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

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Summary

Initial velocities for the urokinase (EC 3.4.99.26)-catalysed conversion of glutamic acid plasminogen to plasmin (EC 3.4.21.7) have been determined at various urokinase and glutamic acid plasminogen concentrations. As has been found for the corresponding reaction with lysine plasminogen this conversion obeys the Michaelis rate equation. The apparent Michaelis constants are of the same order of magnitude for lysine and glutamic acid plasminogens. The difference in conversion rates for the reactions has been shown to be connected with their having different catalytic constants. The data were analysed according to two reaction schemes, in one of which only one peptide bond is split during the glutamic acid plasminogen-plasmin conversion and in the other of which the cleavage of two peptide bonds with the obligatory formation of an intermediate plasminogen is assumed. The results favour the former.

Introduction

It is generally accepted that in the urokinase (EC 3.4.99.26)-catalysed conversion of human NH_2-terminal glutamic acid plasminogen (Glu-plasminogen) to plasmin (EC 3.4.21.7) at least one peptide bond in the COOH-terminal part of the molecule, is cleaved. There is disagreement, however, as to whether this is the only cleavage essential for plasmin formation. Some authors [1—3] have described the formation of a modified plasminogen prior to the appearance of plasmin in the time course of the conversion. This led to the suggestion that the process occurs in two sequential steps in which urokinase first splits off a peptide from the NH_2-terminal part of the plasminogen molecule to form a modified plasminogen and thereafter cleaves the arginyl-valyl bond to form plasmin. Others [4,5] have suggested that plasmin itself is responsible for the formation of the modified plasminogen and recently a plasmin with no loss of peptide

compared to Glu-plasminogen has been reported to be the product of urokinase-catalysed Glu-plasminogen conversion conducted in the presence of the plasmin inhibitor trasylol [4]. The conversion of Glu-plasminogen to modified plasminogen is accompanied by major changes in the conformation of the protein [6,7]. Conformational changes are also induced when Glu-plasminogen binds various ligands such as L-lysine, 6-aminohexanoic acid and *trans*-4-aminomethyl-cyclohexane-1-carboxylic acid [6—9]. If these conformationally altered molecules are subsequently converted to plasmin by urokinase the rates are drastically increased compared with that observed for the corresponding conversion of the unmodified protein [3,5,7,9,12].

Some advocates of the two-step sequential mechanism for Glu-plasminogen conversion to plasmin have suggested that the first step is rate determining [10]; others [3] believe that the second step fills this role. To explain that the overall reaction is slow compared to the conversion of modified plasminogen, which presumably is the second step of the overall reaction, it is suggested that the first step is rate determining; on the other hand it seems necessary to assume a rate-determining second step to account for the presence of large amounts of modified plasminogen during the reaction. Clearly a better understanding of the urokinase-catalysed Glu-plasminogen-plasmin conversion is required to resolve these apparently conflicting conclusions. Consequently kinetic studies of the process have been undertaken and are described here. The kinetics of the urokinase-catalysed conversion of lysine plasminogen (Lys-plasminogen) have been dealt with previously [11]. One of the specific aims of this study was to investigate whether urokinase binds Glu-plasminogen much stronger than it does Lys-plasminogen. The problem mentioned above could be resolved in this case since only a very limited amount of urokinase would be free to react with the modified plasminogen as long as most of the Glu-plasminogen was present. The results obtained show, however, that the binding characteristics are approximately the same for the two plasminogens, and that the difference in conversion rate is determined by the rate(s) of the ensuing reaction step(s).

Materials and Methods

Human urokinase. "Reagent" preparations from Leo, Denmark containing 10 000 Ploug units/mg [12]. 1 mol of urokinase is approx. $4.2 \cdot 10^{12}$ Ploug units [13].

Human plasminogens. Batches of human Glu- and Lys-plasminogen were prepared from 700 to 900 ml of stored human plasma. The plasminogens were purified at 4° C in a four step procedure: (1) affinity chromatography in 0.1 M phosphate buffer, pH 7.6, on a lysine-Sepharose column from which the proteins were eluted with a solution of 0.01 M 6-aminohexanoic acid in the same buffer [14]; (2) gel filtration in 0.05 M Tris · HCl buffer, pH 8.0, on Sephadex G-25; (3) ion-exchange chromatography on DEAE-Sephadex A-50, from which the proteins were eluted with a linear gradient of NaCl obtained from a solution of 0.3 M NaCl, 0.05 M Tris · HCl buffer, pH 8.0, and the equilibration buffer 0.05 M Tris · HCl, pH 8.0. Glu-plasminogen and Lys-plasminogen were eluted at I = 0.14 and I = 0.23, respectively; (4) dialysis/ultrafiltration against

0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8, in an Amicon stirred cell with a diaflo PM10 membrane until the preparations were suitably concentrated ($c \approx 20$—50 μM). The Lys-plasminogen preparations sometimes contained traces of plasmin. This impurity was removed by affinity chromatography on a trasylol-Sepharose column. The plasminogen preparations, which were stored at —20° C, all showed a single peak against both monospecific rabbit anti-plasminogen and rabbit anti-human serum (Dacopatts, Copenhagen) in crossed immuno electrophoresis performed as described by Ganrot [15]. NH_2-terminal amino acids were determined by the method of Gros and Labouesse [16].

Determination of plasminogen concentrations. In reaction mixtures consisting of 120 nM urokinase (500 Ploug units/ml), approx. 20 μM plasminogen, 0.05 M Tris · HCl, 0.1 M NaCl, 0.1 M L-lysine and 25% (v/v) glycerol, pH 7.8, 25° C, the concentration of plasmin formed after 2 h was determined by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate [17] and with trasylol using α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) as the plasmin substrate [18]. The results of titrations of aliquots removed from the reaction mixtures at different times showed that the plasminogen-plasmin conversion was complete after 2 h and that the concentration of plasmin remained constant for at least a further 24 h in these reaction mixtures. The concentration of plasminogen to be determined was therefore taken to be equal to the concentration of plasmin determined after 2 h of reaction. There never were difference between the results of the two titration methods.

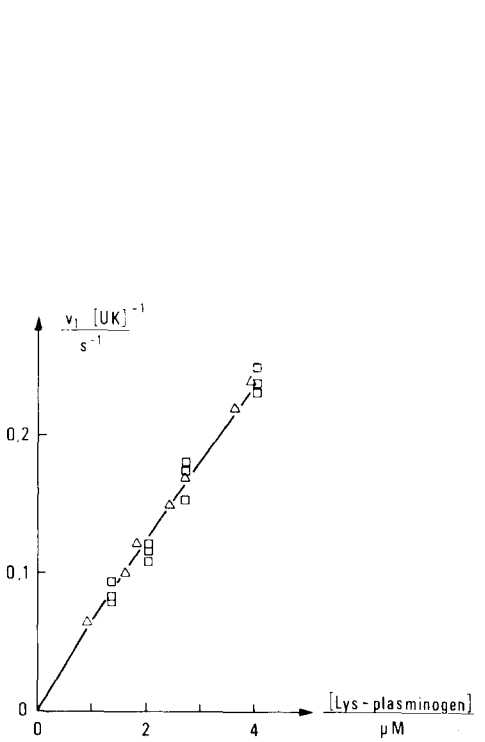
The kinetic experiments were performed and analysed in the manner described earlier [11]. The method is based upon the facts that plasmin catalyses the hydrolysis of Bz-Arg-OEt and that this is a well-characterized process. In a solution containing urokinase, plasminogen and Bz-Arg-OEt, plasminogen is converted to plasmin, which subsequently hydrolyses Bz-Arg-OEt. The amount of Bz-Arg-OH produced is recorded as a function of time and analysed to the corresponding rate of plasmin formation [11]. Pure Glu-plasminogen, pure Lys-plasminogen and mixtures of Glu- and Lys-plasminogen of various known concentrations were used as substrates for urokinase. The influence of the presence of 0.1 M L-lysine on these reactions was also studied. Most experiments were performed in triplicate under conditions in which the ratio of the concentration of urokinase to the concentration of plasminogen(s) did not exceed 10^{-3}. This is generally accepted as a necessary requirement in satisfying the assumptions of the steady-state hypothesis in enzyme kinetics.

Results and Discussion

Urokinase is ane enzyme and its reaction with Lys-plasminogen is a normal saturation kinetic process. The Michaelis equation (Eqn. 1) is therefore obeyed.

$$v_1 = \frac{k_l[\text{urokinase}][\text{Lys-plasminogen}]}{K_l + [\text{Lys-plasminogen}]}$$

v_1 is the verlocity of the reaction, k_l (= 2.6 s^{-1}) is the catalytic constant, and K_l (= 40 μM) is the apparent Michaelis constant [11]. Fig. 1 shows that the same initial velocities of urokinase-catalysed conversion of Lys-plasminogen were obtained both with and without 0.1 M L-lysine present in the reaction mix-



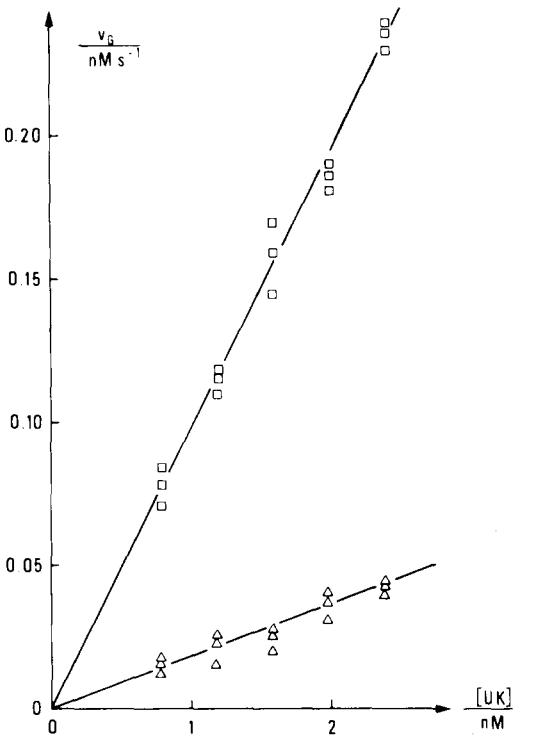


Fig. 1. The initial velocity for the conversion of Lys-plasminogen at unit concentration of urokinase (v_l / [UK] s^{-1}) plotted against the concentration of Lys-plasminogen (μM). Experimental conditions: buffer 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8, 25°C. △, no L-lysine; □, with 0.1 M L-lysine. The curve is drawn through points calculated using Eqn. 1 with k_l = 2.6 s^{-1} and K_l = 40.7 μM.

Fig. 2. The initial velocity for the conversion of Glu-plasminogen, (v_g, nM s^{-1}), plotted against the concentration of urokinase (nM). Experimental conditions: buffer 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8, 25°C; concentration of Glu-plasminogen: 2.4 μM. △, no L-lysine; □, with 0.1 M L-lysine.

tures. The results of the experiments with Glu-plasminogen as urokinase substrate, also both with and without 0.1 M L-lysine present, are illustrated in Figs. 2 and 3. Fig. 2 shows the relationships between the initial velocities and the concentrations of urokinase at fixed Glu-plasminogen concentration. These are both linear. Fig. 3 shows the dependences of the initial velocities upon the concentrations of Glu-plasminogen at unit urokinase concentration. In each case there is a deviation from a linear relationship. These curves might be sections of rectangular hyperbolae corresponding to the Michaelis rate equation. Incipient saturation of the urokinase apparently occurs at a Glu-plasminogen concentration of approx. 8 μM. The initial velocities at unit urokinase concentration were fitted to the Michaelis equation using a modified version of the programme given by Cleland [19]. The values of the kinetic parameters obtained, which are given in Table I, are rather poorly estimated. The absorbance of Glu-plasminogen at 253 nm, the wavelength at which the measurements were made, prohibits the use of concentrations of Glu-plasminogen high enough to give more accurate values. The results do show, however, that the apparent Michaelis constant for the conversion of Glu-plasminogen by urokinase is of the same

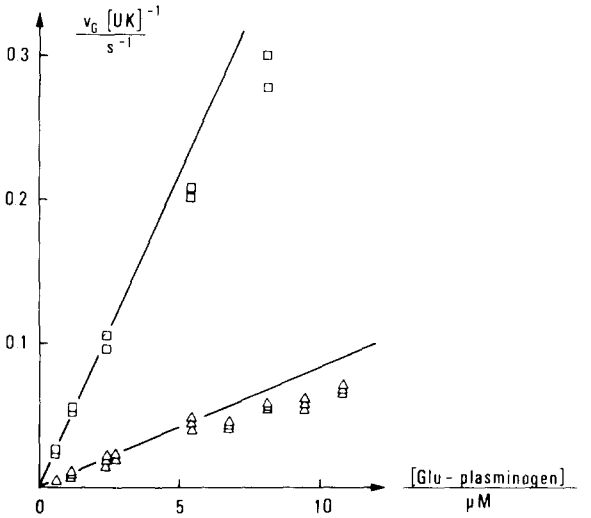


Fig. 3. The initial velocity for the conversion of Glu-plasminogen at unit concentration of urokinase (v_g/[UK] s^{-1}) plotted against the concentration of Glu-plasminogen (μM). Experimental conditions: buffer 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.8, 25°C. △, no L-lysine; □, with 0.1 M L-lysine. The lines were obtained from v_g/[UK] = ($k_c/K_{m,app.}$) × [Glu-plasminogen] (Table I).

order of magnitude as that for the conversion of Lys-plasminogen; that is, that saturation of urokinase with Glu-plasminogen does not occur at much lower concentrations that it does with Lys-plasminogen. Since the presence of 0.1 M L-lysine does not change this the marked increase of the verlocity which occurs when it is added can hardly be due to significant changes in the conditions of the binding of Glu-plasminogen to urokinase. Rather the rate(s) of the ensuing step(s) of the reaction must increase. This corresponds to an increase of the catalytic constant. The initial velocities obtained for plasmin formation in reaction mixtures containing both Glu- and Lys-plasminogens are given in Table II. The concentrations used were small compared to the apparent Michaelis constants, but of the same order of magnitude as the physiological concentration of plasminogen (≈2.2 μM) [20,21]. Under these conditions the initial velocities are seen to be equal to the sums of those obtained for pure Lys-plasminogen and pure Glu-plasminogen each measured at the same concentration as it occurs in the mixtures. These are thus no indications of inhibition of any of the reactions by the other substrate.

TABLE I

KINETIC PARAMETERS FOR UROKINASE-CATALYSED PLASMIN FORMATION

Substrate	The catalytic constant (k_c, s^{-1})	The apparent Michaelis constant ($K_{m,\,app.}$, μM)	$k_c/K_{m,\,app.}$ ($M^{-1} \cdot s^{-1}$)
Glu-plasminogen	$k_g = 0.26 \pm 0.07$	$K_g = 32 \pm 11$	$(8.3 \pm 0.5) \cdot 10^3$
Glu-plasminogen, 0.1 M L-lysine	$k'_g = 1.5 \pm 0.3$	$K'_g = 35 \pm 8$	$(4.4 \pm 0.2) \cdot 10^4$
Lys-plasminogen [11]	$k_l = 2.6 \pm 0.3$	$K_l = 40 \pm 6$	$(6.3 \pm 0.2) \cdot 10^4$

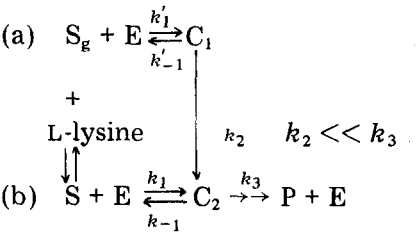
TABLE II

INITIAL VELOCITIES (v, nM/s) OBTAINED AT A CONCENTRATION OF UROKINASE (1.59 nM) USING MIXTURES OF GLU- AND LYS-PLASMINOGEN AS SUBSTRATE

Concentration of Lys-plasminogen (μM)	Concentration of Glu-plasminogen (μM)		
	0	1.2	2.4
0	—	0.015 ± 0.002	0.029 ± 0.003
0.55	0.055 ± 0.003	0.071 ± 0.004	0.085 ± 0.003
1.1	0.106 ± 0.004	0.123 ± 0.002	0.137 ± 0.004

In this study initial velocities of the conversion of plasminogen(s) to plasmin have been determined; only very small amounts of plasmin were formed in the measuring periods and excess of Bz-Arg-OEt, a plasmin substrate, was present. It is therefore assumed in the following analysis that the effect of plasmin on the plasminogen(s) has no significant influence on the conversion reactions. A reaction scheme which is consistent with the results is

Scheme I



S_g is Glu-plasminogen, S is L-lysine-modified Glu-plasminogen or Lys-plasminogen, E is the enzyme, urokinase, the C_i data are urokinase-plasminogen complexes, P is plasmin and the k_i values are rate constants. According to this scheme the conversion of Glu-plasminogen and L-lysine-modified Glu-plasminogen (and Lys-plasminogen) follows two different reaction paths. Binding of Glu-plasminogen to urokinase leads to the formation of the enzyme-substrate complex, C_1, which then in a slow reaction step undergoes a conformational change to the complex C_2. The arginyl-valyl bond is subsequently split (probably more than one reaction step) and plasmin is formed. Both L-lysine-modified Glu-plasminogen and Lys-plasminogen have undergone a conformational change before the reaction with urokinase. Thus the slow step $C_1 \rightarrow C_2$ need not be a step in the conversion of these substrates. The steady-state kinetic parameters corresponding to the reactions of Scheme I are:
Path a: For the conversion of S_g with velocity v_g,

$$v_g = k_g[E_0][S_g]/(K_g + [S_g]), \tag{2}$$

the apparent Michaelis constant, $K_g = \dfrac{(k'_{-1} + k_2)k_3}{k'_1(k_2 + k_3)}$ (3)

the catalytic constant, $k_g = \dfrac{k_2 k_3}{k_2 + k_3}$. (4)

It is assumed that $k_2 << k_3$, and therefore that

$K_g = (k'_{-1} + k_2)/k'_1$ and $k_g = k_2$.

Path b: For the conversion of S with velocity v,

$$v = k[E_0][S]/(K + [S]) \quad (5)$$

the apparent Michaelis constant, $K = (k_{-1} + k_3)/k_1$, (6)

the catalytic constant, $k = k_3$. (7)

In both cases the velocity is proportional to the catalytic constant but, in accordance with the experimental results, it is higher when the substrate is a modified plasminogen rather than Glu-plasminogen. According to the reaction scheme the catalytic constant for the L-lysine-influenced Glu-plasminogen conversion should be the same as that for the Lys-plasminogen conversion. Brockway and Castellino [22] report the dissociation constant for the L-lysine-Glu-plasminogen complex to be $K_i = 0.068$ M. At a concentration of L-lysine of 0.1 M about 68% of the Glu-plasminogen present should therefore be complexed with L-lysine, so that the expected value of the catalytic constant is 1.8 s^{-1}. This agrees well with the value actually determined (Table I). The assay system seems not to be influenced by 0.1 M L-lysine (Fig. 1).

The proposed model can account for the observation that the apparent Michaelis constants are all of the same order of magnitude in one or more of a number of ways. This is so for example, if $k'_{-1}/k'_1 = k_{-1}/k_1$ and $k_3 << k_{-1}$. Reaction Scheme I contains only one proteolytic step, so that it therefore assumes that plasmin formed from Glu-plasminogen directly possesses a NH_2-terminal glutamic acid residue. This is in accordance with recently published results [4].

A mechanism which has often been proposed for the urokinase-catalysed conversion of Glu-plasminogen involves the cleavage of two peptide bonds in a specific order, so that a modified plasminogen is formed and accumulated as an intermediate [2,3]. Reaction Scheme II represents the simplest possible model for such a mechanism.

Scheme II

(a) $S_g + E \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} C_1 \xrightarrow{k'_2} S + E$

(b) $S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C_2 \xrightarrow{k_2} P + E.$

According to this model the initial velocity for the formation of plasmin would not normally follow the Michaelis rate equation. The concentration of the substrate for Reaction b is zero when the reaction is initiated. The overall reaction must therefore pass through an acceleration phase, a steady-state phase and a deceleration phase until it finally stops when all Glu-plasminogen has been converted to plasmin. If the acceleration phase is very short and is followed by a general steady-state phase in which the rate of formation of modified plasminogen in Reaction a and the rate of plasmin formation in Reaction b are equal, and if rate measurements are made when the system is in this condition, then

the results will obey the Michaelis rate equation. Writing $i(t)$ for the concentration of the I'th component at time t and v_a and v_b as the velocities of the first and the second reaction step, respectively, the rate equation for the overall reaction may be derived as follows:

$$e(0) = e(t) + c_1(t) + c_2(t) = c_1(t)[e(t)/c_1(t) + 1 + c_2(t)/c_1(t)] = c_2(t)[e(t)/c_2(t) + c_1(t)/c_2(t) + 1]. \quad (8)$$

In steady-state conditions

$$d(c_1(t))/dt = 0 = k'_1 s_g(t) e(t) - (k'_{-1} + k'_2) c_1(t), \quad (9)$$

$$d(c_2(t))/dt = 0 = k_1 s(t) e(t) - (k_{-1} + k_2) c_2(t) \quad (10)$$

and

$$d(s(t))/dt = 0 = k'_2 c_1(t) - k_1 s(t) e(t) + k_{-1} c_2(t). \quad (11)$$

Since

$$v_a = k'_2 c_1(t) = v_b = k_2 c_2(t) \quad (12)$$

$$v_b = \frac{k_2 e(0)}{1 + k_2/k'_2 + K_b/s(t)} = \frac{k'_2 e(0)}{1 + k'_2/k_2 + K_a/s_g(t)} = v_a \quad (13)$$

where $K_b = (k'_{-1} + k'_2)/k'_1$ and $K_a = (k_{-1} + k_2)/k_1$.

Eqn. 13 may also be written:

$$v = \frac{k_2 k'_2/(k_2 + k'_2) e(0)}{1 + K_b k'_2/[(k_2 + k'_2) s(t)]} = \frac{k'_2 k_2/(k_2 + k'_2) e(0)}{1 + K_a K_2/[(k_2 + k'_2) s_g(t)]} \quad (14)$$

leading to

$$s(t)/s_g(t) = K_b k'_2/K_a k_2. \quad (15)$$

Since $s_g(0) = s_g(t) + s(t) + p(t)$ (16)

and therefore (Eqns. 15 and 16), since

$$s_g(0) - p(t) = s(t)(1 + K_a k_2/K_b k'_2), \quad (17)$$

the final steady-state rate equation is (Eqns. 13 and 17)

$$v = \frac{k_2 k'_2/(k_2 + k'_2) e(0)}{1 + [K_b k'_2 + K_a k_2]/[(k_2 + k'_2)(s_g(0) - p(t))]}. \quad (18)$$

This is a Michaelis rate equation in which the catalytic constant is

$$k_c = k_2 k'_2/(k_2 + k'_2) \quad (19)$$

and the apparent Michaelis constant is

$$K_{m,app} = (K_b k'_2 + K_a k_2)/(k_2 + k'_2) = k_c(K_b/2 + K_a/k'_2). \quad (20)$$

From Eqn. 15

$$\frac{s(t)}{s(t) + s_g(t)} = \frac{K_b k'_2}{K_b k'_2 + K_a k_2}, \quad (21)$$

indicating that a large fraction of the plasminogen present will be modified plasminogen only if

$$K_a/k'_2 << K_b/k_2. \tag{22}$$

In this case the velocity of the overall reaction is less than that which Reaction b would have at the same substrate and enzyme concentrations. This is seen by noting that, for the overall reaction (Eqn. 19)

$$k_c = k_2k'_2/(k_2 + k'_2) = k_2/(1 + k_2/k'_2) = ak_2, \quad a < 1 \tag{23}$$

so that (Eqns. 20 and 22)

$$K_{m,app} = [K_b/k_2 + K_a/k'_2]k_c \approx k_cK_b/k_2 = aK_b, \quad a < 1 \tag{24}$$

and (Eqn. 18)

$$v = ak_2e(0)/[1 + aK_b/(s_g(0) - p(t))]$$

For Reaction b

$$v_b = k_2e(0)/[1 + K_b/(s(0) - p'(t))] \tag{25}$$

Hence

$$v_b/v = \frac{1 + aK_b/(s_g(0) - p(t))}{a + aK_b/(s(0) - p'(t))} \tag{26}$$

where $s_g(0) = s(0)$. Regarding only the initial parts of the reactions, where $p'(t) << s_g(0)$, the ratio becomes

$$v_b/v = \frac{1 + aK_b/s(0)}{a + aK_b/s(0)} > 1, \text{ since } a < 1. \tag{27}$$

Thus it may be concluded that if K_a/k'_2 is very much smaller than K_b/k_2 the velocity of the overall reaction will be smaller than the velocity of Reaction b alone and that modified plasminogen will be accumulated in the overall reaction.

The rate measurements described above give

$$K_{m,app.}/k_c = K_b/k_2 + K_a/k'_2 = K_g/k_g = 123\ \mu M\ s^{-1}$$

and

$$K_b/k_2 = K_I/K_I = 15.3\ \mu M\ s^{-1},$$

so that
$(K_a/k'_2)/(K_b/k_2) = 7$; which is not "very much smaller than" unity. Further the values of the kinetic parameters for Reaction a calculated from Eqns. 19 and 20 using the values of Table I, are $k'_2 = 0.29\ s^{-1}$ and $K_a = 31\ \mu M$. Consequently the maximum rate at which Glu-plasminogen can be transformed to modified plasminogen with $s_g(0) \approx 2\ \mu M$ and $e(0) \approx 1$ nM is

$$v_a(t \to 0) = k'_2e(0)/[1 + K_a/s_g(0)] < 2 \cdot 10^{-5}\ \mu M\ s^{-1}.$$

In steady state (Eqns. 15 and 16)

$$s(t)/s_g(t) = 1/7 \leqslant s(t)/(s_g(0) - s(t))$$

so that

$s(t) \geqslant 0.13\, s_g(0) \approx 0.26\ \mu M.$

It would take at least

$t = 0.26/2 \cdot 10^{-5}$ s = 3.6 h to establish this steady state.

Since the data indicate that steady state is not reached very fast if the reaction proceeds according to Scheme II, and since the experimentally determined velocities are constant in the time interval in which the measurements were made, a mechanism for urokinase-catalysed Glu-plasminogen-plasmin conversion in which one peptide bond is split and in which the occurrence, if any, of modified plasminogen is due either to plasminolysis or to secondary urokinase-catalysed hydrolysis, is favoured. Scheme I describes such a mechanism.

The values of the apparent Michaelis constants (Table I) are large compared to the concentration of plasminogen in human blood plasma (≈2.2 μM [20, 21]). This means that at physiological concentrations of plasminogen the capacity of urokinase is poorly utilized, only 5—6% of that present taking part in the reaction at any given time $[(1 + K_g/s_g)^{-1} \approx 0.05—0.06]$. This may be an indication either that urokinase is not a very important physiological activator of plasminogen and/or that the conversion reaction does not normally occur in plasma solution, but on fibrin surfaces where the conditions (the concentrations for example) might be quite different.

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