directly applicable to thousands of other mechanisms which are particular cases of the general model. Previously, the only similar study to the one described here, although more limited, was performed by our research group [12]. The mechanism studied in this latter case is shown in Scheme 2, which is one of the thousands of particular cases of the general model shown in Scheme 1.

Therefore, the aims of the present paper are the following, which are both related and complementary: (1) From the steady-state rate equation of species E_a in Scheme 1 obtained in paper I of this series, to derive novel kinetic parameters furnishing the relative weight of the activation and inhibition routes and also the relative contribution of both inhibitors to the overall inhibition. (2) Using as examples two particular cases of Scheme 1, to establish the best method to apply these results to any of the thousands of mechanisms of zymogen activation overlapped with enzymatic inhibition processes, which can be considered, real or formally, particular cases of the general model shown in Scheme 1 (e.g. Scheme 2). This study will widen and complement our knowledge

Scheme 1



Scheme 2



of the inhibition mechanisms of zymogen activation processess, some of which have been previously described in the scientific literature [12–18].

2 Materials and methods

We have taken as starting point for this paper the equation obtained for the steady-state rate accumulation of E_a in paper I of this series [11] and, from here, usual algebraic relationships and expansions have been used.

3 Theory

3.1 Notation

The following notation will be used in the present paper:

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

[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 towards the activating protease, i.e.:

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

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

$$K_j = k_{-j}/k_j \tag{2}$$

K'_j $(j =$ 3,4,5,6): Equilibrium constants defined as:

$$K'_j = k'_{-j}/k'_j \tag{3}$$

$[\Sigma, E_a]$: Sum of the concentrations of all of the enzymatic species involved in Scheme 1 containing E_a, i.e.:

$$[\Sigma, E_a] = [E_a] + [E_a I'] + [E_a I'^*] + [E_a I] + [E_a I^*] \tag{4}$$

3.2 Steady-state rate of the activated enzyme E_a

At relatively high values of *rt*, i.e. in the steady-state, the following equation for the rate of formation of E_a (see paper I of this series; [11]) is obtained:

$$\alpha = \frac{k_1 k_2 k_{-3} k_{-4} k'_{-3} k'_{-4} k_{-5} k_{-6} k'_{-5} k'_{-6} [Z]_0 [E]_0}{F_5 F'_4} \tag{5}$$

where:

$$\begin{aligned} \mathrm{F_5} = {} & \mathrm{k_{-3}k_{-4}k'_{-5}k'_{-6}\,(k_{-1} + k_2) + k_1k_{-3}k_{-4}k'_{-5}k'_{-6}\,[Z]_0} \\ & \mathrm{+k_3k'_{-5}k'_{-6}\,(k_{-1} + k_2)\,(k_4 + k_{-4})\,[I]_0} \\ & \mathrm{+ k'_5k_{-3}k_{-4}\,(k_{-1} + k_2)\left(k'_6 + k'_{-6}\right)\left[I'\right]_0} \end{aligned} \tag{6}$$

$$\mathrm{F'_4 = k'_{-3}k'_{-4}k_{-5}k_{-6} + k_5k'_{-3}k'_{-4}\,(k_6 + k_{-6})\,[I]_0 + k'_3k_{-5}k_{-6}\left(k'_4 + k'_{-4}\right)\left[I'\right]_0} \tag{7}$$

Eq. 5 was obtained under the following initial conditions:

$$[Z]_0, [I]_0, [I']_0 >> [E]_0 \tag{8}$$

$$[I], [I']_0 >> [Z]_0 \tag{9}$$

In addition, we shall consider a reaction time, *rt*, for which $[E_a]$ remains much less than $[Z]_0$, i.e.:

$$[E_a] << [Z]_0 \quad \text{during time } rt \tag{10}$$

Note that α can also be expressed as follows:

$$\alpha = \frac{V_{\max}[Z]_0}{K_m + [Z]_0} r \tag{11}$$

where $V_{\max} = k_2[E]_0$ (the maximum rate of accumulation of E_a in the steady-state which, mathematically, corresponds to an initial zymogen concentration so that $[Z]_0 >> K_m$ in the absence of inhibitors) and r is a dimensionless parameter given by the following equation:

$$r = \frac{1}{1 + p + p' + q} \tag{12}$$

where the following notation has been used:

$$p = \left\{ \frac{1}{K_5}\left(1+\frac{1}{K_6}\right) + \frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\frac{K_m}{K_m+[Z]_0}\left(1+\frac{1}{K_5}\left(1+\frac{1}{K_6}\right)[I]_0\right)\right\}[I]_0 \tag{13}$$

$$p' = \left\{ \frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right) + \frac{1}{K'_5}\left(1+\frac{1}{K'_6}\right)\frac{K_m}{K_m+[Z]_0}\left(1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I']_0\right)\right\}[I']_0 \tag{14}$$

and

$$q = \frac{K_m}{K_m+[Z]_0}\left\{\frac{1}{K_5K'_5}\left(1+\frac{1}{K_6}\right)\left(1+\frac{1}{K'_6}\right) + \frac{1}{K_3K'_3}\left(1+\frac{1}{K_4}\right)\left(1+\frac{1}{K'_4}\right)\right\}[I]_0[I']_0 \tag{15}$$

Note that $0 \leq r \leq 1$, in agreement with Eq. 12 and bearing in mind that p, p' and q are non-negative quantities.

4 Results and discussion

The mathematical expression giving the steady-state rate of E_a (Eq. 11) and the expressions of r, p, p' and q allow us to know the relative weight of the activation and inhibition routes in the process, as well as to suggest additional dimensionless parameters, other than r, giving the efficiency of the inhibition and the relative contribution of both inhibitors to the overall inhibition of zymogen activation.

4.1 Quantification of the relative weight of the activation and inhibition routes

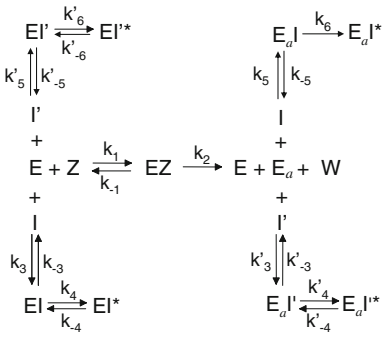
The previously defined dimensionless parameter r, whose value is between 0 and 1, quantifies the relative weight of the activation and inhibition routes. We shall consider different cases depending on the value of *r:*

(a): *r*= 1

This is the maximum value of r and is only attained if:

$$p + p' + q = 0 \tag{16}$$

Scheme 3



This condition is fulfilled when $[I]_0 = [I']_0 = 0$, i.e. in the absence of both inhibitors, where the steady-state rate is:

$$\alpha = \frac{V_{\max}[Z]_0}{K_m + [Z]_0} \tag{17}$$

(b): $r \approx 1$

This case will occur when the following condition is fulfilled, according to Eq. 12:

$$1 >> p + p' + q \tag{18}$$

for which it is sufficient, but necessary that the following three conditions be simultaneously fulfilled:

$$1 >> p, 1 >> p' \quad \text{and} \quad 1 >> q \tag{19}$$

Obviously, p and p' can be null. In this case the steady-state rate will be:

$$\alpha \approx \frac{V_{\max}[Z]_0}{k_m + [Z]_0} \tag{20}$$

(c): $r = 0$

If one or more of the following constants k_{-3} (but not k_3), k_{-4} (but not k_4), k'_{-3} (but not k'_3), k'_{-4} (but not k'_4), k'_{-5} (but not k'_5), k'_{-6} (but not k'_6), k_{-5} (but not k_5), k_{-6} (but not k_6) are null, i.e. if one or more of the following equilibrium constants, K_3, K_4, K'_3, K'_4, K'_5, K'_6, K_5 and K_6 are null (which means that at least one of the two inhibitors is irreversible), then $r = 0$, and so the steady-state rate of E_a formation, i.e.:

$$\alpha = 0 \tag{21}$$

An example, between other, of a particular case of Scheme 1 in this situation is shown in Scheme 3, in which $k_{-6} = 0$ and so $K_6 = 0$. This scheme corresponds to the situation in which the inhibitor I is irreversible in two steps when it acts on E_a.

Table 1 Values of p, p', q, r, α and η_T for different values of initial inhibitors concentrations

$[I]_0$(mM)	$[I']_0$(mM)	p	p'	q	r	α(nM s^{-1})	η_T
1	1	0.07554	16.35	0.6511	0.05531	26.34	0.9447
0.1	1	0.007450	16.35	0.06511	0.05738	27.33	0.9426
1	0.1	0.07554	1.116	0.06511	0.4431	211.0	0.5569
0.1	0.1	0.007450	1.116	0.006511	0.4694	223.5	0.5306
0	1	0	16.35	0	0.05762	27.44	0.9424
1	0	0.07554	0	0	0.9298	442.7	0.07023
0	0	0	0	0	1	476.2	0

The following arbitrary values of the rate constants and initial conditions have been used: $k_1 = 10^4$ 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}, $[E]_0 = 1\mu$M, and $[Z]_0 = 0.1$ mM

(d): $r \approx 0$

The parameter r will be much less than unity and so approximately equal to zero in those cases in which at least one of the three following relationships is fulfilled, in agreement with Eq. 12.

$$\left.\begin{array}{l} p >> 1 \\ p' >> 1 \\ q >> 1 \end{array}\right\} \tag{22}$$

Figure 1a illustrates the variation of r upon the concentration of both inhibitors, at a fixed zymogen concentration. Table 1 shows the values of p, p', q, r and α for different initial concentrations of both inhibitors and a fixed initial zymogen concentration and fixed rate constants.

The previously defined parameter r can be used to quantify the relative weight of activation and inhibition routes in the general model (Scheme 1) and in each of its derived mechanisms. The more different r is from its maximum value, 1 (corresponding to the absence of inhibitors), the greater the inhibitory action of the inhibitors. Thus, r provides the relative weight of the action of the activating enzyme, E on Z, and the action of I and I' on E and E_a. We suggest the name of *efficiency of activation* for r, which can also be expressed as a percentage.

4.1.1 Relative weight of activation and inhibition processess in some particular cases of the general model

4.1.1.1. Particular case: Scheme 2

As previously indicated for the general model, α indicates the steady-state formation rate of E_a and can be written as a function of r, as indicated in Eq. 11. If Eqs. 12–15 from Scheme 1 are applied to Scheme 2, type 0/0 uncertainties would result, e.g. if we are to obtain K'_5 ($K'_5 = k'_{-5}/k'_5$). In this case, we proceed as follows: make zero the minimal set of rate constants that transform the general mechanism into another

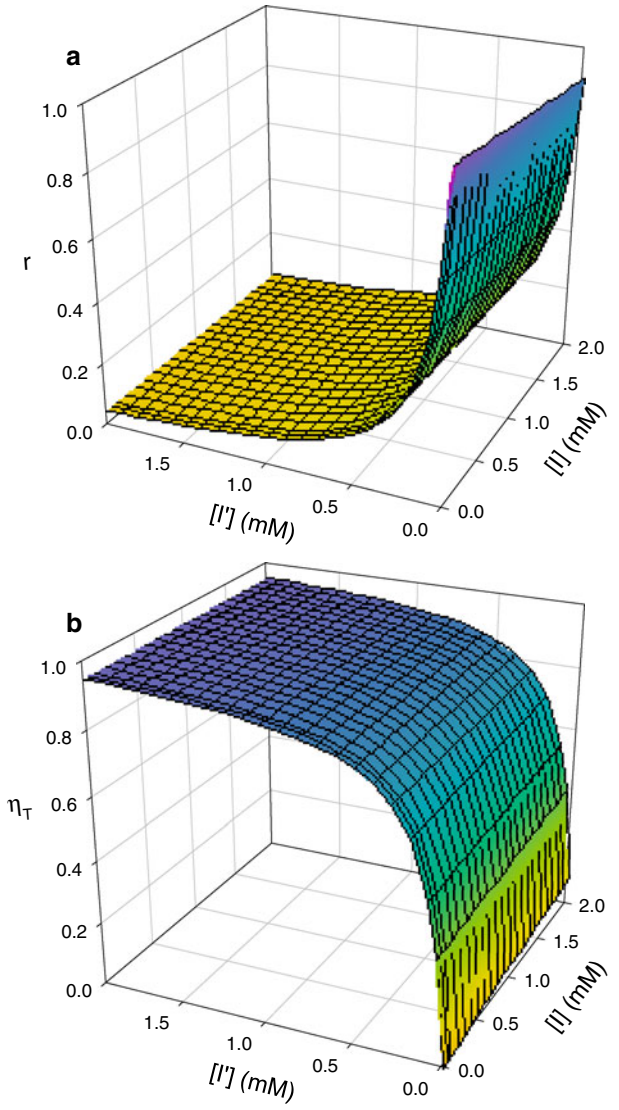
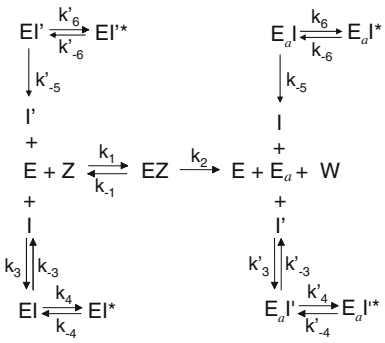


Fig. 1 Three-dimensional plot of r (**a**) and η_T (**b**) *vs.* $[I]_0$ and $[I']_0$ [Eqs. 26 and 45, respectively] for a fixed value of $[Z]_0$. The values of the rate constants and the fixed $[E]_0$-value are the same as indicated in Table 1

that is kinetically equivalent to the mechanism under study (intermediate mechanism, Scheme 4), whenever the same initial conditions as in the general model hold. In the resulting equations, make zero the rest of the rate constants, if there are any, to obtain the true mechanism under consideration (Scheme 2).

In this way, the first constants that are to be cancelled to obtain the equivalent intermediate mechanism (Scheme 4) are k'_5 and k_5. The result is:

Scheme 4



$$K_5 \to \infty \tag{23}$$

$$K_5' \to \infty \tag{24}$$

If Eqs. 23 and 24 are inserted into Eqs. 13–15 one obtains:

$$p = \frac{1}{K_3}\left(1 + \frac{1}{K_4}\right)\frac{K_m}{K_m + [Z]_0}[I]_0 \tag{25}$$

$$p' = \frac{1}{K_3'}\left(1 + \frac{1}{K_4'}\right)[I']_0 \tag{26}$$

and

$$q = \frac{K_m}{K_m + [Z]_0}\left\{\frac{1}{K_3 K_3'}\left(1 + \frac{1}{K_4}\right)\left(1 + \frac{1}{K_4'}\right)\right\}[I]_0[I']_0 \tag{27}$$

Note that in this case the following relationship is fulfilled:

$$q = pp' \tag{28}$$

and so Eq. 12 can be rewritten as:

$$r = \frac{1}{1 + p + p' + pp'} \tag{29}$$

Now, we shall consider different cases depending on the value of r.

(a): r= 1

This is the maximum value of r and is only attained if:

$$p + p' + pp' = 0 \tag{30}$$

for which it is necessary and sufficient that:

$$p = p' = 0 \tag{31}$$

This condition is only attained if $[I]_0 = [I]'_0 = 0$, i.e. in the absence of both inhibitors. The steady-state rate will be:

$$\alpha = \frac{V_{\max}[Z]_0}{K_m + [Z]_0} \tag{32}$$

(b): $r \approx 1$

This case will occurr when the following condition is fulfilled, in agreement with Eq. 29:

$$1 >> p + p' + pp' \tag{33}$$

for which it is necessary and sufficient that the two following conditions are simultaneously fulfilled:

$$1 >> p \text{ and } 1 >> p' \tag{34}$$

Obviously, both p and p' may be null. In this case, the steady-state rate will be:

$$\alpha \approx \frac{V_{\max}[Z]_0}{K_m + [Z]_0} \tag{35}$$

(c): $r = 0$

If one or more of the following constants, k_{-3} (but not k_3), k_{-4} (but not k_4), k'_{-3} (but not k'_3) and k'_{-4} (but not k'_4) from Scheme 2 are null, i.e. if one or more of the following equilibrium constants, K_3, K_4, K'_3 and K'_4 are null (which means that at least one of the two inhibitors is irreversible), then $r = 0$ and so:

$$\alpha = 0 \tag{36}$$

(d): $r \approx 0$

The parameter r will be less than unity in those cases in which the following condition is fulfilled, in agreement with Eq. 29:

$$p + p' + pp' >> 1 \tag{37}$$

for which it is necessary and sufficient that one and/or the two next conditions are fulfilled:

$$\left.\begin{array}{l} p >> 1 \\ p' >> 1 \end{array}\right\} \tag{38}$$

Particular cases from Scheme 1 in which $I \equiv I'$ (particular cases AC, BC and ABC)

In those cases in which both inhibitors match (particular cases AC, BC and ABC), the concept of the inhibitory action of I or I' should be replaced by the inhibitory action of the inhibitor on E and E_a. If we denote the only inhibitor by I, then p is given by Eq. 25 and p' by the following expression:

$$p' = \frac{1}{K_3'}\left(1 + \frac{1}{K_4'}\right)[I]_0 \tag{39}$$

and so:

$$p + pp' = \frac{K_m}{K_m + [Z]_0}\frac{1}{K_3}\left(1 + \frac{1}{K_4}\right)\left\{1 + \frac{1}{K_3'}\left(1 + \frac{1}{K_4'}\right)[I]_0\right\}[I]_0 \tag{40}$$

The kinetic parameter r is now given by:

$$r = \frac{1}{1 + \left[\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1 + \frac{1}{K_4}\right)\left\{1 + \frac{1}{K_3'}\left(1 + \frac{1}{K_4'}\right)\right\} + \frac{1}{K_3'}\left(1 + \frac{1}{K_4'}\right)\right][I]_0} \tag{41}$$

4.1.1.2. Particular case: Scheme 5

The kinetic parameters p, p' and q are given by:

$$p = \frac{K_1}{K_1 + [Z]_0}\frac{[I]_0}{K_3} \tag{42}$$

$$p' \to \infty \tag{43}$$

$$q \to \infty \tag{44}$$

and so, according to Eq. 29, $r = 0$.

4.2 Total inhibition efficiency

We shall define the *total inhibition efficiency*, η_T, as follows:

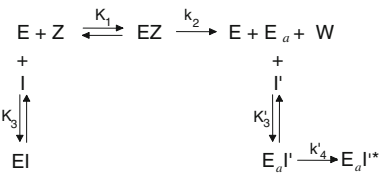
$$\eta_T = 1 - r \tag{45}$$

From Eqs. 45 and 29 one obtains:

$$\eta_T = \frac{p + p' + q}{1 + p + p' + q} \tag{46}$$

η_T takes values between 0 and 1, zero in the absence of both inhibitors and 1 when at least one of the two inhibitors is irreversible. The larger the values of p, p' and q,

Scheme 5



the greater is η_T. Table 1 shows the value of η_T for different values of initial concentrations of inhibitors and fixed values of zymogen concentration and rate constants. Figure 1b illustrates the variation of η_T versus the concentration of both inhibitors at a fixed zymogen concentration.

4.2.1 Total inhibition efficiency in some particular cases

4.2.1.1. Particular case: Scheme 2

In this case, the total inhibition efficiency given by Eq. 46 for the general model, is reduced to the following expression, where p and p' are given by Eqs. 25 and 26:

$$\eta_T = \frac{p + p' + pp'}{1 + p + p' + pp'} \tag{47}$$

Particular cases in which $I \equiv I'$ (particular cases AC, BC and ABC)

In those cases in which $I \equiv I'$ (particular cases AC, BC and ABC), the kinetic parameter η_T is given by the following expression, taking into account Eqs. 25 and 26:

$$\eta_T = \frac{\left[\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K_3'}\left(1+\frac{1}{K_4'}\right)[I]_0\right\}+\frac{1}{K_3'}\left(1+\frac{1}{K_4'}\right)\right][I]_0}{1+\left[\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K_3'}\left(1+\frac{1}{K_4'}\right)[I]_0\right\}+\frac{1}{K_3'}\left(1+\frac{1}{K_4'}\right)\right][I]_0} \tag{48}$$

4.2.1.2. Particular case: Scheme 5

In this case, in view of Eqs. 46 and 42–44:

$$\eta_T = 1 \tag{49}$$

If we make $p + p' + q = x$, then $\eta_T = x(1 + x)$. If $p' \to \infty$ and $x \to \infty$ are taken into account, it results Eq. 49 for η_T. The same result would have been obtained using Eq. 45 and taking into account that, in this case, $r = 0$.

4.3 Absolute and relative contribution of both inhibition routes to the total inhibition

Eq. 46 may be also written as:

$$\eta_T = \frac{p}{1 + p + p' + q} + \frac{p'}{1 + p + p' + q} + \frac{q}{1 + p + p' + q} \tag{50}$$

This equation suggests the definition of new parameters, indicating the contribution of both inhibitors to the total inhibition efficiency. Particularly, we shall define

the contribution of each inhibitor, I and I', as if they were acting alone, η and η', respectively, as:

$$\eta = \frac{p}{1 + p + p' + q} \tag{51}$$

$$\eta' = \frac{p'}{1 + p + p' + q} \tag{52}$$

Analogously, we shall define the contribution of the presence of both inhibitors to the total inhibition efficiency, as:

$$\eta_s = \frac{q}{1 + p + p' + q} \tag{53}$$

The parameter η_s represents the contribution (in per unit) of the presence of both inhibitors to the total inhibition efficiency, i.e. the synergistic effect of the presence of both inhibitors.

Obviously, from Eqs. 51–53:

$$\eta_T = \eta + \eta' + \eta_s \tag{54}$$

We shall define now the relative contributions δ, δ' and δ_s to the total inhibition as:

$$\delta = \frac{\eta}{\eta_T} \tag{55}$$

$$\delta' = \frac{\eta'}{\eta_T} \tag{56}$$

$$\delta_s = \frac{\eta_s}{\eta_T} \tag{57}$$

Taking into account Eqs. 51–53:

$$\delta = \frac{p}{p + p' + q} \tag{58}$$

$$\delta' = \frac{p'}{p + p' + q} \tag{59}$$

$$\delta_s = \frac{q}{p + p' + q} \tag{60}$$

Clearly:

$$\delta + \delta' + \delta_s = 1 \tag{61}$$

The physical meaning of the sum $p + p' + q$ is the contribution of both inhibitors to the total inhibition, in agreement with Eqs. 58–60. Thus, p is the contribution of I in the absence of I', whereas p' is the contribution of I' in the absence of I. However,

the inhibitory action in the presence of both inhibitors, $p + p' + q$, is greater than the sum of the individual action of each inhibitor, i.e., there is a synergistic effect which is measured by the term q.

Lastly, we shall define the relative weight of the inhibitory action of I with respect to that of I' as:

$$g = \frac{p + q}{p' + q} \tag{62}$$

This parameter can have any non-negative value. A value of $g = 0$ means that I does not exist or I' is irreversible at any step (so p' would be infinite). If, on the contrary, $g \to \infty$, then I' does not exist or I is irreversible at any step (so p would be infinite). A value of $g = 1$ means that $p = p'$, so either both inhibitors have quantitatively the same effect on the total inhibition, or neither of the inhibitors exists ($p = p' = 0$). A value of g less or greater than unity means that the inhibitory action of I is lower or higher than that of I', respectively. Table 2 shows the values of η, η', η_s, δ, δ', δ_s and g for the same conditions as indicated in Table 1.

4.3.1 Absolute and relative contributions of both inhibition routes to the total inhibition in some particular cases

4.3.1.1. Particular case: Scheme 2 In this case, the contribution of each inhibitor to the total inhibition, as if they were acting alone, given by Eqs. 51 and 52 for the general model, is reduced to the following expressions:

$$\eta = \frac{p}{1 + p + p' + pp'} \tag{63}$$

$$\eta' = \frac{p'}{1 + p + p' + pp'} \tag{64}$$

Analogously, the contribution of the presence of both inhibitors to the total inhibition, according to Eq. 53, will be:

$$\eta_s = \frac{pp'}{1 + p + p' + pp'} \tag{65}$$

where p and p' are given by Eqs. 25 and 26.

In turn, Eqs. 58–60, which correspond to the relative contributions δ, δ' and δ_s in the general model, are reduced to the following expressions:

$$\delta = \frac{p}{p + p' + pp'} \tag{66}$$

$$\delta' = \frac{p'}{p + p' + pp'} \tag{67}$$

$$\delta_s = \frac{pp'}{p + p' + pp'} \tag{68}$$

Table 2 Values of η, η', η_s, δ, δ', δ_s and g for the conditions indicated in Table 1

$[I]_0$ (mM)	$[I']_0$ (mM)	$\eta \times 10^2$	$\eta' \times 10^2$	$\eta_s \times 10^2$	$\delta \times 10^2$	$\delta' \times 10^2$	$\delta_s \times 10^2$	$g \times 10^2$
1	1	0.4178	90.45	3.601	0.4422	95.75	3.812	4.273
0.1	1	0.04275	93.85	0.3737	0.04535	99.56	0.3964	0.4420
1	0.1	3.347	49.46	2.885	6.010	88.81	5.180	11.91
0.1	0.1	0.3497	52.40	0.3057	0.6592	98.76	0.5761	1.243
0	1	0	94.24	0	0	100	0	0
1	0	7.023	0	0	100	0	0	∞
0	0	0	0	0	–	–	–	–

Finally, Eq. 62 which gives the relative weight of the inhibitory action of I with respect to that of I' in the general model is now reduced to:

$$g = \frac{p(1+p')}{p'(1+p)} \tag{69}$$

Particular cases from Scheme 2 in which $I \equiv I'$ (particular cases AC, BC and ABC)

In those cases in which $I \equiv I'$ (particular cases AC, BC and ABC), the concept of the inhibitory action of I or I' should be replaced by the inhibitory action of the inhibitor on E and E_a. If we denote by I the only inhibitor, p is given by Eq. 25 and p' by Eq. 26 and so the kinetic parameters η, η', η_s, δ, δ', δ_s and g are given by:

$$\eta = \frac{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)[I]_0}{1+\left[\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)\right][I]_0} \tag{70}$$

$$\eta' = \frac{\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0}{1+\left[\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)\right][I]_0} \tag{71}$$

$$\eta_s = \frac{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3K'_3}\left(1+\frac{1}{K_4}\right)\left(1+\frac{1}{K'_4}\right)[I]_0^2}{1+\left[\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)\right][I]_0} \tag{72}$$

$$\delta = \frac{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)}{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)} \tag{73}$$

$$\delta' = \frac{\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)}{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)} \tag{74}$$

$$\delta_s = \frac{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3K'_3}\left(1+\frac{1}{K_4}\right)\left(1+\frac{1}{K'_4}\right)[I]_0}{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)} \tag{75}$$

$$g = \frac{\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)\left\{1+\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)[I]_0\right\}}{\frac{1}{K'_3}\left(1+\frac{1}{K'_4}\right)\left\{1+\frac{K_m}{K_m+[Z]_0}\frac{1}{K_3}\left(1+\frac{1}{K_4}\right)[I]_0\right\}} \tag{76}$$

4.3.1.2. Particular case: Scheme 5

Taking into account that in this case, Eqs. 42–44 are fulfilled, the following expressions are obtained for η, η', η_s, δ, δ', δ_s and g:

$$\eta = 0 \tag{77}$$

$$\eta' = \frac{1}{1+p} \tag{78}$$

$$\eta_s = \frac{p}{1+p} \tag{79}$$

$$\delta = 0 \tag{80}$$

$$\delta' = \frac{1}{1+p} \tag{81}$$

$$\delta_s = \frac{p}{1+p} \tag{82}$$

$$g = \frac{p}{1+p} \tag{83}$$

Acknowledgments 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 and Ciencia, both of them from Junta de Comunidades de Castilla-La Mancha (JCCM, Spain).

References

1. E.R. Stadtman, P.B. Chock, Interconvertible enzyme cascades in metabolic regulation. Curr. Top. Cell. Regul. **13**, 53–95 (1978)
2. R. Varon, B.H. Havsteen, E. Valero, M. Molina, F. Garcia-Canovas, M. Garcia-Moreno, Kinetic analysis of the transient phase and steady-state of open multicyclic enzyme cascades. Acta Biochim. Pol. **52**, 765–780 (2005)
3. E.A. Newsholme, R.A.J. Challiss, B. Crabtree, Substrate cycles: their role in improving sensitivity in metabolic control. TIBS **9**, 277–280 (1984)
4. E. Valero, R. Varon, F. Garcia-Carmona, Kinetic study of an enzymic cycling system coupled to an enzymic step: determination of alkaline phosphatase activity. Biochem. J. **309**, 181–185 (1995)
5. H. Holzer, P.C. Heinrich, Control of proteolysis. Annu. Rev. Biochem. **49**, 63–91 (1980)
6. M.E. Fuentes, R. Varon, M. Garcia-Moreno, E. Valero, Kinetics of intra- and intermolecular zymogen activation with formation of an enzyme-zymogen complex. FEBS J. **272**, 85–96 (2005)
7. M.E. Fuentes, R. Varon, M. Garcia-Moreno, E. Valero, Kinetics of autocatalytic zymogen activation measured by a coupled reaction: pepsinogen autoactivation. Biol. Chem. **386**, 689–698 (2005)
8. A.J. Barrett, N.D. Rawlings, J.F. Woessner, Handbook of Proteolytic Enzymes, 2nd edn. (Elsevier Academic Press, London, 2004)
9. C. Dash, A. Kulkarni, B. Dunn, M. Rao, Aspartic peptidase inhibitors: implications in drug development. Crit. Rev. Biochem. Mol. Biol. **38**, 89–119 (2003)
10. G. Salvesen, H. Nagase, in Proteolytic Enzymes: A Practical Approach, ed. by R.J. Beynond, J.S. Bond (Oxford University Press, Oxford, 1989)
11. J. Masia-Perez, J. Escribano, E. Valero, E. Arribas, M. Garcia-Moreno, J.L. Muñoz-Muñoz, R. Gomez-Ladron de Guevara, R. Varon, A general model for non-autocatalytic zymogen activation in the presence of two different and mutually exclusive inhibitors. I. Kinetic analysis. J. Math. Chem. (2010). doi:10.1007/s10910-010-9696-0
12. E. Valero, M. Garcia-Moreno, J. Masia, M.J. Garcia-Meseguer, R. Varon, Kinetic behaviour of proenzymes activation in the presence of different inhibitors for both the activating and the activated enzyme. J. Theor. Biol. **245**, 175–192 (2007)

13. M.C. Manjabacas, E. Valero, M. Garcia-Moreno, F. Garcia-Canovas, J.N. Rodriguez, R. Varon, Kinetic analysis of the control through inhibition of autocatalytic zymogen activation. Biochem. J. **282**, 583–587 (1992)
14. M.C. Manjabacas, E. Valero, M. Garcia-Moreno, R. Varon, Kinetic analysis of an autocatalytic process coupled to a reversible inhibition. The inhibition of the system trypsinogen-trypsin by *p*-aminobenzamidine. Biol. Chem. Hoppe-Seyler **376**, 577–580 (1995)
15. M.C. Manjabacas, E. Valero, M. Garcia-Moreno, C. Garrido, R. Varon, Kinetics of an autocatalytic zymogen reaction in the presence of an inhibitor coupled to a monitoring reaction. Bull. Math. Biol. **58**, 19–41 (1996)
16. M.C. Manjabacas, E. Valero, M. Moreno-Conesa, M. Garcia-Moreno, M. Molina, R. Varon, Linear mixed irreversible inhibition of the autocatalytic activation of zymogens. Kinetic analysis checked by simulated progress curves. Int. J. Biochem. Cell Biol. **34**, 358–369 (2002)
17. A. Muñoz-Lopez, A. Sotos-Lomas, E. Arribas, J. Masia-Perez, F. Garcia-Molina, M. Garcia-Moreno, R. Varon, Kinetic analysis of a general model of activation of aspartic zymogens involving a reversible inhibitor. I. Kinetic analysis. J. Enzyme Inhib. Med. Chem. **22**, 157–163 (2007)
18. R. Varon, M.A. Minaya-Pacheco, F. Garcia-Molina, E. Arribas, E. Arias, J. Masia, F. Garcia-Sevilla, Competitive and uncompetitive inhibitors simultaneously acting on an autocatalytic zymogen activation reaction. J. Enzyme Inh. Med. Chem. **21**, 635–645 (2006)