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Kinetic analysis of the mechanism of plasminogen activation by streptokinase

M.E. Fuentes, E. Valero, M. García-Moreno, E. Vique and R. Varón*

Grupo de Modelización en Bioquímica, Departamento de Química-Física, Escuela Politécnica Superior de Albacete, Universidad de Castilla-La Mancha, Albacete, Spain
E-mail: Ramon.Varon@uclm.es

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A kinetic study is made of plasminogen activation to plasmin catalyzed by streptokinase. The goal of the present paper is the resolution of the mechanism corresponding to the activation process by a global way, considering the mechanism as a whole and under less restrictive assumptions that those used by other authors. The kinetic equations describing the evolution with time of species involved in the system have been obtained. These equations are valid for both the transient phase and the steady state of the reaction. A kinetic data analysis procedure to evaluate the kinetic parameters, based on the derived kinetic equations has been suggested, for the first time, in the present paper. The validity of the results obtained has been checked by using simulated progress curves of the species involved. Finally, we have demonstrated that the time course equations obtained can be applied directly to different mechanisms of zymogen activation that could be considered to be particular cases of the general studied mechanism.

KEY WORDS: Enzyme kinetics, zymogen activation, mechanism, plasminogen, streptokinase

1. Introduction

A number of proteolytic enzymes are synthesized 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 change into the active form [1]. This is a phenomenon of great importance to our understanding of the behaviour of many fundamental physiological processes, such as digestion [2], cell death [3,4], differentiation [5], immune response [6], blood coagulation [7], fibrinolysis [8,9] and response to injury [10]. Other examples are the evolution of viruses, oncogenes and metastatic cells [11].

Activation of plasminogen to form plasmin is the central event in the blood clot lysis by the fibrinolytic system. Whereas several different proteases

*Corresponding author.

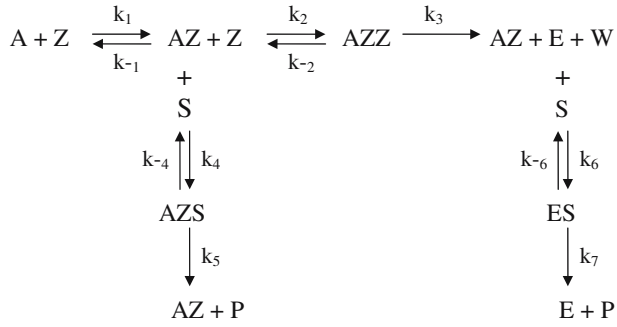
are able to activate plasminogen *in vitro*, including factor XIIa [12], factor XIa [13], plasma kallikrein [14] and even trypsin [15]. The most widely studied *in vivo* plaminogen activators are tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which are clinically employed for thrombolytic therapy. Bacterial plasminogen activators (streptokinase and staphylokinase) are also very important because of their *in vivo* thromboytic potential [16].

Activation kinetics of human Lys-plasminogen and Glu-plasminogen by different types of streptokinase at pH 6.0 and 30°C have been described using a new mathematical model [17] and a more general model has been developed, studying also plasminogen activation kinetics under physiological conditions of pH 7.4 and 37°C [18]. However, the results obtained by these authors have some limitations. Thus, Wohl et al. [17, 18] by assuming rapid equilibrium obtained an equation for the product formation which is only valid for the steady state. Ranby [19] supposed the independence of the reaction steps, while Galindo et al. [20] considered the transient phase duration of the activated enzyme to be negligible compared with the transient phase duration of the activating enzyme. Varón et al. [8] obtained the time-dependent concentrations of all species involved in the general mechanism of human plasminogen activation proposed by Wohl et al. [18], which are valid for the whole course of the reaction, i.e. the transient phase and the steady state. More recently Boxrud et al. [21] charaterized the conformational activation steps in the kinetic mechanism of activation of plasminogen by streptokinase described by scheme 1. The coupling of conformational plasminogen activation to the proteolytic activation pathway of streptokinase-induced plasmin formation was defined in a companion paper [22]. These contributions have significantly contributed to the experimental kinetic study of the plasminogen activation to plasmin by streptokinase. In both above contributions, the time course equation of the product of the reaction was obtained under very exigent assumptions. In the present paper, we have analysed the mechanism in scheme 1 under less limited conditions and we have obtained time course equations of the species involved in the system, which are valid for both the transient phase and the steady state. The validity of the results obtained has been checked by using simulated progress curves of the species involved.

2. Theory

2.1. Notation and definitions

$[A]$, $[Z]$, $[AZ]$, $[AAZ]$, $[AZS]$, $[E]$, $[ES]$, $[P]$, $[W]$: instantaneous concentrations of the species A, Z, AZ, AAZ, AZS, E, ES, P and W, respectively.
Z_0, S_0 and A_0: initial concentrations of the species Z, S and A respectively.



Scheme 1. Activation mechanism of plasminogen to plasmin by streptokinase [21, 22]. *A* is the activating enzyme, streptokinase; *Z* is the zymogen, plasminogen; *E* is the activated enzyme, plamin; *AZ* is a conformationally activated complex, which can bind the chromogenic substrate (*S*), and generate product (*P*), in two ways, on the one hand by means of the formation of a ternary complex, *AZS*, by binding with the substrate, and on the other through the formation of the active enzyme and so by the formation of the complex active enzyme-substrate, *ES*; *AZZ* is a ternary complex and *W* is one or more peptides released from *Z* during the formation of *E*.

The dissociation constants of the AZ and AZZ complexes will be, respectively:

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

and

$$K_2 = \frac{k_{-2}}{k_2}.$$

The presence of ES and AZS complexes allows the definition of two Michaelis–Menten constants for the activation of zymogen towards its active enzyme as follows:

$$K_{m,4} = \frac{k_{-4} + k_5}{k_4}$$

and

$$K_{m,6} = \frac{k_{-6} + k_7}{k_6}.$$

2.2. *Time course differential equations*

The kinetic behaviour of the species involved in the mechanism shown in scheme 1 is described by the following nonlinear set of differential equations:

Table 1
Particular cases of Scheme 1. In the third column we detail the rate constants which in Scheme 1 must be null to obtain the corresponding equations of each particular case.

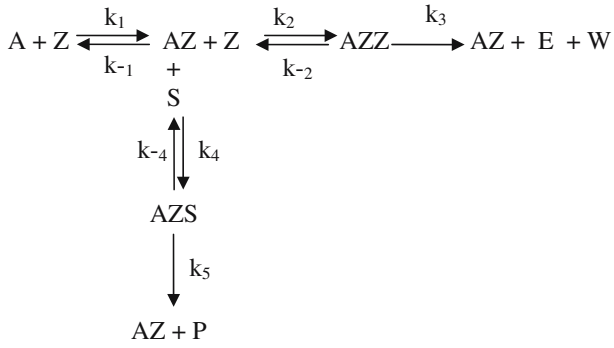
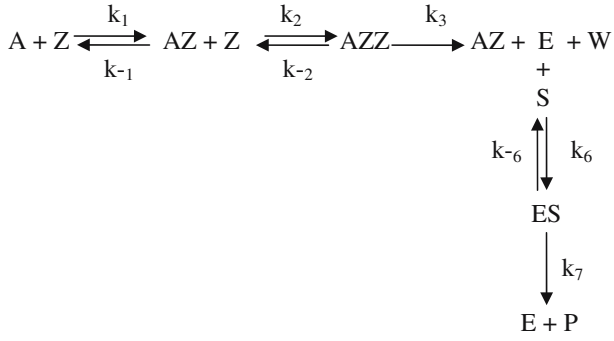
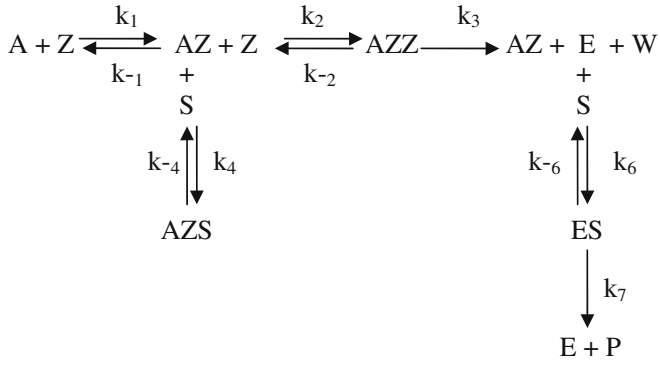
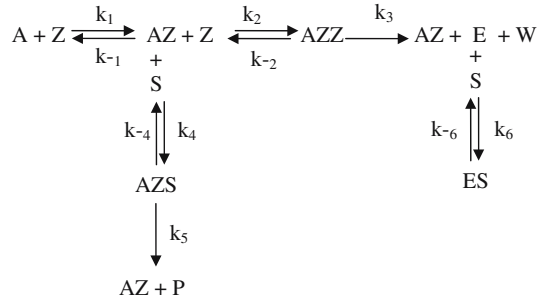
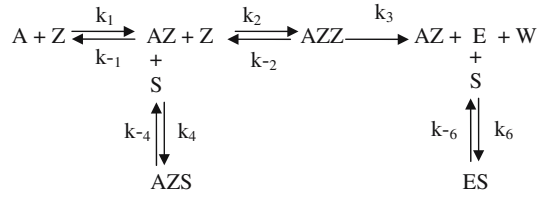
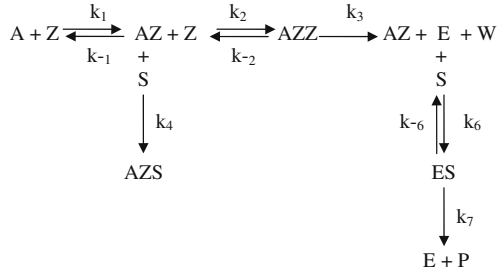
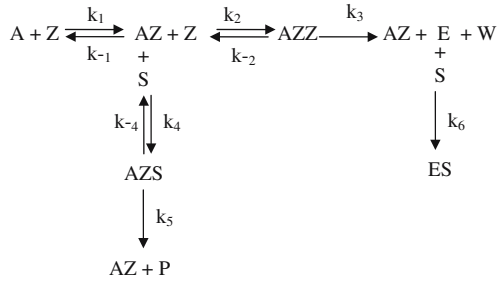
Scheme no.	Scheme	Particular values of the rate and/or equilibrium constants
2		$k_6 = 0$
3		$k_4 = 0$
4		$k_5 = 0$

Table 1
(continued)

Scheme no.	Scheme	Particular values of the rate and/or equilibrium constants
5		$k_7 = 0$
6		k_5 and $k_7 = 0$
7		k_5 and $k_{-4} = 0$
8		k_7 and $k_{-6} = 0$

$$\frac{d[A]}{dt} = -k_1[Z][A] + k_{-1}[AZ], \tag{1}$$

$$\frac{d[Z]}{dt} = -k_1[Z][A] + k_{-1}[AZ] - k_2[Z][AZ] + k_{-2}[AZZ], \tag{2}$$

$$\frac{d[AZ]}{dt} = k_1[Z][A] - (k_{-1} + k_4[S] + k_2[Z])[AZ] + (k_{-2} + k_3)[AZZ] + (k_{-4} + k_5)[AZS], \tag{3}$$

$$\frac{d[AZZ]}{dt} = k_2[Z][AZ] - (k_{-2} + k_3)[AZZ], \tag{4}$$

$$\frac{d[AZS]}{dt} = k_4[S][AZ] - (k_{-4} + k_5)[AZS], \tag{5}$$

$$\frac{d[E]}{dt} = k_3[AZZ] - k_6[S][E] + (k_{-6} + k_7)[ES], \tag{6}$$

$$\frac{d[ES]}{dt} = k_6[S][E] - (k_{-6} + k_7)[ES], \tag{7}$$

$$\frac{d[S]}{dt} = -k_4[AZ][S] + k_{-4}[AZS] - k_6[E][S] + k_{-6}[ES], \tag{8}$$

$$\frac{d[P]}{dt} = k_5[AZS] + k_7[ES], \tag{9}$$

$$\frac{d[W]}{dt} = k_3[AZZ]. \tag{10}$$

To obtain analytical solutions we shall assume that the concentration of substrates Z and S remains approximately constant during the reaction time registered, i.e.:

$$[Z] \approx Z_0 \tag{11}$$

and

$$[S] \approx S_0. \tag{12}$$

Because the mass balance equations for this scheme reaction are:

$$Z_0 = [Z] + [AZ] + 2[AZZ] + [AZS] + [E] + [ES], \tag{13}$$

$$S_0 = [S] + [AZS] + [ES] + [P] \tag{14}$$

these assumptions will be possible if the following conditions are fulfilled:

$$[AZ] + [AZZ] + 2[AZS] + [E] + [ES] \ll Z_0 \tag{15}$$

and

$$[AZS] + [ES] + [P] \ll S_0. \tag{16}$$

Under these assumptions the differential equation system that describes the mechanism shown in scheme 1 becomes:

$$\frac{d[A]}{dt} = -k_1 Z_0[A] + k_{-1}[AZ], \tag{17}$$

$$\frac{d[AZ]}{dt} = k_1 Z_0[A] - (k_{-1} + k_4 S_0 + k_2 Z_0)[AZ] + (k_{-2} + k_3)[AZZ] + (k_{-4} + k_5)[AZS], \tag{18}$$

$$\frac{d[AZZ]}{dt} = k_2 Z_0[AZ] - (k_{-2} + k_3)[AZZ], \tag{19}$$

$$\frac{d[AZS]}{dt} = k_4 S_0[AZ] - (k_{-4} + k_5)[AZS], \tag{20}$$

$$\frac{d[E]}{dt} = k_3[AZZ] - k_6 S_0[E] + (k_{-6} + k_7)[ES], \tag{21}$$

$$\frac{d[ES]}{dt} = k_6 S_0[E] - (k_{-6} + k_7)[ES], \tag{22}$$

$$\frac{d[P]}{dt} = k_5[AZS] + k_7[ES], \tag{23}$$

$$\frac{d[W]}{dt} = k_3[AZZ]. \tag{24}$$

The differential equations (17)–(24) constitute a linear system that can be solved by any integration method. To do this we used the Laplace transformation method [23]. The results obtained for the species AZS and E, taking into account that at $t = 0$ the only species present in the reaction medium are A, Z and S, are:

$$[AZS] = A_{1,0} + A_{1,2}e^{\lambda_2 t} + A_{1,3}e^{\lambda_3 t} + A_{1,4}e^{\lambda_4 t}, \tag{25}$$

$$[ES] = A_{2,0} + A_{2,1}e^{\lambda_1 t} + A_{2,2}e^{\lambda_2 t} + A_{2,3}e^{\lambda_3 t} + A_{2,4}e^{\lambda_4 t} + B_{2,0}t. \tag{26}$$

The expressions corresponding to $A_{i,j} (i = 1, 2; j = 0 - 4)$ and $B_{2,0}$ are given in Appendix A, equations (A1)–(A9) and (A10), respectively. λ_1 is the root of the polynomial equation:

$$\lambda + G_1 = 0, \tag{27}$$

i.e.

$$\lambda_1 = -G_1 \tag{28}$$

being given the expression for G_1 in Appendix A, equation (A11). λ_2–λ_4 are the roots of the polynomial equation:

$$\lambda^3 + F_1\lambda^2 + F_2\lambda - F_3 = 0 \tag{29}$$

being given the expressions for $F_i (i = 1-3)$ in Appendix A, equations (A12)–(A14). $\lambda_i (i = 2–4)$ are related to F_i by equations (A15)–(A17) in the Appendix A. From Eqs. (A15)–(A17) and owing to the secular determinant of the set of differential lineal equations with constant coefficients describing the kinetics of the enzyme species in the reaction mechanism under study is dominant diagonal, $\lambda_i (i = 2–4)$ are negative or one of them is negative and the other two are complex conjugated with a negative real part [24].

Inserting now equations (25) and (26) into equation (23) and integrating with the initial condition $[P] = 0$ at $t = 0$, the following expression is obtained for the accumulation of the chromogenic product in the reaction medium:

$$[P] = a + bt + ct^2 + \gamma_1 e^{\lambda_1 t} + \gamma_2 e^{\lambda_2^t} + \gamma_3 e^{\lambda_3 t} + \gamma_4 e^{\lambda_4 t} \tag{30}$$

being given the expressions for a, b, c and $\gamma_i (i = 1–4)$ in Appendix A, equations (A18)–(A24).

3. Results and discussion

We have obtained kinetic equations that describe the time dependence of the species involved in scheme 1 for both the transient phase and the steady state. On the basis of these kinetic equations, a novel procedure is suggested to evaluate the kinetic parameters of the system. The validity of the results obtained has been checked by using simulated progress curves of the species involved.

3.1. Validity of the time course equations here derived

Kinetic equations for the species involved in the mechanism shown in scheme 1 were derived by solving the set of ordinary, linear (with constant coefficients), differential equations (17)–(24). These kinetic equations are valid whenever conditions (11) and (12) hold, and for this reason they are approximate analytical solutions. This is a common practice in enzyme kinetics, where to derive approximate analytical solutions corresponding either to the transient phase or to the steady-state of an enzymatic reaction, substrate concentration (Z and S in this case) is usually assumed to remain approximately constant [23,25,26] and therefore, the results obtained are only valid under these conditions. It is obvious that if the reaction is allowed to progress, the final concentration of zymogen (and also the concentration of the chromogenic substrate) will be zero. Thus, as is usual in assays on enzyme kinetics, the reaction can only be allowed to evolve to a small extent during the assays compared with the total reaction time taken for the substrate to vanish [27]. Obviously, as more $[Z]$ and $[S]$ diminish, the equations obtained become less accurate.

Taking into account that the zymogen and the substrate are continuously consumed in the reaction medium from the beginning of the reaction, the equations here obtained will be less accurate as longer the reaction times registered. The reaction time over which equations obtained are valid depends so on initial concentrations of both the zymogen and the substrate and on the rate constants.

In order to achieve conditions (11) and (12), it would be required that

$$Z_0 \gg A_0 \tag{31}$$

and

$$S_0 \gg Z_0;\ E_0. \tag{32}$$

These conditions can easily be obtained experimentally.

Besides these assumptions, during the time of reaction registered the following conditions must be fulfilled:

$$[E] \ll Z_0 \tag{33}$$

and

$$[P] \ll S_0, \tag{34}$$

i.e.

$$\frac{[E]}{Z_0} \ll 1 \tag{35}$$

and

$$\frac{[P]}{S_0} \ll 1. \tag{36}$$

It is obvious that if the reaction is allowed to progress, for higher $[E]$, higher will be the committed error.

To illustrate the degree of validity of our approach, in figure 1 we show the time progress curve of the quotients $[E]/Z_0$ and $[P]/S_0$ and the product $[P]$ obtained by numerical integration of the entire differential equation system directly obtained from the mechanism shown in scheme 1 (equations 1–10) (solid lines) compared with data of $[P]$ obtained from the equation here derived equation (30) (dotted line), for an arbitrary set of rate constants values and Z_0, S_0 and A_0-values. Note that at higer $[E]/Z_0$ and $[P]/S_0$ values, less accurate the equations here obtained become.

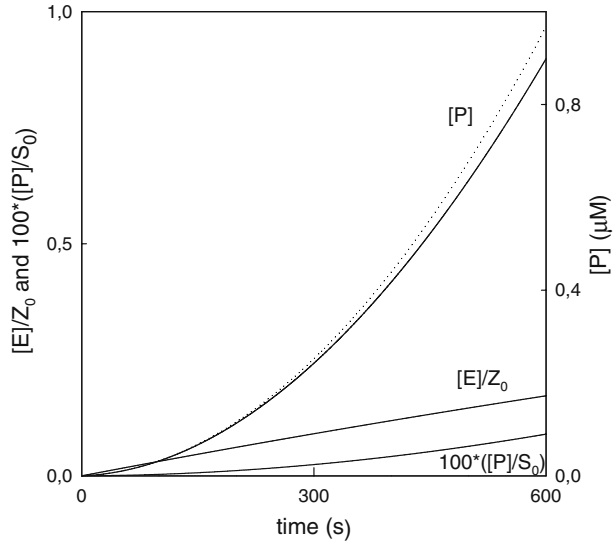


Figure 1. Progress curves corresponding to $[E]/Z_0, [P]/S_0$ and $[P]$ accumulation involved in the mechanism shown in scheme 1, obtained from numerical integration (solid lines) and progress curve of $[P]$ obtained from equation (30) (dotted line). The values of the rate constants used were: $k_1 = 1.1 \times 10^5\ \mathrm{M^{-1}\,s^{-1}}$, $k_{-1} = 1.2 \times 10^1\ \mathrm{s^{-1}}$, $k_2 = 1.0 \times 10^5\ \mathrm{M^{-1}\,s^{-1}}$, $k_{-2} = 1.0 \times 10^1\ \mathrm{s^{-1}}$, $k_3 = 0.1\ \mathrm{s^{-1}}$, $k_4 = 0.3\ \mathrm{M^{-1}\,s^{-1}}$, $k_{-4} = 0.1\ \mathrm{s^{-1}}$, $k_5 = 0.5\ \mathrm{s^{-1}}$, $k_6 = 0.2\ \mathrm{M^{-1}\,s^{-1}}$, $k_{-6} = 0.1\ \mathrm{s^{-1}}$ and $k_7 = 0.4\ \mathrm{s^{-1}}$. The initial concentrations used were: $Z_0 = 1.0 \times 10^{-4}$ M, $S_0 = 1.0 \times 10^{-3}$ M, and $A_0 = 1.0 \times 10^{-6}$ M. For grater clarity, taking into account the very small values obtained for $[P]/S_0$, these data have been multiplied by 100.

3.1.1. Parabolic behaviour

The time course equation for the concentration of the product here obtained is of four-exponential type. Nevertheless, because $\lambda_i (i = 1\text{–}4)$ are negative or complex conjugated with a negative real part, the exponential terms in equation (30) can be neglected from a relative short-time after the onset of the reaction, so that the kinetics of the accumulation of $[P]$ become parabolic from this time. In this way, the kinetic equation for P becomes:

$$[P] = a + bt + ct^2. \tag{37}$$

Figure 2 shows a comparison of the time course of product accumulation obtained from equation (30) and from equation (37), for an arbitrary set of rate constants values and Z_0, S_0 and A_0-values. In the inset it can be seen that at time near the coordinate origin these plots do not coincide.

Equation (30) is excessive complex to be applied in experimental assays due to it has four exponential terms and by the complexity of the coefficients, and equation (37) although simpler still has the complexity of the coefficients. For these reasons, we will make reasonable assumptions to simplify these equations.

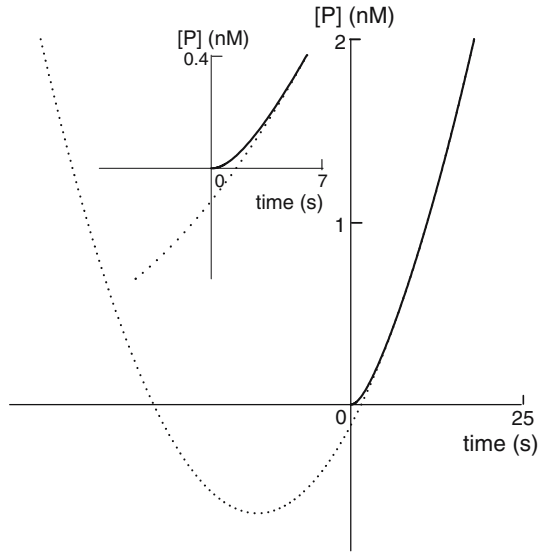


Figure 2. Progress curves corresponding to Z accumulation, obtained from equation (30) (solid line) and from equation (37) (dotted line). Conditions as figure 1. The inset shows an expansion of this graph near the coordinate origin.

One of these assumptions is the rapid equilibrium assumption which we can see in the following paragraph.

3.1.2. Rapid equilibrium assumptions

If moreover we assume rapid equilibrium conditions for the following reversible reaction steps involved in scheme 1, then the equations obtained above are further simplified:

$$A + Z \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} AZ,$$

$$AZ + Z \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} AZZ$$

it does mean that [28]:

$$k_1[Z], k_{-1}, k_2[Z], k_{-2} \to \infty \tag{38}$$

and

$$k_1[Z], k_{-1}, k_2[Z], k_{-2} \quad \text{mutually not very different.} \tag{39}$$

Following the procedure reported by Varón et al. [28] for rapid equilibrium conditions, it can be easily deduced that two of the $\lambda_i (i = 2\text{–}4)$, for example

λ_3 and λ_4, are much higher than the other two. Therefore, the two exponential terms with relatively high $\lambda(\lambda_3$ and $\lambda_4)$ can be neglected from the onset of the reaction, so that the kinetic behaviour of the species involved in scheme 1 becomes approximately bi-exponential from this time.

Taking this into account, equation (30) can be expressed as follows:

$$[P] = a^* + b^*t + c^*t^2 + \gamma_1^* e^{\lambda_1^* t} + \gamma_2^* e^{\lambda_2^* t}, \tag{40}$$

where a^*, b^*, c^*, γ_1^* and γ_2^* are the expressions resulting of inserting in a, b, c, γ_1 and γ_2 the conditions of rapid equilibrium, respectively, and they are given in Appendix A, equations (A25)–(A29), and λ_2^* is the expression of λ_2 under rapid equilibrium conditions, being given in the Appendix A, equation (A26). The expression of λ_1 does not change under equilibrium conditions.

Since the λ-values in equation (40) are negative, in the steady state, i.e. for times relatively long, the exponential terms may be neglected and equation (40) can be rewritten as follows:

$$[P] = a^* + b^*t + c^*t^2. \tag{41}$$

The time for which equation (40) applies can be very small if rapid equilibrium conditions prevail, so that practically from the start of the reaction (i.e. at $t \approx 0$) equation (41) is valid, since the transient phase will be undetectable in these cases.

Figure 3 shows a comparison of the time progress curve of $[P]$ obtained by numerical integration of the entire differential equation system obtained directly form the mechanism shown in scheme 1 [equations (1)–(10) (curve i, solid line) with the curve obtained from the equation here derived equation (30)] (curve ii, dotted line), and the equations under rapid equilibrium assumption [equation (40) (curve iii, dash line) and (41) (curve iv, dash-dot-dot line)], for arbitrary Z_0, S_0 and A_0-values. In case of figure 3A, the set of rate constants values chosen takes into account assumptions (38) and (39) (i.e. conditions of rapid equilibrium), and in figure 3B these assumptions were not taken into account. Note that in figure 3A the four progress curves are near superposed and in the case of figure 3B progress curves obtained with equations (9) and (30) are superposed on one hand, and on the other progress curves obtained in conditions of rapid equilibrium, i.e. equations (40) and (41) are superposed.

In figure 4 we show an experimental product accumulation curve as well as the corresponding fitting to equation (41). The values of the parameter thus obtained were: $a^* = 5.45\ \mu$M, $b^* = 5.58 \times 10^{-1}\ \mu$M s^{-1}, $c^* = 3.90 \times 10^{-2}$ μM s^{-2}, $r = 0.9998$, showing in this way the goodness of the fitting.

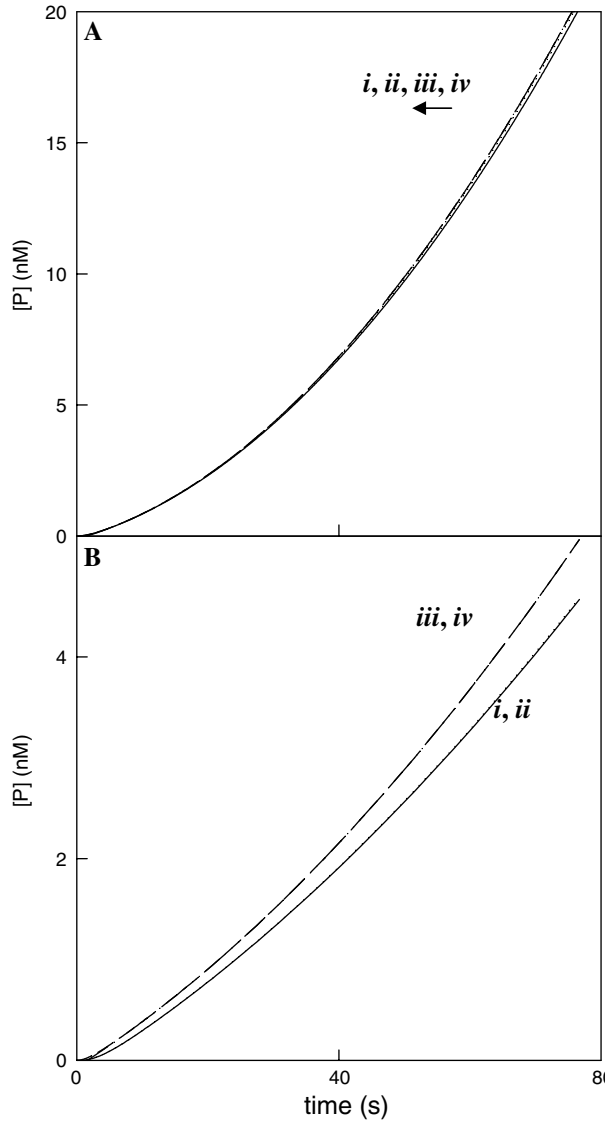


Figure 3. Progress curves corresponding $[P]$ accumulation involved in the mechanism shown in scheme 1, obtained from numerical integration (curve i, solid line) and from equation (30) (curve ii, dotted line), equation (40) (curve iii, dash line) and equation (41) (curve iv, dash-dot-dot line). (A) conditions are the same as figure 1, and (B) the initial concentrations used were the same as figure 1, the values of the rate constants used being: $k_1 = 1.1 \times 10^3\,\mathrm{M^{-1}\,s^{-1}}$, $k_{-1} = 0.5\,\mathrm{s^{-1}}$, $k_2 = 1.0 \times 10^3\,\mathrm{M^{-1}\,s^{-1}}$, $k_{-2} = 0.4\,\mathrm{s^{-1}}$, $k_3 = 0.1\,\mathrm{s^{-1}}$, $k_4 = 0.3\,\mathrm{M^{-1}\,s^{-1}}$, $k_{-4} = 0.1\,\mathrm{s^{-1}}$, $k_5 = 0.5\,\mathrm{s^{-1}}$, $k_6 = 0.2\,\mathrm{M^{-1}\,s^{-1}}$, $k_{-6} = 0.1\,\mathrm{s^{-1}}$ and $k_7 = 0.4\,\mathrm{s^{-1}}$.

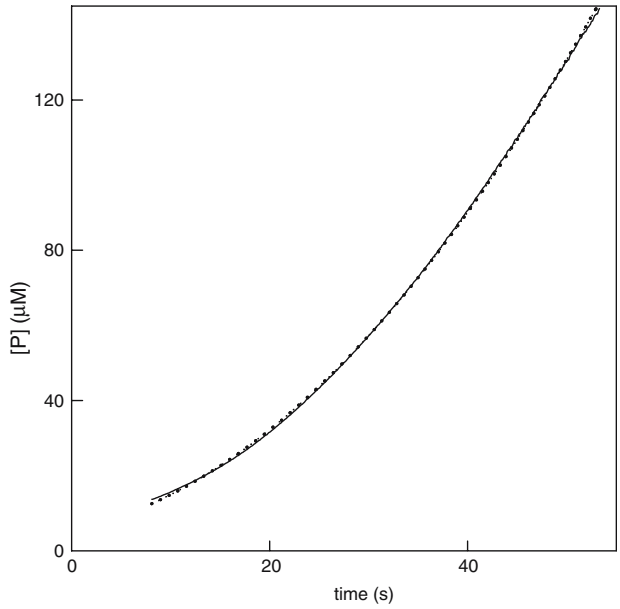


Figure 4. Product accumulation curve of plasminogen activation kinetics monitored by means of the plasmin activity to D-Val-Leu-Lys-Nan. The line represent the experimental data and the points the corresponding fitting to equation (41).

3.2. *Kinetic data analysis*

In this section, we are going to propose an experimental design and a kinetic data analysis to evaluate the kinetic parameters of the autocatalytic system, from the equations here obtained. The procedure we suggest is valid whenever conditions (11), (12) and (33) are fulfilled during the reaction time registered, and it consists of the following steps.

(1) In first place experimental progress curves corresponding to the accumulation of the product at different initial substrate and zymogen concentrations should be obtained. Curves thus obtained must be fitted by nonlinear regression to equation (41), for times relatively long, where the system can be considered in steady state. Thus, values of a^*, b^* and c^* are obtained.

(2) Plotting $\left(\frac{A_0 Z_0^2 S}{2c^*}\right)$-values at different initial substrate and zymogen concentrations versus $[S]$, and fitting these data by linear regression to a quadratic polynomial, as follows:

$$y = A + B\,[S] + C\,[S]^2 \tag{42}$$

the values of A, B and C can be evaluated, which have the following properties:

$$A = \frac{K_{m,6}}{k_3 k_7}\left(Z_0^2 + K_2 Z_0 + K_1 K_2\right), \tag{43}$$

$$B = \frac{1}{k_3 k_7}\left\{Z_0^2 + \left(1 + \frac{K_{m,6}}{K_{m,4}}\right) K_2 Z_0 + K_1 K_2\right\}, \tag{44}$$

$$C = \frac{K_2}{k_3 k_7 K_{m,4}}. \tag{45}$$

(3) Plotting the A-values above obtained versus Z_0, and fitting these data by linear regression to a quadratic polynomial, as follows:

$$y = \frac{K_{m,6}}{k_3 k_7} Z_0^2 + \frac{K_{m,6} K_2}{k_3 k_7} Z_0 + \frac{K_{m,6} K_1 K_2}{k_3 k_7}. \tag{46}$$

The kinetic parameters K_1, K_2 and $K_{m,6}/k_3 k_7$ can be evaluated.

(4) Plotting the B-values obtained in step (2) versus Z_0, and fitting these data by linear regression to a quadratic polynomial, as follows.

$$y = \frac{1}{k_3 k_7} Z_0^2 + \frac{\left(1 + \frac{K_{m,6}}{K_{m,4}}\right) K_2}{k_3 k_7} Z_0 + \frac{K_1 K_2}{k_3 k_7}. \tag{47}$$

The kinetic parameters $K_{m,4}$, $K_{m,6}$ and $k_3 k_7$ can be evaluated.

(5) The kinetic parameter k_7 can be evaluated in a classical way in a separated set of assays by varying the initial chromogenic substrate concentration at a fixed concentration of plasmin. Fitting data thus obtained to the Michaelis–Menten equation, k_7 is easily obtained from $V_{\max}$ once known the plasmin concentration used and the molar extinction coefficient for the experimentally monitored species. Once the kinetic parameter k_7 is known we immediately can evaluate the kinetic parameter k_3 from the value of $k_3 k_7$ obtained in the above step (4).

Note that this analysis lets to obtain the equilibrium constants K_1 and K_2; the first-order constants k_3 and k_7 and the global constants $K_{m,4}$ and $K_{m,6}$.

3.3. *Particular cases of scheme 1*

Different processes can be considered particular cases of the model shown in scheme 1 if one or more of the rate constants of first- and pseudo-first order are null. In table 1, seven particular cases of scheme 1 are listed.

In scheme 2 the enzyme E has not activity over the substrate, in scheme 3 the complex AZ has not activity over the substrate, in scheme 4, the substrate is

a reversible uncompetitive inhibitor of the streptokinase, in scheme 5, the substrate is a reversible competitive inhibitor of the enzyme, in scheme 6, the substrate is a reversible uncompetitive inhibitor of streptokinase and a reversible competitive inhibitor of the enzyme. In scheme 7, the substrate is an irreversible uncompetitive inhibitor of streptokinase. In scheme 8, the substrate is an irreversible competitive inhibitor of the enzyme.

The time course equations of the product for each of the schemes in table 1 can be obtained from the corresponding system of differential equations describing its kinetics. However, the same result can more easily be obtained by setting the changes indicated on the third column in table 1 into equation (30).

For example, the time course equation for the product corresponding to the particular case given by scheme 4 is obtained making null the rate constant k_5 in equation (30).

3.4. Comparison of our results with those obtained by other authors

Equation (41) obtained by us for the time course of product concentration has been obtained under a minimum number of assumptions which have been indicated in equations (11) and (12), and under rapid equilibrium assumptions (in the steps of union of the zymogen to A and AZ) from the onset of the reaction.

Other authors [21, 22] have reported for this same mechanism, also under the same rapid equilibrium conditions, equation (B1) shown in Appendix B that describes the increase in absorbance due to the product accumulation. After dividing equation (B1) by ε, the absortivity molar coefficient of the product, it is possible to obtain equation (B2) describing the time course of product concentration, but under more restrictive assumptions. To obtain the time course equations corresponding to this mechanism (scheme 1), these authors divided it into two parts, assuming that at the onset of the reaction (i.e. at $t = 0$), one of the parts is already in the steady-state. However, the use of more assumptions has led them to obtain a simpler equation than the one obtained by us, although less closed to reality.

Figure 5 shows a comparison of the results obtained for $[P]$ accumulation obtained from numerical integration of the rigorous set of differential equations directly obtained from the mechanism shown in scheme 1 [equations (1)–(10), and from the equation here derived (equation 41)] and from the equation previously reported in the literature [Equation (B2), Appendix B] [21], using the same values for the rate constants and initial conditions. It can be seen that the curve obtained from numerical integration without any assumption is nearer to the equation here derived (equation (41)) than to the one previously reported (adapted to $[P]$ formation) (equation (B2)).

Note that in, in Appendix B, the expression corresponding to v_2 equation (B1) was not given by Boxrud et al. [21] and Boxrud and Bock [22], and so we

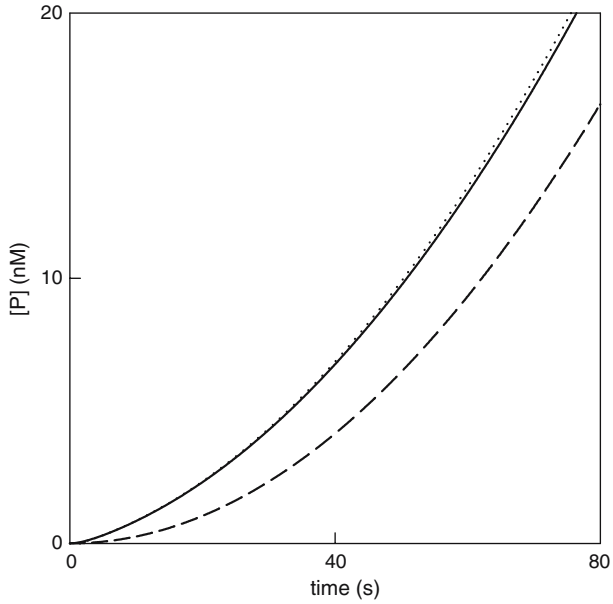


Figure 5. Progress curves corresponding to Z accumulation involved in the mechanism shown in scheme 1, obtained from numerical integration (solid line) and from equation (41) (dotted line) and from the equation proposed by Boxrud et al. [21] [equation (B2), appendix B] (dash line). Conditions as figure 1.

have used for v_2 the equation obtained by Wohl et al. [18] after adapting it to scheme 1. We have omitted here this procedure. It can be demonstrated that multiplied by 2 and ε our expression of c^* given in equation (A27), in Appendix A, it coincides with equation (B5), in Appendix B, obtained by Wohl et al. [18].

4. Experimental procedures

4.1. Material

Plasminogen from human plasma (6.6 units $\times$ mg protein^{-1}), plasmin from human plasma (3–6 units $\times$ mg protein^{-1}), streptokinase buffered aqueous solution (73,520 units $\times$ mg protein^{-1}), D- Valyl-L-leucyl-L-lysine 4-nitroanilide di-hydrochloride (D-Val-Leu-Lys-Nan $\cdot$ 2HCl), Tris (hydroxymethyl)-aminomethane, sodium chloride and L-Lysine were purchased from Sigma (Madrid, Spain). Stock solutions of plasminogen were prepared by solving 25 units of the zymogen in 2.25 ml of 0.05 M Tris/HCl, 0.1 M NaCl, 20 mM L-Lysine buffer, pH = 7.4. All solutions were prepared in ultrapure deionized nonpyrogenic water (Milli Q; Millipore Ibérica S.A., Barcelona, Spain).

Plasminogen activation kinetics were measured at 37°C by spectrophotometrically monitoring, at 405 nm, the appearance of the nitroaniline product of the reaction curves ($\varepsilon = 1 \times 10^4\ M^{-1}\ cm^{-1}$) [29].

4.2. Methods

Spectrophotometric readings were obtained on a Uvikon XS spectrophotometer from Bio-Tek Instruments (Barcelona, Spain). Temperature was controlled by using a Hetofrig Selecta circulating water bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of ±0.1°C.

The experimental progress curves obtained for the autoactivation of plasminogen were fitted by nonlinear regression to equation (41) using the SigmaPlot Scientific Graphing System, Version 8.02 (2002, SPSS Inc., USA).

4.2.1. Numerical integration

Simulated progress curves were obtained by numerical integration of the rigorous set of differential equations directly obtained from the mechanism shown in scheme 1 [equations (1)–(10)], using arbitrary sets of rate constants and initial concentration values. This numerical solution was found by using the classical fourth-order Runge–Kutta formula, but applying an adaptative stepsize control originally invented by Fehlberg [30], Matheux and Fink [31] using a computer program implemented in Visual C++ 6.0 [32]. The above program was run on a PC compatible computer based on a Pentium IV/2 GHZ processor with 512 MB of RAM.

Appendix A

$$A_{1,0} = -\frac{k_1 k_4 Z_0 S_0 A_0 (k_{-2} + k_3)}{\lambda_2 \lambda_3 \lambda_4}, \tag{A1}$$

$$A_{1,2} = \frac{k_1 k_4 Z_0 S_0 A_0 (k_{-2} + k_3 + \lambda_2)}{\lambda_2 (\lambda_2 - \lambda_3)(\lambda_2 - \lambda_4)}, \tag{A2}$$

$$A_{1,3} = \frac{k_1 k_4 Z_0 S_0 A_0 (k_{-2} + k_3 + \lambda_3)}{\lambda_3 (\lambda_3 - \lambda_2)(\lambda_3 - \lambda_4)}, \tag{A3}$$

$$A_{1,4} = \frac{k_1 Z_0 S_0 A_0 (k_{-2} + k_3 + \lambda_4)}{\lambda_4 (\lambda_4 - \lambda_2)(\lambda_4 - \lambda_3)}, \tag{A4}$$

$$A_{2,0} = -\left(A_{2,1} + A_{2,2} + A_{2,3} + A_{2,4}\right), \tag{A5}$$

$$A_{2,2} = \frac{k_1 k_2 k_3 k_6 Z_0^2 S_0 A_0 \left(k_{-4} + k_5 + \lambda_2\right)}{\lambda_2^2 (\lambda_2 - \lambda_1)(\lambda_2 - \lambda_3)(\lambda_2 - \lambda_4)}, \tag{A6}$$

$$A_{2,3} = \frac{k_1 k_2 k_3 k_6 Z_0^2 S_0 A_0 \left(k_{-4} + k_5 + \lambda_3\right)}{\lambda_3^2(\lambda_3 - \lambda_1)(\lambda_3 - \lambda_2)(\lambda_3 - \lambda_4)}, \tag{A7}$$

$$A_{2,4} = \frac{k_1 k_2 k_3 k_6 Z_0^2 S_0 A_0 \left(k_{-4} + k_5 + \lambda_4\right)}{\lambda_4^2(\lambda_4 - \lambda_1)(\lambda_4 - \lambda_2)(\lambda_4 - \lambda_3)}, \tag{A8}$$

$$A_{2,1} = \frac{k_1 k_2 k_3 k_6 Z_0^2 S_0 A_0 \left(k_{-4} + k_5 + \lambda_1\right)}{\lambda_1^2(\lambda_1 - \lambda_2)(\lambda_1 - \lambda_3)(\lambda_1 - \lambda_4)}, \tag{A9}$$

$$B_{2,0} = \frac{k_1 k_2 k_3 k_6 Z_0^2 S_0 A_0 \left(k_{-4} + k_5\right)}{\lambda_1 \lambda_2 \lambda_3 \lambda_4}, \tag{A10}$$

$$G_1 = k_6 S_0 + k_{-6} + k_7, \tag{A11}$$

$$F_1 = k_1 Z_0 + k_{-1} + k_2 Z_0 + k_4 S_0 + k_{-2} + k_3 + k_{-4} + k_5, \tag{A12}$$

$$\begin{aligned} F_2 = {} & k_1 k_2 Z_0^2 + k_1 k_4 Z_0 S_0 + k_1 Z_0 \left(k_{-2} + k_3 + k_{-4} + k_5\right) \\ & + k_{-1}\left(k_{-2} + k_3 + k_{-4} + k_5\right) + k_2 Z_0 \left(k_{-4} + k_5\right), \\ & + k_4 S_0 \left(k_{-2} + k_3\right) + \left(k_{-2} + k_3\right)\left(k_{-4} + k_5\right), \end{aligned} \tag{A13}$$

$$\begin{aligned} F_3 = {} & k_1 k_2 Z_0^2 \left(k_{-4} + k_5\right) + k_1 k_4 Z_0 S_0 \left(k_{-2} + k_3\right) \\ & + k_1 Z_0 \left(k_{-2} + k_3\right)\left(k_{-4} + k_5\right) + k_{-1}\left(k_{-2} + k_3\right)\left(k_{-4} + k_5\right), \end{aligned} \tag{A14}$$

$$\lambda_2 + \lambda_3 + \lambda_4 = -F_1, \tag{A15}$$

$$\lambda_2\lambda_3 + \lambda_2\lambda_4 + \lambda_3\lambda_4 = F_2, \tag{A16}$$

$$\lambda_2\lambda_3\lambda_4 = -F_3, \tag{A17}$$

$$a = -k_5\left(\frac{A_{1,2}}{\lambda_2} + \frac{A_{1,3}}{\lambda_3} + \frac{A_{1,4}}{\lambda_4}\right) - k_7\left(\frac{A_{2,1}}{\lambda_1} + \frac{A_{2,2}}{\lambda_2} + \frac{A_{2,3}}{\lambda_3} + \frac{A_{2,4}}{\lambda_4}\right), \tag{A18}$$

$$b = k_5 A_{1,0} + k_7 A_{2,0}, \tag{A19}$$

$$c = \frac{k_7 B_{2,0}}{2}, \tag{A20}$$

$$\gamma_1 = \frac{k_7 A_{2,1}}{\lambda_1}, \tag{A21}$$

$$\gamma_2 = \left(k_5 A_{1,2} + k_7 A_{2,2}\right)\frac{1}{\lambda_2}, \tag{A22}$$

$$\gamma_3 = \left(k_5 A_{1,3} + k_7 A_{2,3}\right)\frac{1}{\lambda_3}, \tag{A23}$$

$$\gamma_4 = \left(k_5 A_{1,4} + k_7 A_{2,4}\right) \frac{1}{\lambda_4}, \tag{A24}$$

$$a^* = -\left(\gamma_1^* + \gamma_2^*\right), \tag{A25}$$

$$b^* = -\left(\gamma_1^* \lambda_1 + \gamma_2^* \lambda_2^*\right), \tag{A26}$$

$$c^* = \frac{k_3 k_7 K_{m,4} Z_0^2 S_0 A_0}{2\left(K_{m,4} Z_0^2 + K_2 Z_0 S_0 + K_2 K_{m,4} Z_0 + K_1 K_2 K_{m,4}\right)\left(S_0 + K_{m,6}\right)}, \tag{A27}$$

$$\gamma_1^* = -\frac{k_3 k_6 k_7 \left(k_{-4} + k_5 + \lambda_1\right) Z_0^2 S_0 A_0}{\left(Z_0^2 + K_2 Z_0 + K_1 K_2\right) \lambda_1^3 \left(\lambda_2^* - \lambda_1\right)}, \tag{A28}$$

$$\gamma_2^* = \frac{Z_0 S_0 A_0}{\left[Z_0^2\left(k_{-4} + k_5\right) + k_4 K_2 Z_0 S_0 + K_2\left(k_{-4} + k_5\right) Z_0 + K_1 K_2\left(k_{-4} + k_5\right)\right] \lambda_2^*} \times \left(-K_2 k_4 k_5 + \frac{k_3 k_6 k_7 Z_0 \left(k_{-4} + k_5 + \lambda_2^*\right)}{\lambda_2^*\left(\lambda_1 - \lambda_2^*\right)}\right), \tag{A29}$$

$$\lambda_2^* = -\frac{\left(Z_0^2 + K_2 Z_0 + K_1 K_2\right)\left(k_{-4} + k_5\right) + k_4 Z_0 S_0 K_2}{Z_0^2 + Z_0 K_2 + K_1 K_2}. \tag{A30}$$

Appendix B

$$\Delta A_{405\,nm} = \frac{v_2 t^2}{2} + v_1 t, \tag{B1}$$

$$P = \frac{\frac{v_2 t^2}{2} + v_1 t}{\varepsilon}, \tag{B2}$$

$$v_1 = \frac{k_5 [Z]_{\text{free}} A_0 S_0}{\left[K_1 + [Z]_{\text{free}}\left(1 + \frac{S_0}{\frac{k_{-4}+k_5}{k_4}}\right) + \frac{[Z]_{\text{free}}^2}{K_2}\right]\left(\frac{k_{-4}+k_5}{k_4} + S_0\right)}, \tag{B3}$$

$$[Z]_{\text{free}} = \frac{Z_0 - A_0 - K_1 \sqrt{(Z_0 - A_0 - K_1)^2 + 4 Z_0 K_1}}{2}, \tag{B4}$$

$$v_2 = \varepsilon \left[\frac{k_3 k_7 K_{m,4} Z_0^2 S_0 A_0}{\left(K_{m,4} Z_0^2 + K_2 Z_0 S_0 + K_2 K_{m,4} Z_0 + K_1 K_2 K_{m,4}\right)\left(S_0 + K_{m,6}\right)}\right]. \tag{B5}$$

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