Kinetic analysis of the control through inhibition of autocatalytic zymogen activation

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A global kinetic analysis of a model of an autocatalytic zymogen-activation process in which an irreversible inhibitor competes with the zymogen for the active site of the proteinase is presented. Processes like the one here described are of great physiological interest because they are involved in the enzyme regulation of the gastrointestinal-tract enzymes, in blood coagulation, in fibrinolysis and in the complement system. The kinetic equations of both the transient phase and the steady state are derived for this mechanism. In addition, we have introduced a new parameter related to the kinetic behaviour of the system which allows us to predict whether the inhibition route or the activation route prevails in the steady state of the system. Finally, we extend the kinetic equations derived to different particular cases of the system studied.

INTRODUCTION

Proteolytic enzymes are normally synthesized and secreted as inactive precursors, termed proenzymes or zymogens, to protect the cells which produce them. These zymogens must undergo an activation process, usually a limited proteolysis, to attain their catalytic activity.

Zymogen activation is a phenomenon of great importance to our understanding of fundamental biochemical and physiological processes. They are involved in the regulation of enzyme activity, e.g. in digestion [1], metabolism [2], differentiation [3], immunity [4,5], blood coagulation [6], fibrinolysis [7,8] and response to injury [9]. Other examples are the evolution of viruses, oncogenes and metastatic cells [10].

When the activating enzyme and the activated enzyme coincide, the process is an autocatalytic zymogen activation. Physiological examples of these processes are the activation of prekallikrein [11], trypsinogen [1] and pepsinogen [12] by kallikrein, trypsin and pepsin respectively.

The limited proteolysis of zymogens, which frequently occurs in a cascade and is involved in the physiological processes described above, is controlled by natural proteinase inhibitors present in cells and body fluids [10]. Specific inhibitors can be used to test the biological function of certain proteinases and to characterize them [10,13], and such studies permit one to propose inhibitors that might be used as drugs in human therapy [14,15]. Significant reviews on proteinase inhibitors have been recently published [10,16].

Many important proteinase inhibitors interact with the proteinase by competing with a substrate for the active site of the proteinase in an irreversible way under physiological conditions.

Studies upon the simultaneous competition between a trypsin inhibitor, I, and a non-zymogen substrate, S, of the trypsin, according to the following scheme, have been carried out [11,17]:

Nevertheless, a complete kinetic analysis, analogous to the one recently done on the mechanism of an autocatalytic zymogen-activation reaction in which an inhibitor competes in a reversible way with the zymogen for the active site of the proteinase, has yet to be done, in spite of the physiological importance of these processes and the interesting kinetic behaviour of these systems. The mechanism we propose results from the addition to the autocatalytic zymogen activation recently studied [18] of a two-step reaction sequence in which a reversible inhibitor–proteinase complex is initially formed, followed by an irreversible formation of another complex. However, in other cases the inhibition implies a one-step reaction sequence and only a second-order rate constant [17]:

Scheme 1

where E is both the activating proteinase and the activated enzyme, Z is the inactive precursor of E, and W is one or more peptides released from Z during the formation of E.

This mechanism is general enough to include as particular cases most of the inhibition processes of those proteinases acting on their own proenzyme, as we shall see in Results and Discussion section.

KINETIC ANALYSIS

Initial conditions

We assume that the only species present at the onset of the reaction are E, Z and I, and that the concentrations of Z and I remain approximately constant during the course of the reaction. This can be attained if there is a metabolic control of Z and I so

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that they are renewed as they are being consumed. Another way to keep the concentrations of Z and I approximately constant is setting their initial concentrations, $[Z]_0$ and $[I]_0$, much higher than the concentration of E, $[E]$, at any time. A condition for this purpose, easily to be carried out experimentally, is that:

$$[Z]_0, [I]_0 \gg [E]_0 \quad (1)$$

$[E]_0$ being the initial concentration of E. But since in the autocatalytic processes $[E]$ could become higher than $[E]_0$, condition (1) is necessary, but not sufficient, and it must be replaced by the necessary and sufficient condition:

$$[Z]_0, [I]_0 \gg [E] \quad (2)$$

which includes condition (1).

Set of differential equations

Under these assumptions, the evolution of the enzyme species is described by the following linear set of differential equations:

$$\left. \begin{aligned} \frac{d[E]}{dt} &= -(k_{+1}[Z]_0 + k_{+4}[I]_0)[E] + k_{-1}[EZ] \\ &\quad + 2k_{+3}[EE] + k_{-4}[EI] \\ \frac{d[EZ]}{dt} &= k_{+1}[Z]_0[E] - (k_{-1} + k_{+2})[EZ] \\ \frac{d[EE]}{dt} &= k_{+2}[EZ] - k_{+3}[EE] \\ \frac{d[EI]}{dt} &= k_{+4}[I]_0[E] - (k_{-4} + k_1)[EI] \\ \frac{d[EI^*]}{dt} &= k_1[EI] \end{aligned} \right\} \quad (3)$$

We have solved the above set of differential equations (3) by using the Laplace-transformation method. The result for the concentration of the enzyme species E is:

$$[E] = \sum_{h=1}^4 A_h e^{\lambda_h t} \quad (4)$$

where λ_h ($h = 1, 2, 3, 4$) are the roots of the following polynomial $T(\lambda)$:

$$T(\lambda) = \lambda^4 + F_1 \lambda^3 + F_2 \lambda^2 + F_3 \lambda + F_4 \quad (5)$$

the coefficients F_i ($i = 1, 2, 3, 4$) being:

$$F_1 = k_{+1}[Z]_0 + k_{+4}[I]_0 + k_{-1} + k_{+2} + k_{+3} + k_{-4} + k_1 \quad (6)$$

$$F_2 = k_{+1}[Z]_0(k_{+2} + k_{+3} + k_{-4} + k_1) + k_{+4}[I]_0(k_{-1} + k_{+2} + k_{+3} + k_1) + k_{-1}(k_{+3} + k_{-4} + k_1) + k_{+2}(k_{+3} + k_{-4} + k_1) + k_{+3}(k_{-4} + k_1) \quad (7)$$

$$F_3 = k_{+1}[Z]_0(k_{+2} + k_{+3})(k_{-4} + k_1) + k_{+4}[I]_0(k_{-1} + k_{+2}) + k_{+3}k_{+4}[I]_0 + k_{-1}k_{+3}(k_{-4} + k_1) + k_{+2}k_{+3}(k_{-4} + k_1) + \frac{k_{+1}k_{+2}k_{+3}(k_{-4} + k_1)[Z]_0}{r} \left(\frac{1}{k_1} - \frac{1}{k_{-4} + k_1} \right) \quad (8)$$

$$F_4 = k_{+3}k_{+4}k_1(k_{-1} + k_{+2})[I]_0(1-r) \quad (9)$$

$$\text{In eqns. (8) and (9) } r \text{ is the dimensionless quantity given by: } r = \frac{k_{+1}k_{+2}(k_{-4} + k_1)[Z]_0}{k_{+4}k_1(k_{-1} + k_{+2})[I]_0} \quad (10)$$

Hence:

$$A_h = - \frac{\lambda_h^3 + (k_{-1} + k_{+2} + k_{+3} + k_{-4} + k_1)\lambda_h^2 + [k_{+3}(k_{-1} + k_{+2} + k_{-4} + k_1) + (k_{-1} + k_{+2})(k_{-4} + k_1)]\lambda_h + k_{+3}(k_{-1} + k_{+2})(k_{-4} + k_1)}{\prod_{p=1, p \neq h}^4 (\lambda_p - \lambda_h)} [E]_0 \quad (11)$$

Properties of the roots λ_h ($h = 1, 2, 3, 4$)

In this subsection we analyse the properties of the roots λ_h ($h = 1, 2, 3, 4$), considering all the possible different cases that can occur, taking into account the relative values of the rate constants and the zymogen and inhibitor concentrations. We collect all the possibilities through the parameter, r , defined in eqn. (10).

According to eqns. (6) and (7), the coefficients F_1 and F_2 are always positive. According to eqn. (8) the coefficient F_3 is always positive because r is non-negative and $1/k_1$ is higher than $1/(k_{-4} + k_1)$. The coefficient F_4 may be positive, negative or null according to the r value. The following relations among the roots of $T(\lambda)$ and their coefficients, derived from the polynomial theory, yield:

$$\lambda_1 + \lambda_2 + \lambda_3 + \lambda_4 = -F_1 \quad (12)$$

$$\lambda_1 \lambda_2 + \lambda_1 \lambda_3 + \lambda_1 \lambda_4 + \lambda_2 \lambda_3 + \lambda_2 \lambda_4 + \lambda_3 \lambda_4 = F_2 \quad (13)$$

$$\lambda_1 \lambda_2 \lambda_3 + \lambda_1 \lambda_2 \lambda_4 + \lambda_1 \lambda_3 \lambda_4 + \lambda_2 \lambda_3 \lambda_4 = -F_3 \quad (14)$$

$$\lambda_1 \lambda_2 \lambda_3 \lambda_4 = F_4 \quad (15)$$

Since F_1 is always positive, from eqn. (12) it may be shown that at least one of the roots λ_h ($h = 1, 2, 3, 4$) is always negative (or complex with a negative real part, in which case its conjugated complex root also exists).

Case (a): $r = 1$. If this condition is verified, then from eqn. (9) it follows that:

$$F_4 = 0 \quad (16)$$

and therefore, according to eqn. (15), at least one of the roots, let it be λ_4 , is null. Moreover, because of eqn. (16), eqn. (5) becomes:

$$T(\lambda) = \lambda(\lambda^3 + F_1 \lambda^2 + F_2 \lambda + F_3) \quad (17)$$

i.e. the remaining three roots, λ_1 , λ_2 and λ_3 , of $T(\lambda)$ coincide with the roots of the polynomial $\lambda^3 + F_1 \lambda^2 + F_2 \lambda + F_3$. Because F_1 , F_2 and F_3 are positive, none of these roots can be zero, positive or complex with a negative real part, i.e. if $r = 1$, then $T(\lambda)$ has only one null root, the other three roots being either all negative or one negative and the other two conjugated complex with a negative real part.

Case (b): $r < 1$. In this case $F_4 > 0$ and therefore none of the roots of $T(\lambda)$ is null. In its turn, it can be verified that the four coefficients, F_1 , F_2 , F_3 and F_4 , of the polynomial $T(\lambda)$ are positive. Hence, any one of the roots of the polynomial is negative or complex with a negative real part.

Case (c): $r > 1$. In this case, $F_4 < 0$ and therefore, according to eqn. (15), none of the roots is null. Moreover, the same equation admits two possibilities. The first one would be one negative root and three positive roots, and the second one would be three negative roots (or one negative and two conjugated complex roots with a negative real part) and one of them positive.

Regrouping the left side of eqn. (13) and taking into account that F_2 is always positive, it follows that:

$$\lambda_1 \lambda_2 + (\lambda_1 + \lambda_2) \lambda_3 + (\lambda_1 + \lambda_2 + \lambda_3) \lambda_4 > 0 \quad (18)$$

According to eqn. (12) and taking into account that F_1 is

always positive, if we suppose the first possibility and λ_4 is the only negative root, then we could write the following:

$$\lambda_4 < -(\lambda_1 + \lambda_2 + \lambda_3) \quad (19)$$

If we replaced λ_4 by $-(\lambda_1 + \lambda_2 + \lambda_3)$ in eqn. (18), a higher quantity than λ_4 , the left side is now even higher than before and therefore it can be verified that:

$$\lambda_1 \lambda_2 + (\lambda_1 + \lambda_2) \lambda_3 - (\lambda_1 + \lambda_2 + \lambda_3)^2 > 0 \quad (20)$$

However, this result is absurd, because the expansion of the left side gives $-(\lambda_1^2 + \lambda_2^2 + \lambda_3^2 + \lambda_1 \lambda_2 + \lambda_1 \lambda_3 + \lambda_2 \lambda_3)$, which is a negative quantity. Therefore, there is one positive root and the other three are either all negative or one negative and two complex with a negative real part.

RESULTS AND DISCUSSION

The kinetic equation of E has been derived by solving the set of differential equations (3). Analogously the kinetic equations for the other enzyme species can also be obtained from these equations. They are valid for the whole course of the reaction, i.e. for both the transient phase and the steady state. Previously, kinetic analyses of reactions where an inhibitor competes irreversibly with a substrate of a proteinase have been done and have been used for experimental determinations of kinetic parameters [17, 19]. In these cases, the concentration of E always becomes zero for high enough values of t . Nevertheless, any complete kinetic analysis of the interesting case in which the substrate of the proteinase is the zymogen from which it derives has yet to be carried out. In these reactions, as we shall see below, the concentration of E at high enough t values can become zero, a constant different from zero, or increase exponentially. Moreover, the continuous production of E from the zymogen compensates the loss of E through the inhibition, and the experimental monitoring of the reaction becomes easier than in those cases in which the substrate of E is not a zymogen.

Steady-state equations

The behaviour of the system in the steady state ($t \rightarrow \infty$) depends on whether r is equal to, less or higher than, unity (cases a, b and c, respectively, above).

(a) $r = 1$. Then the exponential term $A_4 e^{\lambda_4 t}$ in eqn. (4) becomes A_4 , whereas the other exponential terms become negligible; therefore the concentration of E in the steady state, $[E]_{ss}$, is given by:

$$[E]_{ss} = A_4 \quad (21)$$

If in eqn. (11) with $h = 4$, we take into account that $\lambda_4 = 0$, it follows that:

$$A_4 = \frac{k_{+3}(\kappa_{-1} + \kappa_{+2})(k_{-4} + k_1)[E]_0}{F_3} \quad (22)$$

where F_3 is given by eqn. (8) with $r = 1$. According to eqn. (21), the concentration of E reaches a constant value given by eqn. (22), i.e. in the steady state both processes, the autocatalytic activation and the irreversible inhibition, are balanced.

(b) $r < 1$. In this case the four exponential terms become very small for high-enough t values and, therefore:

$$[E]_{ss} \approx 0 \quad (23)$$

i.e. in this case the irreversible inhibition of E prevails over the autocatalytic activation of Z.

(c) $r > 1$. In this case, the exponential terms $A_j e^{\lambda_j t}$ ($j = 1, 2, 3$) in eqn. (4) become negligible with regard to the exponential term $A_4 e^{\lambda_4 t}$. So the steady-state equation is:

$$[E]_{ss} \approx A_4 e^{\lambda_4 t} \quad (24)$$

i.e., the autocatalytic activation of Z prevails over the irreversible inhibition of E.

Meaning of the r parameter

From the previous discussion a meaning can be given to the parameter r . It is a measure of the relative preponderance of both the activation and the inhibition routes in Scheme 1. According to whether r is equal to, less than or higher than, unity, neither route, activation or inhibition, prevails, as it is deduced from the steady-state behaviour of the system. The limit values of r are 0 and ∞ . The value 0 corresponds to a pure inhibition process, whereas the value ∞ corresponds either to a pure activation process or to an activation process with reversible inhibition.

Note that there are many sets of values of the rate constants and $[Z]_0$ and $[I]_0$ compatible with a same r value, and therefore there are several possible progress curves of E for each r value, but all of them show the same kinetic behaviour with regard to the prevailing route. Under the given experimental conditions of a particular reaction which evolves according to Scheme 1, the rate constants involved in eqn. (10) have fixed values and the r values are exclusively dependent on the ratio $[Z]_0/[I]_0$, so that by varying this ratio one might modify the catalytic behaviour of the system.

Fig. 1 shows variations of $[E]$ with time, obtained from eqn. (4), for three different values of r . For each of the r values used, we have plotted two of the different possible progress curves, and the results agree with our previous findings.

Particular cases of Scheme 1

Different processes can be considered as particular cases of Scheme 1 if one or more of the rate constants are much higher than the other ones (and mutually not very different) or if some rate constants are null. In Table 1 some of the several particular cases of Scheme 1 are summarized. Note that reversible inhibitions of E are also particular cases of Scheme 1. The kinetic equations for each of the above mechanisms can be obtained from the corresponding system of differential equations. However, these kinetic equations can be obtained more easily and

Fig. 1. Plots of $[E]$ versus t obtained from eqn. (4)

Since we are only interested in showing the qualitative kinetic behaviour, the rate constants values used and the initial concentrations are arbitrary. These are: $k_{+1} = 0.5 \mu\text{M}^{-1} \cdot \text{s}^{-1}$, $k_{-1} = 0.02 \text{ s}^{-1}$, $k_{+2} = 0.01 \text{ s}^{-1}$, $k_{+3} = 1 \text{ s}^{-1}$, $k_{+4} = 1 \mu\text{M}^{-1} \cdot \text{s}^{-1}$, $k_{-4} = 0.1 \text{ s}^{-1}$, $k_{+5} = 10^{-3} \text{ s}^{-1}$ and $[Z]_0$ and $[I]_0$ (in μM) are, respectively: (a) 0.3, 0.3; (b) 0.4, 0.4; (c) 0.03, 0.505; (d) 0.06, 1.01; (e) 0.1, 4; (f) 0.2, 8. The r values for each curve are: a and b, 16.8; c and d, 1; e and f, 0.42.

Table 1. Some of the different mechanisms considered as particular cases of Scheme 1

Scheme	Mechanism
2	$ \begin{array}{c} E + Z \xrightleftharpoons[k_{-1}]{K_1} EZ \xrightarrow{k_{+2}} 2E + W \\ \begin{array}{c} + \\ I \\ \downarrow K_4 \quad k_{+4} \\ EI \xrightarrow{k_i} EI^* \end{array} \end{array} $
3	$ \begin{array}{c} E + Z \xrightleftharpoons[k_{-1}]{k_{+1}} EZ \xrightarrow{k_{+2}} 2E + W \\ \begin{array}{c} + \\ I \\ \downarrow k_{+4} \\ EI \end{array} \end{array} $
4	$ \begin{array}{c} E + Z \xrightleftharpoons[k_{-1}]{k_{+1}} EZ \xrightarrow{k_{+2}} EE \xrightarrow{k_{+3}} 2E \\ \begin{array}{c} + \\ I \\ \downarrow k_{+4} \\ EI \end{array} \end{array} $ <p style="text-align: center;"><i>(Note: In the original image, a 'W' is shown as a product of the EZ → EE step in Scheme 4.)</i></p>
5	$ \begin{array}{c} E + Z \xrightleftharpoons[k_{-1}]{k_{+1}} EZ \xrightarrow{k_{+2}} EE \xrightarrow{k_{+3}} 2E \\ \begin{array}{c} + \\ I \\ \downarrow k_{+4} \\ EI \end{array} \end{array} $ <p style="text-align: center;"><i>(Note: In the original image, a 'W' is shown as a product of the EZ → EE step in Scheme 5.)</i></p>
6	$ E + I \xrightleftharpoons[k_{-4}]{k_{+4}} EI \xrightarrow{k_i} EI^* $

more quickly from those of Scheme 1 by setting at infinity or zero those constants which in the Scheme must be much higher than the other ones, or null, to convert it to the corresponding mechanism under study [18,20,21].

The Scheme 2 in Table 1 is a very plausible one. Since in this Scheme the step $EZ \rightarrow EE + W$ requires the cleavage of a peptide bond, whereas the step $EE \rightarrow 2E$ is a simple dissociation process, the relationship $k_{+2} \ll k_{+3}$ is generally assumed [22,23]. This assumption is also justified by numerous kinetic, thermodynamic and structural studies of trypsin and closely related enzymes. The reason for the low k_{+2}/k_{+3} ratio for amide substrates of such enzymes is that a slow conformational change which opens the catalytic site to the specific substrate precedes the bond-cleaving step. The location, course and kinetics of this conformational change have been identified and analysed in detail [24–30]. Almost the same argument supports the existence of a rapid pre-equilibrium involving the binding of Z to E. In addition, direct measurement using the temperature-jump technique shows that

the binding interactions of such enzymes even with protein substrates are much faster than the subsequent steps: activating conformational change, acylation and deacylation [31]. Also, rapid equilibrium conditions for the binding of I to E are usually assumed [17,19]. In order to obtain the kinetic equation corresponding to Scheme 2 we insert in eqn. (4) the conditions:

$$k_{+1}[Z]_0, k_{-1}, k_{+3}, k_{+4}[I]_0, k_{-4} \rightarrow \infty$$

$$k_{+1}[Z]_0, k_{-1}, k_{+3}, k_{+4}[I]_0, k_{-4} \text{ mutually not very different} \quad (25)$$

and the result is:

$$[E] = [E]_0 e^{\lambda t} \quad (26)$$

where

$$\lambda = \frac{k_{+2} K_4 [Z]_0 - k_i K_1 [I]_0}{K_1 [I]_0 + K_4 [Z]_0 + K_1 K_4} \quad (27)$$

with $K_1 = k_{-1}/k_{+1}$ and $K_4 = k_{-4}/k_{+4}$

In its turn, the parameter r is given by:

$$r = \frac{k_{+2} K_4 [Z]_0}{k_i K_1 [I]_0} \quad (28)$$

From eqn. (27) or from eqn. (28) it is deduced that, if

$$k_{+2} K_4 [Z]_0 = k_i K_1 [I]_0,$$

then the irreversible inhibition compensates the autocatalytic activation; if $k_{+2} K_4 [Z]_0 > k_i K_1 [I]_0$, then the autocatalytic activation prevails over the irreversible inhibition and, finally, if $k_{+2} K_4 [Z]_0 < k_i K_1 [I]_0$, then the irreversible inhibition prevails over the autocatalytic activation. The latter condition must prevail in the storage granules of those zymogens susceptible of autocatalytic activation, in order to protect the cells containing the granules from digestion.

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