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Kinetic analysis of a general model of activation of aspartic proteinase zymogens involving a reversible inhibitor. I. Kinetic analysis

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Abstract

Starting from a simple general reaction mechanism of activation of aspartic proteinases zymogens involving a uni- and a bimolecular simultaneous activation route and a reversible inhibition step, the time course equation of the zymogen, inhibitor and activated enzyme concentrations have been derived. Likewise, expressions for the time required for any reaction progress and the corresponding mean activation rates as well as the half-life of the global zymogen activation have been derived. An experimental design and kinetic data analysis is suggested to estimate the kinetic parameters involved in the reaction mechanism proposed.

Keywords: *Enzyme kinetics, aspartic proteinases, autocatalysis, zymogen, activation, inhibition, unimolecular, bimolecular*

Introduction

Proenzyme activation by proteolytic cleavage of one or more peptide bonds requires the presence of an activating enzyme. In the cases in which the activating enzyme is the same as the activated one, the proenzyme activation process is called autocatalytic. Physiological examples are the activation of trypsinogen into trypsin [1,2], the conversion of pepsinogen into pepsin [3–6], prekallikrein into kallikrein [7,8] and proproteinase A into proteinase A [9–11].

Proteolytic enzymes with active site aspartate residues required for catalysis are called aspartic proteinases and they exhibit optimal activity at low pH and are inhibited by pepstatin. Aspartic proteinases have a great versatility and physiological importance [12–14]. They are crucial for survival functions in the human body and, also, they are virulence factors in many diseases. Moreover, they are involved in the replication and survival of many pathways of microorganisms [15–17]. Aspartic proteinases are found in vertebrates, fungi, plants and retroviruses [15,16,18,19]. Some important examples of aspartic proteinases are pepsin [3–6], the secreted aspartic proteinases (SAPs) from *Candida albicans* [20], the HIV proteinase [27] and the yeast proteinase A [10]. All known aspartic proteinases are initially synthesized as zymogens, which must undergo an activation process to produce the active enzyme. The aspartic proteinases from different sources are very similar with respect to their catalytic mechanism, specificity and structure [10].

Many schemes of the mechanism reaction corresponding to the proteinase zymogen activation processes involving both the uni- and bimolecular routes have been suggested and kinetically analysed [3,5,6,21–24]. These schemes differ either in their complexity or in the presence or not of the aspartic

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proteinase at the onset of the reaction, but none of them include the presence of an inhibitor of the enzyme, in spite of the fact that the activation of aspartic proteinase zymogens is controlled by proteinase inhibitors present in cells and body fluids, e.g. pepstatine (isovaleryl-Val-Val-statyl-Ala-statine), a reversible inhibitor of all aspartic proteinases that inhibits cathepsin D, cathepsin G, pepsin, chymosin and renin involved in different physiological processes.

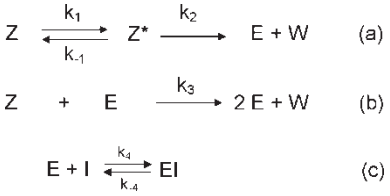
Because aspartic proteinases are involved in different diseases, including acquired immunodeficiency syndrome, malaria, leukemia, and other infectious diseases, their control by different inhibitors as well as the design of new aspartic proteinase inhibitors is a field of increasing interest in recent years [25–31]). Moreover, specific aspartic proteinase inhibitors can be used to test the biological function of certain proteinases, e.g. pepstatine, to show that erythrocyte cathepsin E, as the only aspartic proteinase in erythrocytes, is responsible for the proteolysis and degradation of the membrane proteins [32]. Some additional examples of aspartic proteinase inhibitors are acetyl-pepstatine, an inhibitor of HIV proteinase [33], recombinant squash aspartic peptidase inhibitor (rSQAPI), a reversible inhibitor of aspartic proteinases [34], HLTV-1 retroviral protease inhibitors [35], human immunodeficiency virus (HIV)-1 diaminodiol reversible inhibitors [26], α_2-macroglobulin [36,37].

In this contribution we carry out, for the first time, a complete kinetic analysis of the following reaction scheme of aspartic proteinase zymogen activation including a step of reversible inhibition of the aspartic proteinase: where Z is the zymogen, Z⋆ is the zymogen with all conformation changes in which the active site is unmasked, E is the enzyme, I is the inhibitor and W is the peptide.

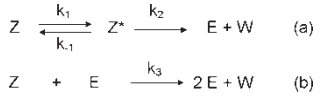
Routes (a) and (b) are the uni- and bi-molecular activation routes, and route (c) is the reversible inhibition step.

Scheme 1 is consistent with the next Scheme 2 recently studied by Varon et al. [24] to which a reversible inhibition step has been added.

The aims of the present paper are: 1) To perform a complete, kinetic analysis of Scheme 1 deriving the time course equations; (2) To acquire the kinetic equations corresponding to Scheme 1 in the absence of the enzyme at the onset of the reaction and/or in absence of the inhibitor from those of Scheme 1; (3) To suggest an experimental design and kinetic data



Scheme 1.



Scheme 2.

analysis to evaluate the kinetic parameters in Scheme 1; (4) To apply our results to acquire the kinetic equations of other reaction schemes of aspartic proteinase zymogen activation that can be formally considered as particular cases of Scheme 1.

Notation and definitions

In the following, some notation and definitions are summarized, some of which are introduced for the first time, and all of them are necessary to facilitate the analysis below.

$[E]$, $[EI]$, $[Z]$, $[Z^\star]$ and $[I]$: Instantaneous concentrations of the species E, EI, Z and Z⋆, respectively, involved in Scheme 1.

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

K_1: The equilibrium constant of the conformational change step, i.e.:

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

K_4: The dissociation equilibrium constant of the complex EI, i.e.:

$$K_4 = \frac{k_{-4}}{k_4} \tag{2}$$

$[E]_a$: Total active enzyme concentration at the time t emanating from the zymogen activation, i.e.:

$$[E]_a = [E] + [EI] - [E]_0 \tag{3}$$

Time course equations

Time course equations of [Z], [Z⋆], [E], [EI] and [I]

The kinetics of E, Z, $Z^\star$ and EI in the aspartic proteinase zymogen activation evolving according to the reaction mechanism in Scheme 1 is described by the following set of differential Equations (6)–(10):

$$\frac{d[Z]}{dt} = -k_1[Z] + k_{-1}[Z^\star] - k_3[Z][E] \tag{4}$$

$$\frac{d[Z^\star]}{dt} = k_1[Z] - k_2[Z^\star] \tag{5}$$

$$\frac{d[E]}{dt} = k_2[Z^\star] + k_3[Z][E] - k_4[E][I] + k_{-4}[EI] \tag{6}$$

$$\frac{d[EI]}{dt} = k_4[E][I] - k_{-4}[EI] \tag{7}$$

$$\frac{d[I]}{dt} = -k_4[E][I] + k_{-4}[EI] \tag{8}$$

This set of differential Equations (4)–(8) is non-linear and, therefore, it has no analytical solution. Nevertheless, if we made the usual and reasonable assumption that the reversible step of the conformational change $Z \rightleftarrows Z^{\star}$ and the binding of the inhibitor to EI are in rapid equilibrium, i.e. that they reach the equilibrium practically from the onset of the reaction and maintain these equilibria during the whole course of the reaction, it is noted at any reaction time, t, that:

$$\frac{[Z]}{[Z^{\star}]} = K_1 \tag{9}$$

$$\frac{[E][I]}{[EI]} = K_4 \tag{10}$$

Moreover, from the mass balance, at any reaction time, t:

$$[Z]_0 + [E]_0 = [Z] + [Z^{\star}] + [E] + [EI] \tag{11}$$

Hence, if in Equation (6), Equations (9), (10) and (11) are inserted, and we use an initial inhibitor concentration, $[I]_0$, much higher than $[E]_0$ and $[Z]_0$, i.e. $[I]_0 \gg [Z]_0, [E]_0$, so that during the whole course of the reaction it is observed that $[I] \simeq [I]_0$, then Equation (6) can be rewritten as:

$$\frac{d[E]}{dt} = \frac{(k_3 K_1 [E] + k_2)\left[[Z]_0 + [E]_0 - [E]\left(1 + \frac{[I]_0}{K_4}\right)\right]}{1 + K_1} \tag{12}$$

Integration of Equation (12) taking into account that (due to fact that the reversible step is in rapid equilibrium) at $t = 0$ is $[E] = [E]_0 K_4/([I]_0 + K_4)$ yields:

$$[E] = \frac{\{([Z]_0 + [E]_0)(K_1 K_4 k_3 [E]_0 + k_2(K_4 + [I]_0))e^{\lambda t} - k_2[Z]_0(K_4 + [I]_0)\}K_4}{\{K_1 K_4 k_3 [Z]_0 + (K_1 K_4 k_3 [E]_0 + k_2(K_4 + [I]_0))e^{\lambda t}\}(K_4 + [I]_0)} \tag{13}$$

where:

$$\lambda = \frac{k_3 K_1([Z]_0 + [E]_0) + k_2\left(1 + \frac{[I]_0}{K_4}\right)}{1 + K_1} \tag{14}$$

From Equations (9)–(11) and (13) we have for the time course of [Z] and [Z⋆] the following expressions:

$$[Z] = \frac{([Z]_0 + [E]_0)K_1}{1 + K_1} - \frac{(([Z]_0 + [E]_0)(K_1 K_4 k_3 [E]_0 + k_2(K_4 + [I]_0))e^{\lambda t} - k_2[Z]_0(K_4 + [I]_0))K_1}{(K_1 + 1)(K_1 K_4 k_3 [Z]_0 + (K_1 K_4 k_3 [E]_0 + k_2(K_4 + [I]))e^{\lambda t})} \tag{15}$$

and

$$[Z^{\star}] = \frac{([Z]_0 + [E]_0)}{1 + K_1} - \frac{(([Z]_0 + [E]_0)(K_1 K_4 k_3 [E]_0 + k_2(K_4 + [I]_0))e^{\lambda t} - k_2[Z]_0(K_4 + [I]_0))}{(K_1 + 1)(K_1 K_4 k_3 [Z]_0 + (K_1 K_4 k_3 [E]_0 + k_2(K_4 + [I]_0))e^{\lambda t})} \tag{16}$$

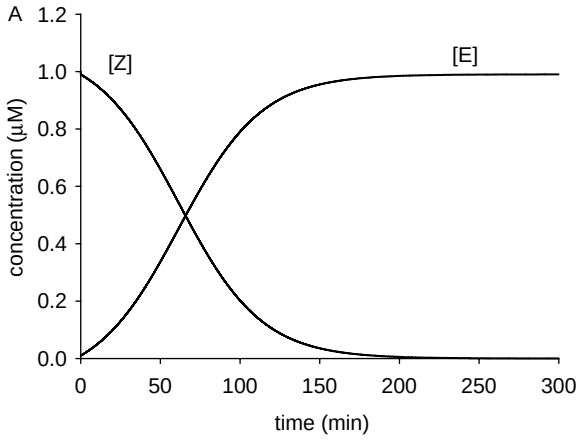


Figure 1. (A) Time course of [E] and [Z] according to Equations (13) and (15) and from the numerical integration of the set of differential Equations (4)–(8). (B) Time course of [Z⋆] and [EI] [EI] according to Equations (16) and (18) and from the numerical integration of the set of differential Equations (4)–(8). The values of the equilibrium constants, rate constants and initial concentrations in (A) and (B) were: $K_1 = 100, K_4 = 0.005\text{M}, k_2 = 0.005\text{s}^{-1}, k_3 = 600\text{M}^{-1}\text{s}^{-1}, [E]_0 = 10\text{nM}, [Z]_0 = 1\mu\text{M}$ and $[I]_0 = 0.1\text{mM}$. In each case the progress curves from the equation and from the simulation overlap in the scale used.

Moreover, from Equation (11) we have, after some algebraic rearrangement:

$$[EI] = ([Z]_0 + [E]_0) - [Z]\left(1 + \frac{1}{K_1}\right) - [E] \qquad (17)$$

If now Equations (13) and (15) are inserted into Equation (17) it becomes:

$$[EI] = \left(1 - \frac{K_4}{[I]_0 + K_4}\right)\left[\frac{([Z]_0 + [E]_0)(k_3K_1K_4[E]_0 + k_2([I]_0 + K_4))e^{\lambda t} - k_2[Z]_0([I]_0 + K_4)}{k_3K_1K_4[Z]_0 + (k_3K_1K_4[E]_0 + k_2([I]_0 + K_4))e^{\lambda t}}\right] \qquad (18)$$

In Figure 1, the time course of the variations of $[E]$, $[Z]$, [EI] and [Z*] are plotted for an arbitrary set of values of the rate constants and the initial concentrations. In each case, they are compared with the simulated progress curve obtained by numerical integration of the set of differential Equations (4)–(8). Note in each case the good agreement.

Time course equations of $[E]_a$

Once the time courses of $[E]$, $[Z]$, $[Z^\star]$ and $[EI]$ have been obtained we can from them derive the time course of the magnitudes of $[E]_a$, $[E]_u$ and $[E]_b$.

$[E]_a$: If Equations (13) and (8) are inserted into Equation (3), we obtain the time course equation of $[E]_a$ (Figure 2). The result is:

$$[E]_a = \frac{(k_3K_1K_4[E]_0 + k_2([I]_0 + K_4))(e^{\lambda t} - 1)}{k_3K_1K_4[Z]_0 + (k_3K_1K_4[E]_0 + k_2([I]_0 + K_4))e^{\lambda t}}[Z]_0 \qquad (19)$$

where λ is given by Equation (14).

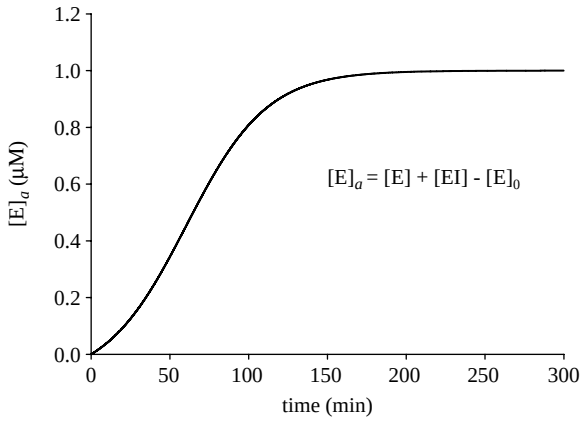


Figure 2. Time course of $[E]_a$, according to Equation (19), for the same set of values of the equilibrium constants, rate constants and initial concentrations were the same as in Fig. 1.

Materials and methods

Simulated progress curves were obtained by numerical integration of the set of differential equations describing the kinetics of the reaction evolving according to the corresponding mechanism under study, using values of the rate constants and initial concentrations, which either have been published in the literature or have been chosen arbitrarily, but realistically. This numerical solution was found by the use of the classical fourth-order Runge-Kutta formula, but applying an adaptative stepsize control that was originally invented by Fehlberg [38,39] using the software WES implemented in Visual C++6.0 [40]. The above program was run on a PC compatible computer based on a Pentium IV/2 GHz processor with 512 Mbytes of RAM.

The plots of the data obtained from the numerical integration, as well as the plots of the Equations (13), (15), (16), (18) and (19) made in Figures 1–5, were carried out using the SigmaPlot Scientific Graphing System, version 8.02 (2002, SPSS Inc).

Results and discussion

We have derived the time course equations for [E], [EI], $[E]_a$, [Z] and [Z*] in the reaction mechanism shown in Scheme 1 [Equations (13)–(18)] suggested by us. This differs from Scheme 2, which has been analysed kinetically in previous contributions, since a reversible inhibitor, I, binds to the aspartic proteinase E [24]. The only assumptions used in the derivation of these equations is that the steps $Z \rightleftarrows Z^*$ and $E + I \rightleftarrows$

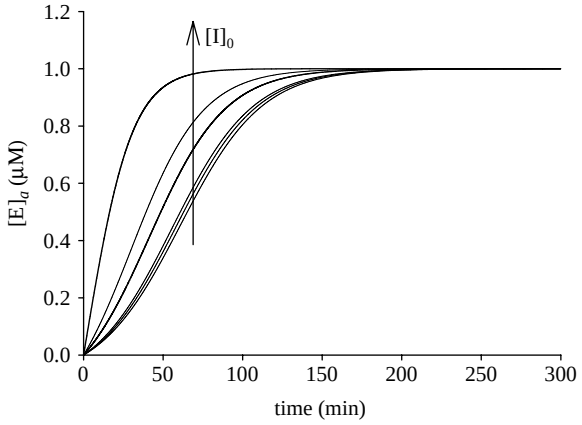


Figure 3. Effect of $[I]_0$ on the time course of $[E]_a$ at fixed $[Z]_0$- and $[E]_0$-values according to Equation (19). For each progress curve the set of values of the equilibrium constants, rate constants, $[Z]_0$ and $[E]_0$ are the same as in Fig. 1. The $[I]_0$-values used in the different progress curves were (mM): 0, 0.05, 1, 5, 10 and 50.

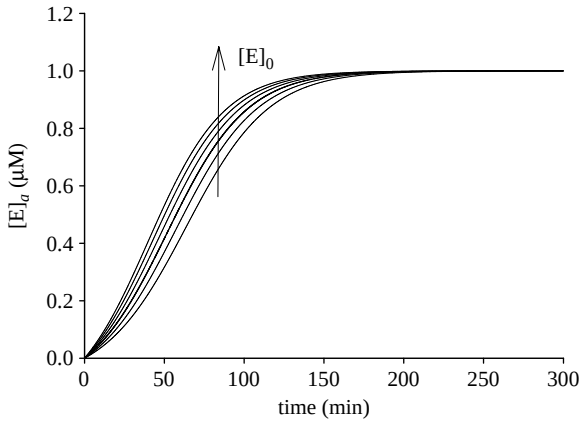


Figure 4. Effect of $[E]_0$ on the time course of $[E]_a$ at fixed $[Z]_0$- and $[I]_0$-values according to Equation (19). For each progress curve the set of values of the equilibrium constants, rate constants, $[Z]_0$ and $[I]_0$ are the same as in Fig. 1. The $[E]_0$-values used in the different progress curves were (nM): 0, 20, 40, 60, 80 and 100.

EI are in rapid equilibrium and that [I] remains approximately constant and approximately equal to $[I]_0$ during the whole course of the reaction. The assumption that step $Z \rightleftarrows Z^\star$ is in rapid equilibrium is reasonable, because this conformational change is very rapid [21,22] and moreover, the cleavage of the propeptide, W, from $Z^\star$ is slow, since it requires the cleavage of a peptide bond [41,42]. Likewise, the assumption of a rapid equilibrium in the binding of a reversible inhibitor to the enzyme is frequently accepted in enzyme kinetics. The values of the initial concentrations of the zymogen, $[Z]_0$, and of the enzyme, $[E]_0$ can take any values except $[Z]_0 = 0$. As commented above, the initial concentration of I must be that $[I]_0 \gg [Z]_0$, $[E]_0$ is observed. The time course equation for $[E]_a$ will allow derivation of the expressions for the instantaneous activation rate and for the period of time that the reaction needs for a given progress.

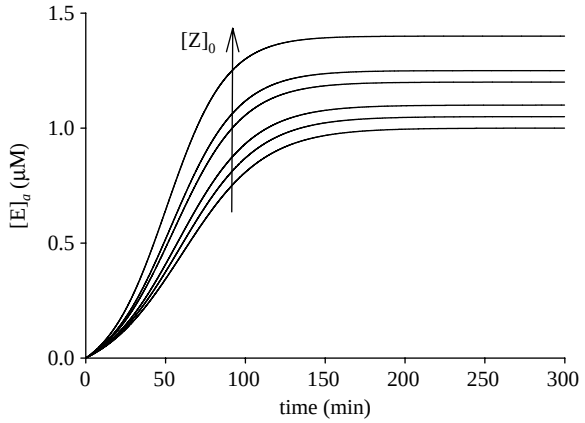


Figure 5. Effect of $[Z]_0$ on the time course of $[E]_a$ at fixed $[I]_0$- and $[E]_0$-values according to Equation (19). For each progress curve the set of values of the equilibrium constants, rate constants, $[I]_0$ and $[E]_0$ are the same as in Fig. 1. The $[Z]_0$-values used in the different progress curves were (μM): 1, 1.05, 1.10, 1.20, 1.25 and 1.40.

Time course of the activation rate

If we denote V as $d[E]_a/dt$ and take into account Equations (3) and (10) we obtain:

$$V = \left(1 + \frac{[I]_0}{K_4}\right)\frac{d[E]}{dt} \tag{20}$$

If now Equation (12) is inserted into Equation (20) this results in:

$$V = \left(1 + \frac{[I]_0}{K_4}\right) \times \frac{(k_3K_1[E] + k_2)\left\{[Z]_0 + [E]_0 - [E]\left(1 + \frac{[I]_0}{K_4}\right)\right\}}{(1 + K_1)} \tag{21}$$

where [E] is given by Equation (13).

The time elapsed for different reaction progresses of the activation

From Equation (19), we see that a total transformation of $[Z]_0$ into $[E]_a$ requires that the theoretical time which must elapse is $t \rightarrow \infty$. The reason is that $[E]_a$ approaches $[Z]_0$ asymptotically. However, in practice, it can be interesting to know the time, t_ξ, the process has proceeded to reach a certain transformation, ξ (expressed as a fraction of $[Z]_0$), of the zymogen into the active enzyme. If we set $[E]_a = \xi[Z]_0$ in Equation (19), then we obtain, after some algebraic rearrangement:

$$t_\xi = \frac{1}{\lambda} \cdot \ln \frac{k_3K_1K_4(\xi[Z]_0 + [E]_0) + k_2([I]_0 + K_4)}{(k_3K_1K_4[E]_0 + k_2([I]_0 + K_4))(1 - \xi)} \tag{22}$$

where λ is given by Equation (14).

Half-life reaction time. An easily obtained kinetic parameter related to the global activation rate is the half-life, $t_{1/2}$ i.e. the reaction time required to reach $[E]_a = 0.5[Z]_0$. This time is obtained by setting $\xi = 0.5$ in Equation (22).

$$t_{0.5} = \frac{1}{\lambda} \cdot \ln \frac{k_3K_1K_4([Z]_0 + 2[E]_0) + 2k_2([I]_0 + K_4)}{(k_3K_1K_4[E]_0 + k_2([I]_0 + K_4))} \tag{23}$$

$t_{0.99}$ reaction time. $t_{0.99}$ time means the time that a reaction needs to reach 99% of its progress. This parameter is frequently used in enzyme kinetics and can be considered as the period of the duration of the reaction. Rigorously, this time should correspond to $\xi = 1$, but this ξ-value yields, according to Equation (22), $t_1 \rightarrow \infty$. Thus, $t_{0.99}$ is the time normally taken as the time which an enzyme

reaction needs to reach completion. This time is obtained by setting $\xi = 0.99$ in Equation (22).

Mean activation rate at different reaction progresses of the activation

If $\xi[Z]_0$ is divided by the time elapsed in the reaction in order for $[E]_a$ to reach this value, given by Equation (22), the mean rate, V_ξ, is obtained with which the reaction reaches the progress ξ.

$$V_\xi = \frac{\lambda \xi [Z]_0}{\ln \frac{k_2([I]_0+K_4)+k_3K_1K_4(\xi[Z]_0+[E]_0)}{(k_3K_1K_4[E]_0+k_2([I]_0+K_4))(1-\xi)}} \tag{24}$$

For example, if in Equation (22) we set $\xi = 0.99$ the mean rate is obtained of the activation of *Z* into *E and EI* to the extent of 99%.

Effect of $[E]_0$, $[Z]_0$ and $[I]_0$ on $[E]_a$

The mechanism shown in Scheme 1 differs from that in Scheme 2 in the presence of a reversible inhibitor. The effect of $[E]_0$, $[Z]_0$ and $[I]_0$ on $[E]_a$ is explicit in Equation (19). In Figures 3–5 these effects are shown graphically for an arbitrary set of values of the equilibrium constants, rate constants.

The case of the absence of the activating enzyme, E, at the beginning of the reaction

The above results concern Scheme 1, in which it has been assumed that the activating enzyme is present at the onset of the reaction. Nevertheless, the analysis carried out here is also applicable to the case, in which $[E]_0 = 0$. It is only necessary to set $[E]_0 = 0$ in the above results to obtain the corresponding ones in the absence of enzyme at the onset of the reaction.

If we set $[E]_0 = 0$ in Equations (13)–(19), and (20)–(24) obtained for the Scheme 1, then:

$$[E] = \frac{k_2([I]_0 + K_4)(e^{\lambda t} - 1)[Z]_0}{k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)e^{\lambda t}} \cdot \frac{K_4}{[I]_0 + K_4} \tag{25}$$

$$\lambda = \frac{k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)}{(1 + K_1)K_4} \tag{26}$$

$$[Z] = \frac{[Z]_0K_1}{1 + K_1} - \frac{K_1(e^{\lambda t} - 1)([I]_0 + K_4)[Z]_0}{(1 + K_1)(k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)e^{\lambda t})} \tag{27}$$

$$[Z^*] = \frac{[Z]_0}{1 + K_1} - \frac{(e^{\lambda t} - 1)([I]_0 + K_4)[Z]_0}{(1 + K_1)(k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)e^{\lambda t})} \tag{28}$$

$$[EI] = \frac{[I]_0}{[I]_0 + K_4} \cdot \frac{k_2([I]_0 + K_4)(e^{\lambda t} - 1)[Z]_0}{k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)e^{\lambda t}} \tag{29}$$

$$[E]_a = \frac{k_2([I]_0 + K_4)(e^{\lambda t} - 1)[Z]_0}{k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)e^{\lambda t}} \tag{30}$$

$$V = \left(1 + \frac{[I]_0}{K_4}\right) \times \frac{(k_3K_1[E] + k_2)\left[[Z]_0 - [E]\left(1 + \frac{[I]_0}{K_4}\right)\right]}{(1 + K_1)} \tag{31}$$

$$t_\xi = \frac{1}{\lambda} \ln \frac{k_3K_1K_4[Z]_0\xi + k_2([I]_0 + K_4)}{k_2([I]_0 + K_4)(1 - \xi)} \tag{32}$$

$$V_\xi = \frac{\lambda \xi [Z]_0}{\ln \frac{k_3K_1K_4[Z]_0\xi + k_2([I]_0+K_4)}{k_2([I]_0+K_4)(1-\xi)}} \tag{33}$$

Under fixed experimental conditions, the kinetic parameters are independent of the presence of the reaction of the activating enzyme at the onset. Nevertheless, in the absence of activating enzyme at the onset of the reaction, all of the results become simplified considerably. Thus, we could work under conditions, in which $[E]_0 = 0$ and then evaluate the equilibrium and rate constants involved in Scheme 1. Once these parameters are known, the kinetics of the activation process in the presence of any initial activating enzyme can be determined readily using the general. equations, if K_1, k_2, k_3 and K_4 are assumed to be $[E]_0$-independent. In the next section, a kinetic data analysis and an experimental design is suggested to evaluate the kinetic parameters involved in Scheme 1.

The case of the absence of inhibitor, I, at the onset of the reaction

In this case, Scheme 1 reduces to Scheme 2 which has been recently analysed by Varon et al. [24] and is a particular case of Scheme 1. It is obvious that the kinetic equations for Scheme 2 can be easily obtained from those corresponding to Scheme 1 by merely setting in them $[I]_0 = 0$. For example, for [Z] it is

obtained that:

$$[Z] = \frac{k_3K_1([Z]_0 + [E]_0) + k_2}{k_3K_1[Z]_0 + (k_2 + k_3K_1[E]_0)e^{\lambda t}} \frac{K_1[Z]_0}{1 + K_1} \quad (34)$$

The case of $[E]_0 = [I]_0 = 0$

Likewise, the kinetic equations for this case are obtained merely by setting $[I]_0 = [E]_0 = 0$ in the above results obtained for Scheme 1. For example, for [E] in this case the following is obtained:

$$[E] = \frac{k_2([I]_0 + K_4)(e^{\lambda t} - 1)[Z]_0}{k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)e^{\lambda t}} \cdot \frac{K_4}{[I]_0 + K_4} \quad (35)$$

where

$$\lambda = \frac{k_3K_1K_4[Z]_0 + k_2([I]_0 + K_4)}{(1 + K_1)K_4} \quad (36)$$

Kinetic data analysis

Recently, Varon et al. [24] starting from Scheme 2 in the absence of enzyme at the onset of the reaction suggested an experimental design and a kinetic data analysis allowing the estimation of K_1, k_2 and k_3. To evaluate the additional kinetic parameters involved in Scheme 1 i.e. K_4 we suggest an experimental design and kinetic data analysis based on the absence of the activating enzyme (but not of the inhibitor) at the onset of the reaction. Thus, the time progress of $[E_a]$ is given by Equation (35). The kinetic data analysis consists of the following three steps;

(1) We obtain, by a discontinuous method, i.e. removing aliquots at time intervals and quantifying the presence of active enzyme, the $[E_a]$-values at different reaction times at a fixed $[Z]_0$-value but different $[I]_0$-values in each of the assays.

(2) We fit the data $[E]_a/[Z]_0$ [see Equation (30)] obtained in each of the assays to all equation type:

$$\frac{[E]_a}{[Z]_0} = \frac{a(e^{\lambda t} - 1)}{b + ae^{\lambda t}} \quad (37)$$

and from these fits obtain the values of the kinetic parameters λ for each of the $[I]_0$-values used in the different assays.

(3) The expression of λ in the absence of the activating enzyme is given by Equation (36). Therefore, because a plot of the λ-values vs $[I]_0$ is a straight line with slope $(k_2/K_4)/(1 + K_1)$ the kinetic parameter can be easily obtained for k_2 and K_1.

Concluding remarks

The two most important characteristics that kinetically distinguish the aspartic proteinases from other groups of proteinases are the following ones:

(1) The activation of their zymogens in the active enzyme follows two simultaneous activation routes: one, an intramolecular (unimolecular) reaction in which the zymogen undergoes autoactivation; and the other, is an intermolecular (bimolecular) reaction in which the zymogen is activated by the active enzyme that it produces. The relative importance of these two routes depends on the experimental conditions.

(2) All aspartic proteases are reversibly inhibited by pepstatin.

Experimental and theoretical kinetic models of zymogen autocatalysis involving reversible inhibition have been reported [1,2,43,44]. However these contributions did not concern themselves with the aspartic proteinase because they ignored the intramolecular activation route. Other contributions deal with the aspartic proteinase because they include the intramolecular activation route [3,5,6,21–24]. Nevertheless, none of the contributions on aspartic proteinase includes an inhibition route overlapping the activation route, for other groups of proteinases [1,2,43,44] which do not exhibit intramolecular activation. Thus, the main aim of this paper was to satisfy this lack of information by analysing the model in Scheme 1, which includes for the first time the two activation pathways of aspartic proteinase zymogens overlapping a reversible inhibition pathway of the enzyme.

The analysis in this work is focused to furnish the kinetic behaviour of the system and its particular cases (those ones in which the activating enzyme, E, and/or the reversible inhibitor, I, are not present at the onset of the reaction) as well as to suggest an experimental design and a kinetic data analysis. Thus, the results obtained here completely characterizes the kinetics of the system.

Nevertheless, there on aspect of the activation of aspartic proteinase zymogens is of great importance: the relative contribution to the activation of the uni- and bimolecular routes. According to van Hazel et al. [10], to maintain strict subcellular compartmentalization of hydrolytic activities, efficient regulation of auto activation is required. Thus there are two counteracting demands: the threshold for intracellular auto activation must be low enough to allow this process to take place, but high enough to avoid premature enzymatic activity. Therefore, knowledge of the predominant activation pathway represents a useful tool in the study of the physiological conditions of the activation process of a zymogen at intra- and extracellular levels, as well as in the study of possible regulation mechanisms of autocatalytic zymogen activation processes.

The study of the relative weight of both activating routes and their dependence on experimental conditions requires the application of the results obtained in the analysis made in part I and to this task we devote part II of this series.

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