Biochimica et Biophysica Acta, 480 (1977) 275—281
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BBA 68001

KINETIC STUDIES OF THE UROKINASE CATALYSED CONVERSION OF NH_2-TERMINAL LYSINE PLASMINOGEN TO PLASMIN

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(Received July 2nd, 1976)

Summary

A method for determining initial velocities of the urokinase (EC 3.4.99.26) catalysed conversion of NH_2-terminal lysine plasminogen to plasmin (EC 3.4.21.7) is presented. This reaction has been coupled with the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester, which is catalysed by plasmin, and its rate has been determined from the time course of the overall reaction.

The proenzyme-enzyme conversion was found to obey the Michaelis-Menten rate equation. The following values of the kinetic parameters were obtained: the apparent Michaelis constant, $K_m = 40.7 \pm 6.2$ μM; the catalytic constant, $k_c = 2.59 \pm 0.31$ s^{-1}, and $k_c/K_m = 6.36 \cdot 10^4 \pm 0.24 \cdot 10^4$ $M^{-1} \cdot s^{-1}$.

Introduction

The rate equation describing the behaviour of most enzymes is

$$v = k_c e_o/(1 + K_m/s) \quad (1)$$

where v is the steady state velocity, e_o is the total concentration of enzyme and s is the concentration of substrate. k_c and K_m are the fundamental kinetic parameters, the catalytic and the (apparent) Michaelis constant, respectively. The dependence of these kinetic parameters on, for example, the concentration of other substrates and various inhibitors, and on such variables as pH, temperature and ionic strength is the main kinetic source of information about the mechanism of the reaction being studied.

The conversion of plasminogen into plasmin (EC 3.4.21.7) is an important step in fibrinolysis. Investigation of the kinetics of this process is complicated because no simple direct method exists for measuring continuously the con-

Abbreviations: Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester; Bz-Arg-OH, α-*N*-benzoyl-L-arginine.

centration of plasminogen or plasmin and thereby for estimating the rate of the reaction. This paper presents a method for determining the initial velocity of plasmin formation from plasminogen. Urokinase (EC 3.4.99.26) catalysed proteolysis of NH_2-terminal lysine plasminogen has been coupled with the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt), which is catalysed by the plasmin formed, and the rate of the proteolytic reaction has been determined from the rate of α-*N*-benzoyl-L-arginine (Bz-Arg-OH) appearance.

Theory

Two reactions occur in a reaction mixture in which Bz-Arg-OEt is present in addition to NH_2-terminal lysine plasminogen and urokinase. They are

$$\text{(I)}\ NH_2\text{-Lys-plasminogen} \xrightarrow{\text{urokinase}} \text{plasmin.}$$

$$\text{(II)}\ \text{Bz-Arg-OEt} \xrightarrow{\text{plasmin}} \text{Bz-Arg-OH + ethanol}$$

In reaction I NH_2-terminal lysine plasminogen is converted into plasmin and in reaction II Bz-Arg-OEt is hydrolysed by this plasmin. A plausible mechanism for reaction II [1] is

$$\text{(IIa)}\ E + \text{Bz-Arg-OEt} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C_1 \xrightarrow{k_2} C_2 + \text{ethanol.}$$

$$e(t) \quad s(t) \qquad c_1(t) \quad c_2(t) \quad p_1(t)$$

$$C_2 \xrightarrow{k_3} E + \text{Bz-Arg-OH,}$$

$$c_2(t) \quad e(t) \quad p_2(t)$$

where E is free plasmin, and C_1 and C_2 are plasmin-substrate complexes. The concentration of the *i*th-reactant at time t is indicated by $i(t)$. The velocity of reaction I, v_1, gives the rate of formation of plasmin. If the total concentration of plasmin at time t is $e_0(t)$, then

$$v_1 = \mathrm{d}(e_0(t))/\mathrm{d}t = \dot{e}_0(t). \tag{2}$$

Since $e_0(t)$ is the total concentration of the enzyme catalyzing reaction II, it follows that

$$e_0(t) = e(t) + c_1(t) + c_2(t) \tag{3}$$

The following differential equations describe the system:

$$\dot{e}(t) = v_1 - k_1 s(t) e(t) + k_{-1} c_1(t) + k_3 c_2(t) \tag{4}$$

$$\dot{c}_1(t) = k_1 s(t) e(t) - (k_{-1} + k_2) c_1(t) \tag{5}$$

$$\dot{c}_2(t) = k_2 c_1(t) - k_3 c_2(t) \tag{6}$$

$$\dot{p}_1(t) = k_2 c_1(t) \tag{7}$$

$$\dot{p}_2(t) = k_3 c_2(t). \tag{8}$$

It follows from Eqns. 3 and 6 that

$$e(t) = e_0(t) - c_1(t) - c_2(t) \tag{9}$$

and

$$c_1(t) = \dot{c}_2(t)/k_2 + c_2(t)k_3/k_2 \tag{10}$$

from which

$$\dot{c}_1(t) = \ddot{c}_2(t)/k_2 + \dot{c}_2(t)k_3/k_2. \tag{11}$$

If these equations are now used to eliminate $e(t)$, $c_1(t)$ and $\dot{c}_1(t)$ from Eqn. 5

$$e_0(t) = \ddot{c}_2(t)/k_1k_2s(t) + \dot{c}_2(t)[(k_1s(t) + k_{-1} + k_2 + k_3)/k_1k_2s(t)] \tag{12}$$
$$+ c_2(t)\left[1 + \frac{(k_{-1} + k_2)k_3}{k_1s(t)(k_2 + k_3)}\right]\left[\frac{k_3 + k_2}{k_2}\right].$$

The concentration of Bz-Arg-OH is measured as a function of time to give a rate

$$v = \dot{p}_2(t) = k_3c_2(t); \quad c_2(t) = v/k_3. \tag{13}$$

Substituting for $c_2(t)$ and introducing the steady-state kinetic parameters of reaction IIa [1], given by $k_c = k_2k_3/(k_2 + k_3)$, the catalytic constant; $K_s = (k_{-1} + k_2)/k_1$, the Michaelis constant: $K_m = K_sk_3/(k_2 + k_3)$, the apparent Michaelis constant, into Eqn. 12 then leads to

$$e_0(t) = \ddot{v}/k_1k_2k_3s(t) + \dot{v}[1 + K_s/s(t) + k_3/k_1s(t)]/(k_2k_3) + v(1 + K_m/s(t))/k_c. \tag{14}$$

This can be re-written as

$$e_0(t) = f_1\ddot{v} + f_2\dot{v} + f_3v. \tag{15}$$

Eqn. 15 gives the relation between the rate of reaction II, $v = \dot{p}_2(t)$, and the concentration at time t of plasmin, $e_0(t)$, the product formed in reaction I.

Materials and Methods

α-*N*-Benzoyl-L-arginine ethyl ester was obtained from Sigma Chemical Company, U.S.A. Urokinase was the Leo, Copenhagen, Denmark, "Reagent" preparation containing 7700 Ploug units/mg. (1 Ploug unit = 1.31 CTA units [2].) According to White and Barlow [3] and to Lesuk et al. [4] 1 mol urokinase $\approx 5.5 \cdot 10^{12}$ CTA units, and hence $\approx 4.2 \cdot 10^{12}$ Ploug units.

In all kinetic experiments the buffer used was 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8.

Human plasminogen was prepared from stored plasma by the method based on that of Deutsch and Mertz [5], as described by Thorsen and Müllertz [6]. All preparations from stored plasma showed a double plasminogen peak against monospecific rabbit antiplasminogen in crossed immunoelectrophoresis performed as described by Ganrot [7]. The two plasminogens were separated by ion-exchange chromatography on DEAE-Sephadex A 50. The equilibration buffer was 0.05 M Tris · HCl, pH 8.0 and the proteins were eluted with a linear gradient of NaCl obtained from a solution of 0.5 M NaCl, 0.05 M Tris · HCl, pH 8.0. The two plasminogens were eluted at ionic strengths $I = 0.14$ and $I = 0.23$, respectively. The separate preparations were finally dialysed against

0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8 and suitably concentrated by dialysis/ultrafiltration in an Amicon stirred cell with a diaflo PM10 membrane. They were then stored at −20° C. The plasminogen eluted at $I = 0.14$ showed β_2-mobility on gel electrophoresis and NH_2-terminal glutamic acid as determined by the method of Gros and Labouesse [8], while the plasminogen eluted at $I = 0.23$ showed γ-mobility and NH_2-terminal lysine.

The velocities of urokinase catalysed conversion of NH_2-terminal lysine plasminogen into plasmin were determined at concentrations of plasminogen in the range 1—20 μM and concentrations of urokinase in the range of 0.7—24 nM. At zero time the concentration of Bz-Arg-OEt was 0.667 mM, and no Bz-Arg-OH or plasmin was present. The reaction was initiated by adding an appropriate amount of urokinase stock solution (1000 Ploug units/ml in Tris buffer, 25° C) to a mixture of Bz-Arg-OEt and NH_2-terminal lysine plasminogen in the same buffer, 25° C. After mixing the appearance of Bz-Arg-OH was followed by recording the reaction mixture's change in absorbance at $\lambda = 253$ nm. Recording was terminated when 15% of the initial Bz-Arg-OEt was hydrolysed. Values of t and $p_2(t)$ μM were read at fifty points on each recorded progress curve. The ten polynomials

$$y_n(t) = a_0 + \sum_{i=1}^{n} a_i t^i \qquad n = 1,10 \tag{16}$$

were then fitted to these data using the method of least squares. The standard error of estimate of each fit was obtained from

$$s_n^2 = \frac{\sum_{t=t_1}^{t_{50}} (p_2(t) - y_n(t))^2}{50 - (n+1)} \cdot \tag{17}$$

Of each ten fitted equations that with the least estimated standard error, $y_i(t)$, was taken to express the concentration of Bz-Arg-OH as a function of time; that is, $p_2(t) = y_i(t)$.

The value of the kinetic parameters for plasmin catalysed hydrolysis of Bz-Arg-OEt, 25° C, pH 7.8, are [1]: $k_c = 12\ s^{-1}$; $k_2 \geqslant 564\ s^{-1}$; $k_3 = 12\ s^{-1}$; $K_s = 12$ mM and $K_m = 0.25$ mM. During the course of these experiments the concentration of Bz-Arg-OEt remained in the range 0.667—0.567 mM and therefore (Eqns. 14 and 15)

$$f_3 = [1 + 0.25/(0.667 - p_2(t))]/12\ s \tag{18}$$

That is, $0.115\ s \leqslant f_3 \leqslant 0.120\ s$. Further, $f_2 = [1 + K_s/s(t) + k_3/k_1 s(t)]/[k_2 k_3]$, and therefore since $[1/(k_1 k_2 s(t)] > 0$

$$f_2 \leqslant (1 + K_s/s(t))/k_2 k_3 \leqslant 3.3 \cdot 10^{-3} s^2 = \phi_2 \tag{19}$$

and

$$f_1 = 1/k_1 k_2 k_3 s(t) < K_s/k_2^2 k_3 s(t) \leqslant 5.5 \cdot 10^{-6}\ s^3 = \phi_1. \tag{20}$$

The functions $f_3 \dot{y}_i(t)$, $\phi_2 y_i(t)$ and $\phi_1 y_i(t)$ were computed at $t = t_1, t2 \ldots t_{50}$. In all cases the values of $\phi_2 \dot{y}_i(t)$ and $\phi_1 \ddot{y}_i(t)$ were negligibly small (if not zero)

compared to the corresponding value of $f_3\dot{y}_i(t)$, so that (Eqn. 15)

$$e_0(t) = f_3\dot{y}_i(t). \tag{21}$$

The initial velocity of reaction I was then estimated from the slope of the first part of the curve $e_0(t)$ plotted against t.

Results and Discussion

Initial velocities were determined at 20 concentrations of NH_2-terminal lysine plasminogen in the range 0.9—20 μM. Three urokinase concentrations, 0.71 nM, 1.19 nM and 1.59 nM were used. The reciprocal of the initial velocity at unit urokinase concentration, $(v_1/[UK])^{-1}$s, is plotted against the reciprocal of plasminogen concentration in Fig. 1. This Lineweaver-Burk plot indicates that the simple steady-state rate equation (Eqn. 1) is obeyed. The values of the kinetic parameters obtained from a fit to Eqn. 1 [9] are $k_{c,1} = 2.59 \pm 0.31\ s^{-1}$, $K_{m,1} = 40.7 \pm 6.2\ \mu M$ and $k_{c,1}/K_{m,1} = 6.36 \cdot 10^4 \pm 0.24 \cdot 10^4\ M^{-1} \cdot s^{-1}$.

If the reaction I is assumed to occur according to the rate expression (Eqn. 1)

$$v_1 = k_{c,1}[UK]/(1 + K_{m,1}/s_1) \tag{22}$$

where s_1 is the concentration of plasminogen and [UK] is the concentration of urokinase, then the expected time course of the overall reaction may be calculated. If only the initial part of the reaction, for which s_1 and thus also v_1 are approximately constant, is considered the plasmin concentration becomes

$$e_0(t) = v_1 t. \tag{23}$$

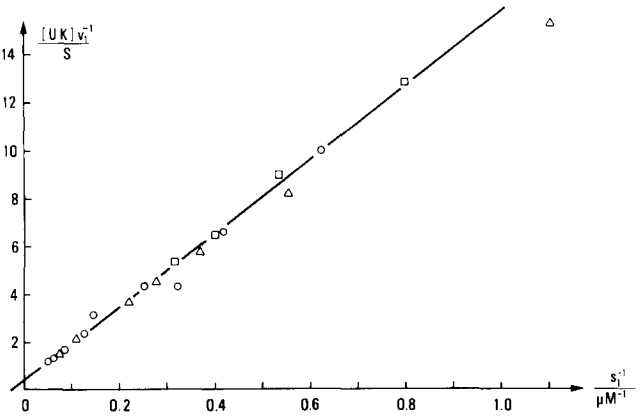

Fig. 1. Lineweaver-Burk plot of the urokinase catalysed formation of plasmin from NH_2-terminal lysine plasminogen. Ordinate: reciprocal of initial velocity at unit urokinase concentration, $(v_1/[UK])^{-1}$/s. It is assumed that 1 mole urokinase = $4.2 \cdot 10^{12}$ Ploug units [2—4]. Abscissa: reciprocal of concentration of NH_2-terminal lysine plasminogen, $s_1^{-1}/\mu M^{-1}$. Experimental conditions: buffer 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8, 25°C. Urokinase concentrations were: 0.71 nM (△———△); 1.19 nM (○———○); and 1.59 nM (□———□).

The solution to Eqn. 15 is then

$$v = \dot{p}_2(t) = (v_1/f_3)(t - f_2/f_3). \tag{24}$$

From Eqn. 24

$$p_2(t) = (v_1/f_3)(t^2/2 - f_2 t/f_3). \tag{25}$$

Since $f_2/f_3 < 3 \cdot 10^{-2}$ s (Eqns. 18, 19) only the $t^2/2$ term is significant for $t \geqslant 10$ s, so that

$$p_2(t) = v_1 t^2/2f_3 = \frac{k_{c,1}[\mathrm{UK}]t^2}{(1 + K_{m,1}/s_1)2f_3} \tag{26}$$

In all cases the experimental time course of the overall reaction and that calculated from Eqn. 26 using the values $k_{c,1}$ = 2.59 s and $K_{m,1}$ = 40.7 μM, were found to agree well. One of these is illustrated in Fig. 2, in which the concentration of Bz-Arg-OH, $p_2(t)$, obtained experimentally, theoretically (Eqn. 26), and from the fitted polynomial is plotted against time. The initial concentration of plasminogen was 3.13 μM, the concentration of urokinase was

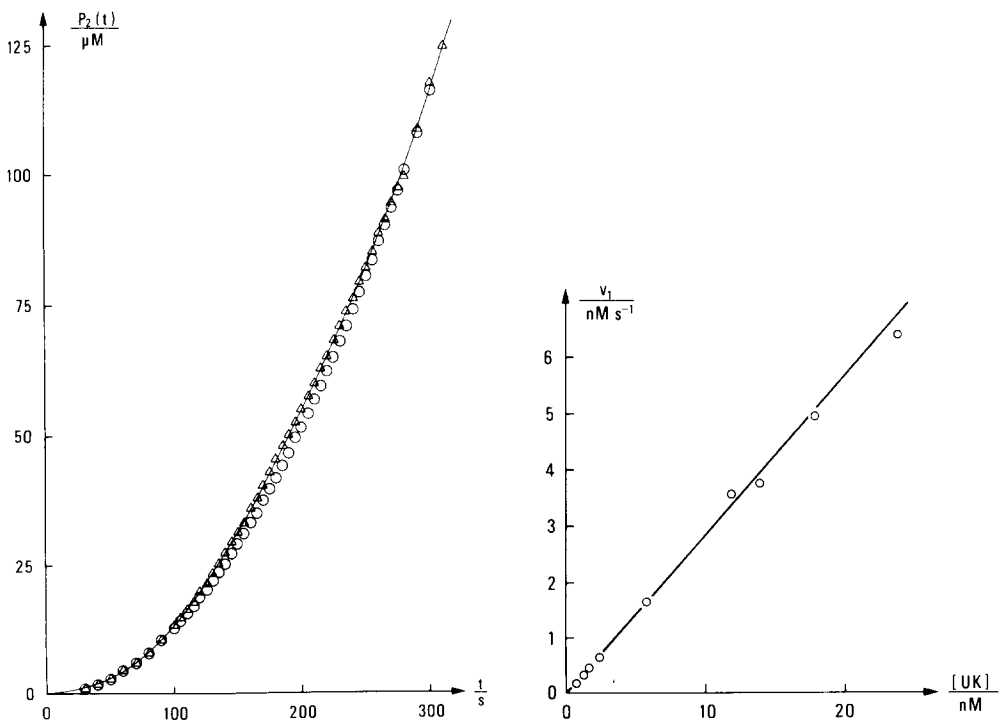

Fig. 2. The time course of the overall reaction obtained in an experiment with an initial concentration of NH_2-terminal lysine plasminogen of 3.13 μM and a concentration of urokinase of 1.59 nM. Ordinate: concentration of Bz-Arg-OH, $p_2(t)/\mu$M, obtained experimentally (△———△); theoretically (Eqn. 26) (○———○); and given by the fitted polynomial, $y_i(t)$, (Eqns. 16, 17) (———). Abscissa: time, t/s.

Fig. 3. (○———○), the initial velocity, v_1/nM s^{-1}, plotted against the urokinase concentration, [UK]/nM. Initial concentration of NH_2-terminal lysine plasminogen 5.0 μM. The line shown is that obtained from a calculation using Eqn. 22.

1.59 nM, and the measuring period was ≈300 s so that the decrease in plasminogen concentration during the experiment was approximately $e_o(300) \approx 90$ nM. Since this is less than 3% of the initial concentration, the assumption that v_1 does not decrease during the experiment should be fulfilled. The applied in all of the experiments.

The dependence of the initial velocity of reaction I on the urokinase concentration was investigated at a concentration of plasminogen of 5 μM. The result is in reasonable accordance with Eqn. 22. The measured initial velocities are on the whole proportional to the urokinase concentration (Fig. 3). At high urokinase concentrations a tendency for them to diverge from linearity would be expected, since the determination of initial velocity is based upon measurements made at least 5—10 s after initiation of the reaction, and at higher enzyme concentrations a substantial amount of the substrate could be converted into product in that time. Further the steady-state hypothesis may not be satisfied if the ratio of substrate to enzyme concentration is much less than 10^3.

From Eqn. 25, with which the experimental results agree,

$$v_1 = p_2(t)f_3/(t^2/2 - f_2 t/f_3). \tag{27}$$

The initial velocity of reaction I as determined from the time course of the overall reaction should be given by Eqn. 27 if the rate does not decrease perceptibly during the measurement. Provided that the kinetics of the coupled reaction (reaction II) are well-known the equation should be generally applicable to the study of the kinetics of reactions in which an enzyme is formed from a proenzyme.

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