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Zymogen-activation kinetics

Modulatory effects of *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid and poly-D-lysine on plasminogen activation

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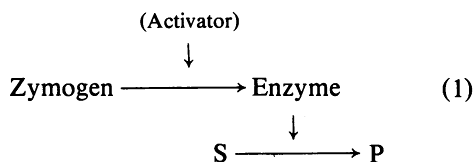
The kinetics of plasminogen activation catalysed by urokinase and tissue-type plasminogen activator were investigated. Kinetic measurements are performed by means of a specific chromogenic peptide substrate for plasmin, D-valyl-L-leucyl-L-lysine 4-nitroanilide. Two methods are proposed for the analysis of the resulting progress curve of nitroaniline formation in terms of zymogen-activation kinetics: (1) a graphical transformation of the parabolic curve and (2) transformation of the curve for nitroaniline production into a linear progress curve by the addition of a specific inhibitor of plasmin, bovine pancreatic trypsin inhibitor. The two methods give similar results, suggesting that the reaction between activator and plasminogen is a simple second-order reaction at least at plasminogen concentrations up to about 10 μM. The kinetics of both Glu_1-plasminogen (residues 1–790) and Lys_{77}-plasminogen (residues 77–790) activation were investigated. The results confirm previous observations showing that trans-4-(aminomethyl)cyclohexane-1-carboxylic acid at relatively low concentrations enhances the activation rate of Glu_1-plasminogen but not that of Lys_{77}-plasminogen. At higher concentrations both Glu_1- and Lys_{77}-plasminogen activation are inhibited. The concentration interval for the inhibition of urokinase-catalysed reactions is shown to be very different from that of the tissue-plasminogen activator system. Evidence is presented indicating that (i) binding to the active site of urokinase ($K_D = 2.0$ mM) is responsible for the inhibition of the urokinase system, (ii) binding to the active site of tissue-plasminogen activator is approx. 100-fold weaker, and (iii) inhibition of the tissue-plasminogen activator system, when monitored by plasmin activity, is mainly due to plasmin inhibition. Poly-D-lysine (M_r 160000) causes a marked enhancement of plasminogen activation catalysed by tissue-plasminogen activator but not by urokinase. Bell-shaped curves of enhancement as a function of the logarithm of poly-D-lysine concentration are obtained for both Glu_1- and Lys_{77}-plasminogen activation, with a maximal effect at about 10 mg/litre. The enhancement of Glu_1-plasminogen activation exerted by *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid is additive to that of poly-D-lysine, whereas poly-D-lysine-induced enhancement of Lys_{77}-plasminogen activation is abolished by *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid. Analogies are drawn up between the effector functions of poly-D-lysine and fibrin on the catalytic activity of tissue-plasminogen activator.

Abbreviations used: Val-Leu-Lys-Nan, D-valyl-L-leucyl-L-lysine 4-nitroanilide; <Glu-Gly-Arg-Nan, L-pyroglutamylglycyl-L-arginine 4-nitroanilide; Ile-Pro-Arg-Nan, D-isoleucine-L-prolyl-L-arginine 4-nitroanilide; *t*-AMCA, *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid; e.l.i.s.a., enzyme-linked immunosorbent assay.

A zymogen-activation cascade is essential for the physiological response of many biological systems to external stimuli. Each step of the cascade (organized as described in eqn. 1) provides an amplification unit in the sense that the catalytic activity of a single activator molecule results in production of several enzyme molecules with new catalytic activity:



(1)

The fibrinolytic process is built up in this way. The physiological response of the system is the proteolytic degradation of fibrin (S) to soluble fragments (P). This reaction is catalysed by plasmin (EC 3.4.21.4). Detailed information about the molecular properties of plasmin, its zymogen (plasminogen), and the action of plasmin on fibrin is now available (see, e.g., Collen, 1980; Castellino, 1981). The activation of plasminogen is catalysed by urokinase (EC 3.4.21.31) or tissue-type plasminogen activator (EC 3.4.21.-) and considerable progress in the description of the molecular structures and properties of these activators has been reported (Steffens *et al.*, 1982; Pennica *et al.*, 1983). In spite of such knowledge, the regulatory mechanisms involved in the fibrinolytic system remain to be elucidated. The physiological stimuli and the mechanism by which they trigger the fibrinolytic system are not known. The exact mechanism by which the activator activity is turned on and off is an unsolved problem. Likewise the regulatory control of each of the subsequent steps in the fibrinolytic system needs clarification.

It is known that fibrin is of central importance for the regulatory control of fibrinolysis in general (Camiolo *et al.*, 1971; Wallén, 1978; Hoylaerts *et al.*, 1982; Rijken *et al.*, 1982; Rånby, 1982; Suenson *et al.*, 1984). However, an interpretation of the results is complicated by the fact that fibrin is an insoluble polymer that may be rapidly degraded by plasmin during the course of the experiments designed to investigate its modulatory effect.

Non-physiological polycations like poly-lysine and polyornithine (Allen, 1982), and anti-fibrinolytic acids like *t*-AMCA, 6-aminohexanoic acid and lysine (see, e.g., Castellino, 1981) have been reported to induce modulatory effects on the plasminogen activation reaction with some resemblance to those induced by fibrin. The aim of the present study was to investigate the effect of these molecules on the plasminogen activation kinetics in order to study the regulatory mechanism of the fibrinolytic process in a model system where the experimental complication associated with fibrin are avoided.

Materials and methods

Val-Leu-Lys-Nan,2HCl (substrate S-2251), <Glu-Gly-Arg-Nan,HCl (substrate S-2444), Ile-Pro-Arg-Nan,2HCl (substrate S-2288) and *t*-AMCA were from Kabi (Stockholm, Sweden). Bovine pancreatic trypsin inhibitor (Trasylol) was from Bayer (Leverkusen, Germany) and poly-D-lysine (M_r 160000) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Human Glu_1-plasminogen (residues 1–790) and Lys_{77}-plasminogen (mainly residues 77–790, but with minor amounts of residues 78–790 and 68–790) were prepared as previously described (Thorsen *et al.*, 1981). Low-M_r urokinase was a gift from Dr. B. Åsted (Department of Gynecology and Obstetrics, University of Lund, Allmänna Sjukhuset, Malmö, Sweden). Tissue-type plasminogen activator was prepared from culture medium of human melanoma cells by immunoadsorption to a monoclonal antibody as previously described (Selmer *et al.*, 1983).

The concentrations of urokinase and plasmin were determined from their catalytic activities with synthetic peptide substrates by using the kinetic parameters compiled by Lottenberg *et al.*, (1981). The concentration of tissues plasminogen activator was determined by means of e.l.i.s.a. (Brender & Selmer, 1983), with as reference a tissue-plasminogen-activator preparation the concentration of which was determined by amino acid analysis by Dr. L. Sottrup-Jensen (Department of Molecular Biology and Plant Physiology, University of Århus, Denmark).

Measurement of plasminogen activation kinetics were performed in 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.4, with 0.01% Tween 80 at 25°C. The formation of nitroaniline was monitored at 410 nm with a Beckman Acta VI spectrophotometer. The activator activity was determined by either of the two methods described in the Results and discussion section.

Theory

The theory for the steady-state kinetics of the system described in eqn. (1) consisting of activator (A), zymogen (Z) and a specific substrate for the activated enzyme (E) has been described by Christensen & Müllertz (1977) and Kosow (1975). The progress curve for the accumulation of product (P) is described by a parabolic relationship (eqn. 2):

$$p = a_0 \frac{k_a z_0}{K_z + z_0} \cdot \frac{k_e s_0}{K_s + s_0} \cdot \frac{t^2}{2} \qquad (2)$$

where the concentrations of the components P, A, Z and S are indicated by p, a, z and s respectively; t is the time, and K_z and k_a represent K_m and $k_{cat.}$ for the activator-catalysed conversion of Z to E. K_s and k_e represent similar kinetic constants for the conversion of S to P catalysed by E. To obtain eqn. (2) it is necessary to assume that initial-rate conditions apply, so that $z_0 \gg a_0$; $z_0 \gg e$; $s_0 \gg p$. Both urokinase and tissue-plasminogen activator in the absence of fibrin have been reported to have low affinities for plasminogen (Christensen & Müllertz, 1977; Christensen, 1977; Rånby, 1982). This means that $K_z \gg z_0$ under the experimental conditions employed in the present study, and that eqn. (2) simplifies to eqn. (3):

$$p = a_0 z_0 \frac{k_a}{K_z} \cdot \frac{k_e s_0}{K_s + s_0} \cdot \frac{t^2}{2} \tag{3}$$

The reaction mixture may contain small amounts of E and/or P from the beginning of the experiment, in which case the progress curve is described by eqn. (4):

$$p = p_0 + e_0 \frac{k_e s_0}{K_s + s_0} t + a_0 z_0 \frac{k_a}{K_z} \cdot \frac{k_e s_0}{K_s + s_0} \frac{t^2}{2} \tag{4}$$

Plasminogen-activation rates have been obtained from parabolic progress curves by plotting p against t^2 (Kosow, 1975; Drapier *et al.*, 1979).

An alternative graphical procedure involves a plot of $\Delta p/\Delta t$ against time ($\bar{t}$), where $\Delta p/\Delta t$ represents an increase in p, ($\Delta p = p_2 - p_1$) over a given fixed time interval ($\Delta t = t_2 - t_1$) and where $\bar{t}$ is the mean of the time interval ($\bar{t}$) = $(t_2 + t_1)/2\bar{t}$. Insertion in eqn. (4) yields eqn. (5):

$$\frac{\Delta p}{\Delta t} = e_0 \frac{k_e s_0}{K_s + s_0} + a_0 z_0 \frac{k_a}{K_z} \cdot \frac{k_e s_0}{K_s + s_0} \bar{t} \tag{5}$$

$\Delta p/\Delta t$ is proportional to the plasmin activity (concentration) at time $\bar{t}$, and the slope of the plot is proportional to the activator activity.

A linear progress curve in p, the slope of which is proportional to the activator activity, may also be recorded directly when an irreversible inhibitor for the activated enzyme is added to the reaction mixture. In the presence of an activator for the zymogen and an inhibitor (I) for the enzyme the system will reach a steady state:

$$\frac{de}{dt} = \frac{k_a}{K_z} a_0 z_0 - k_i' e\, i_0 = 0 \tag{6}$$

where $(k_a/K_z) a_0 z_0$ is the activation rate, and where e is the steady-state concentrations of activated enzyme; i_0 is the concentration of inhibitor ($i_0 \gg e_t$) and k_i' is the rate constant for the inhibition. $k_i' = k_i[K_s/(K_s + s_0)]$ in the presence of substrate under conditions where competition between inhibitor and substrate exists (Petersen & Clemmensen, 1981). Since e is also proportional to the enzyme activity (eqn. 7):

$$\frac{dp}{dt} = k_e e \frac{s_0}{K_s + s_0} \tag{7}$$

we have eqn. 8:

$$\frac{dp}{dt} = a_0 z_0 \frac{k_a}{K_z} \frac{k_e s_0}{k_i K_s i_0} \tag{8}$$

Results and discussion

Determination of plasminogen-activation rate from progress curves of plasmin-catalysed product formation (method I)

Fig. 1(*a*) shows progress curves of nitroaniline formation in a system, such as that shown in eqn. (1), containing urokinase, Lys$_{77}$-plasminogen and a specific synthetic plasmin substrate, Val-Leu-Lys-Nan. The parabolic curves recorded at five different concentrations of urokinase are shown. A special data-handling technique is required for the analysis of zymogen-activation kinetics, when monitored in this way, preferably the transformation of the parabolic progress curve into a simpler expression of activator activity. Data obtained from the primary curves of Fig. 1(*a*) are plotted as $\Delta p/\Delta t$ against $\bar{t}$ in Fig. 1(*b*). A linear increase in $\Delta p/\Delta t$ (plasmin activity) with time is obtained. It is shown (Fig. 1*b* inset) that the urokinase activity, calculated from the slope of the $\Delta p/\Delta t$-versus-$\bar{t}$ curves, is proportional to the urokinase concentrations. Both the p-versus-t^2 (Kosow, 1975; Drapier *et al.*, 1979) and the $\Delta p/\Delta t$-versus-$\bar{t}$ plot presented here result in transformation of the parabolic progress curve into linear curves. However, the latter is advantageous, as it avoids some of the possible systematic errors associated with the former transformation. These errors include an inaccuracy in the determination of t^2 due to the presence of a lag phase in the activation reaction and compensation for background absorbance due to p_0 or light-scattering. The $\Delta p/\Delta t$-versus-$\bar{t}$ plot provides the additional advantage that $\Delta p/\Delta t$ is directly proportional to the concentration of the product (the activated enzyme), and that the $\Delta p/\Delta t$ curve can be monitored by means of a simple analog circuit or by means of on-line computation.

Determination of plasminogen activation rate from steady-state plasmin concentration in the presence of pancreatic trypsin inhibitor (method II)

Transformation of a parabolic progress curve into a linear one may also be attained by chemical

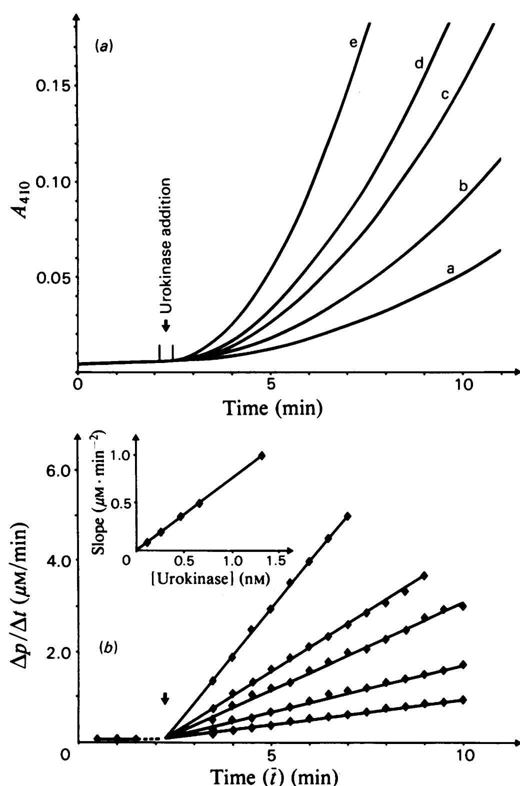


Fig. 1. *Urokinase-catalysed Lys_{77}-plasminogen activation monitored by means of the plasmin activity with Val-Leu-Lys-Nan*

(*a*) Parabolic progress curves of nitroaniline formation measured at 410nm in a system containing 0.31 µM-Lys_{77}-plasminogen and 0.36mM-Val-Leu-Lys-Nan. Plasminogen activation was initiated at the arrow by addition or urokinase. Progress curves for the activation reaction in the presence of a, 0.13nM-, b, 0.26nM-, c, 0.52nM-, d, 0.65nM- and e, 1.3nM-urokinase are recorded. (*b*) $\Delta p/\Delta t$-versus-$\bar{t}$ plots of data obtained from the corresponding progress curves of (*a*). The increase in nitroaniline concentration, Δp, over a given fixed time interval, Δt (1 min) is plotted against the mean value, $\bar{t}$, of this particular time interval. Inset: activator activity as a function of urokinase concentration. The activator activity is obtained from the slope of the curves shown.

means as demonstrated in Fig. 2. This Figure shows the effect on progress curves of nitroaniline formation when various concentrations of pancreatic trypsin inhibitor are added to a system containing urokinase, Lys_{77}-plasminogen and Val-Leu-Lys-Nan. In the presence of a suitable concentration of inhibitor, a constant steady-state level of plasmin is established, as indicated by a linear progress curve. The plasmin activity at that stage, and thus the slope of the curve, is proportional to the urokinase activity. The slopes obtained with 0.4 µM-pancreatic trypsin inhibitor present, and with various fixed concentrations of (i) urokinase, (ii) Lys_{77}-plasminogen or (iii) Val-Leu-Lys-Nan, are shown as the insets to Fig. 2. In accordance with eqn. (8), the slope is proportional to the concentrations of each of these components. Although the simple determination of activator activity from the slope of a linear progress curve is clearly advantageous, the indirect nature of the assay stipulates certain limits to its applicability. The steady-state concentration of plasmin, and hence the rate measured by the assay, decreases with an increase in inhibitor concentration (Fig. 2; eqn. 8). Consequently the sensitivity of the assay is not very high at high concentrations of inhibitor. On the other hand, decreasing the inhibitor concentration results in prolongation of the time it takes for the system to reach steady state. This is apparent from the progress curves shown in Fig. 2. A quantitative description of the transition of the plasmin activity towards the steady-state level is given by eqn. (9):

$$\frac{dp}{dt} = \frac{k_a k_e a_0 z_0 s_0}{k_i K_s i_0}\left[1 - \exp\left(-\frac{k_i K_s}{K_s + s_0} i_0 t\right)\right] \quad (9)$$

where $[(k_i K_s)/(K_s + s_0)]i_0$ is the apparent first-order constant for the inactivation of plasmin. The transition time is inversely proportional to this constant. It follows that an increase in s_0 results in both an increased rate of nitroaniline formation and an increased transition time.

Determination of second-order rate constants, k_a/K_z, for urokinase-catalysed plasminogen activation

Experiments performed at various concentrations of plasminogen with either method I (not shown) or method II (Fig. 2) suggest that the activation rate is proportional to the plasminogen concentrations as predicted by eqns. (5) and (8). This applies to both Glu_1- and Lys_{77}-plasminogen. The results are fully consistent with the previous observation (Christensen, 1977; Christensen & Müllertz, 1977; Peltz *et al.*, 1982) of a high K_m for plasminogen, and suggest the presentation of kinetic data in terms of apparent second-order constants (k_a/K_z). Such values calculated by means of eqns. (5) and (8) assuming $k_e = 14\,s^{-1}$ and $K_s = 0.12$ mM (Lottenberg *et al.*, 1981), $k_i = 5.6 \times 10^4\,M^{-1}\,s^{-1}$ (Petersen & Clemmensen, 1981) are listed in Table 1. Under the conditions employed in the present study, comparable results are obtained by the two methods.

Effect of t-AMCA on the catalytic activity of urokinase and tissue-plasminogen activator

Fig. 3(*a*) shows the inhibitory effect of *t*-AMCA on the catalytic activity of urokinase, plasmin and

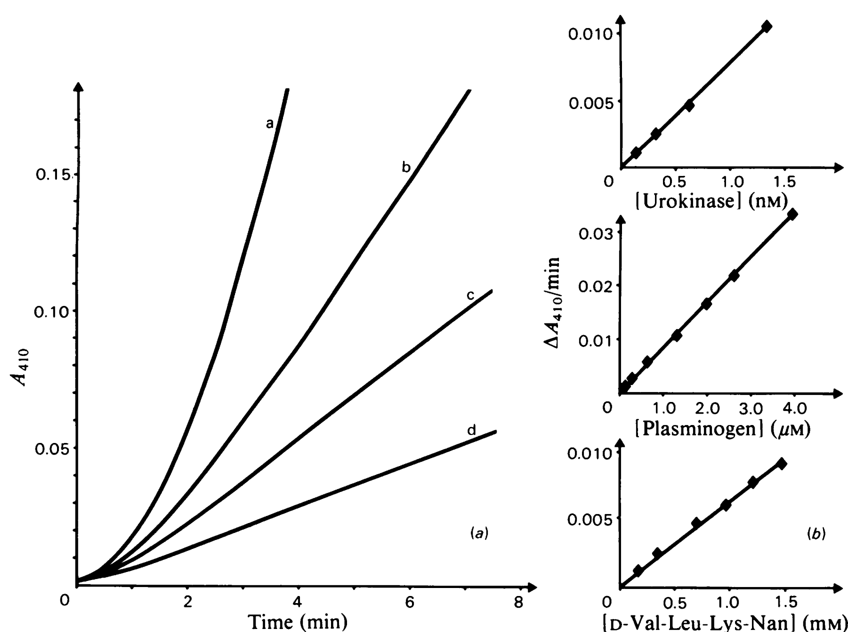


Fig. 2. *Urokinase-catalysed Lys_{77}-plasminogen activation in the presence of pancreatic plasmin inhibitor monitored by means of the plasmin activity with Val-Leu-Lys-Nan*

(*a*) Progress curves of nitroaniline formation measured at 410nm and a system containing 2.0nM-urokinase, 0.31 μM-Lys_{77}-plasminogen, 0.72mM-Val-Leu-Lys-Nan and various concentrations of pancreatic trypsin inhibitor: a, 0 μM; b, 0.1 μM, c, 0.2 μM; and d, 0.4 μM. (*b*) Steady-state plasmin activity during plasminogen activation and subsequent plasmin inhibition, measured in three series of experiments where the concentration of (i) urokinase, (ii) plasminogen or (iii) Val-Leu-Lys-Nan was varied. Other conditions were as described in curve d of (*a*).

Table 1. *Apparent second-order constants (k_a/K_z) for the reaction between activator and plasminogen*

Abbreviation used: Plg, plasminogen. References: (*a*) Christensen (1977); (*b*) Peltz *et al.* (1982); (*c*) Lucas *et al.* (1983); (*d*) Christensen & Müllertz (1977); (*e*) Hoylaerts *et al.* (1982); (*f*) Rijken *et al.* (1982); (*g*) Rånby (1982).

			$10^4 \times k_a/K_z$ ($M^{-1} \cdot s^{-1}$)			
			Present study		Literature	
Activator	Plasminogen	Modulator	Method I	Method II	Value	Reference
Urokinase	Glu_1-Plg	–	0.9	0.7	0.8	(*a*)
					0.7	(*b*)
					2.3	(*c*)
	Lys_{77}-Plg	–	12	7	6.4	(*d*)
					15	(*b*)
					36	(*c*)
Tissue plasminogen activator	Glu_1-Plg	–	0.1		0.1	(*e*)
					0.03	(*f*)
					0.03	(*g*)
		t-AMCA (1mM)	1.2			
		Poly-D-lysine (10mg/l)	1.3			
		t-AMCA (1mM) plus poly-D-lysine (10mg/l)	7			
	Lys_{77}-Plg	–	1.6		1.1	(*e*)
					1	(*g*)
		Poly-D-lysine (10mg/l)	14			

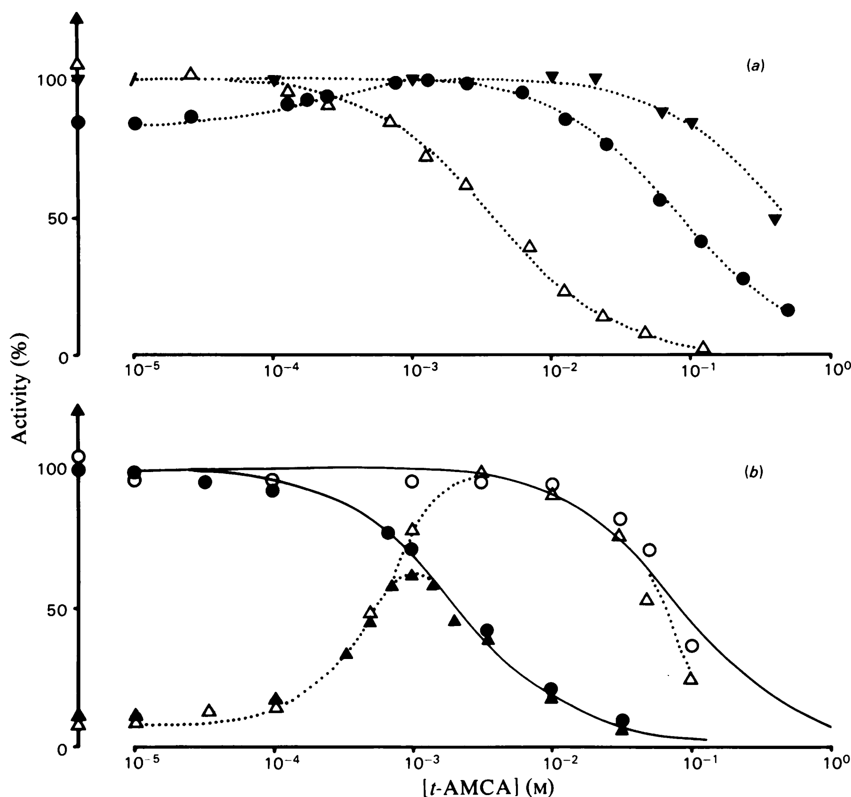


Fig. 3. *Effect of t-AMCA on urokinase and tissue-plasminogen-activator-catalysed reactions*
(*a*) Proteinase activity with synthetic peptide substrates. (△) Catalytic activity of urokinase (48nM) with 50μM-<Glu-Gly-Arg-Nan; (●) catalytic activity of plasmin (20nM) with 0.36mM-Val-Leu-Lys-Nan; and (▼) catalytic activity of tissue-plasminogen activator (6.7nM) with Ile-Pro-Arg-Nan. (*b*) Plasminogen activation catalysed by urokinase [closed symbols, Glu_1-plasminogen (triangles) and Lys_{77}-plasminogen (circles)]. The urokinase activity was determined as described in Fig. 2 (method II). The cuvette contained 2.0nM-urokinase, 0.72mM-Val-Leu-Lys-Nan, 0.4μM-pancreatic trypsin inhibitor, 0.31μM-Glu_1-plasminogen (▲) or Lys_{77}-plasminogen (●). Plasminogen activation catalysed by tissue-plasminogen-activator (open symbols) was determined as described in Fig. 1 (method I). The cuvette contained 2.7nM-tissue-plasminogen activator, 0.36mM-Val-Leu-Lys-Nan and 0.6μM-Glu_1-plasminogen (△) or Lys_{77}-plasminogen (○). Two theoretical binding curves (S-shaped unbroken lines) are shown for comparison: (i) left curve, $K_D = 2.0\,\mu$M, represents the urokinase inhibition curve in the absence of significant substrate competition; (ii) right curve, $K_D = 90\,\mu$M, represents the plasmin inhibition curve in the presence of 0.36mM-Val-Leu-Lys-Nan determined from the data shown in (*a*).

tissue-plasminogen activator with synthetic substrates. Urokinase and tissue-plasminogen-activator activities are studied at substrate concentrations equal to the K_m of the actual specific substrates (determined in separate experiments; results not shown). The inhibition of plasmin is measured at the concentration of Val-Leu-Lys-Nan used in the plasminogen-activation experiments shown in Fig. 3(*b*) ($s_0 = 3K_m$). A modest enhancement of plasmin activity at low concentrations of *t*-AMCA is observed, and has previously been noted by other investigators (Brockway & Castellino, 1971; Christensen, 1978). Apart from this complication, the inhibition pattern is in each case described by a simple binding curve. The K_i values determined by assuming a simple competition between substrate and inhibitor (Lorand & Condit, 1965) are shown in Table 2. The values are in close agreement with those obtained in previous studies (Walton, 1967; Brockway & Castellino, 1971; Christensen, 1978). It is noteworthy that the inhibition of tissue-plasminogen activator is approx. 100-fold weaker than that of urokinase, and that the inhibition of plasmin is intermediate between that of urokinase and tissue-plasminogen activator.

Fig. 3(*b*) shows the effect of *t*-AMCA on plasminogen activation. In agreement with previous observations (see, e.g., Castellino, 1981) a biphasic effect (enhancement followed by inhibition) is ob-

Table 2. *t-AMCA inhibition of fibrinolytic proteinases*
Kinetic measurements were performed in 0.1 M-NaCl/0.05 M-Tris-HCl/0.01% Tween 80, pH 7.4, at 25°C. Other conditions were as described in Fig. 3(*a*). K_i was determined from the results shown in Fig. 3(*a*), assuming competitive inhibition. K_m values were determined in separate experiments, the substrate concentration being varied in the absence of inhibitor.

Proteinase	Substrate	K_m (mM)	K_i (mM)
Urokinase	<Glu-Gly-Arg-Nan	0.05	2.0
Plasmin	Val-Leu-Lys-Nan	0.12	23
Tissue-plasminogen activator	Ile-Pro-Arg-Nan	0.58	~250

served with Glu_1-plasminogen, whereas a monophasic inhibition is observed with Lys_{77}-plasminogen. Both urokinase- and tissue-plasminogen activator-catalysed activation of Glu_1-plasminogen is enhanced at *t*-AMCA concentrations of about 0.6 mM. Above 1 mM the activation rate of Glu_1-plasminogen is equal to that of Lys_{77}-plasminogen with urokinase as well as with tissue-plasminogen activator. However, the inhibitory effect of *t*-AMCA on the urokinase-catalysed system is very different from that exerted on the tissue-plasminogen-activator system. The former is inhibited at a much lower concentration than the latter.

It has been proposed (Clayes & Vermylen, 1974; Thorsen & Müllertz, 1974; Thorsen *et al.*, 1974; Walther *et al.*, 1975) that the enhancement of Glu_1-plasminogen activation is closely associated with conformational changes ('loosening') in the molecular structure induced by binding of ligands such as *t*-AMCA and other anti-fibrinolytic reagents. The concentration of *t*-AMCA at which the enhancement attains its half-maximal value (Fig. 3*b*) is close to the value ($K_{0.5} = 0.7$ mM) obtained for the ligand-induced change in Glu_1-plasminogen conformation (Brockway & Castellino, 1972; Violand *et al.*, 1978; Markus *et al.*, 1979). The sharpness of the curve describing the enhancement effect (Fig. 3*b*) might indicate co-operative binding of the ligand to Glu_1-plasminogen.

The inhibitory effect of *t*-AMCA at higher concentrations can in each case be explained as simple competitive binding of this ligand to the active site of a serine proteinase present in the reaction mixture. The results obtained with urokinase (Fig. 3*a* and Table 2) suggest that inhibition of the activation reaction is due to interaction of the ligand with the active site of urokinase ($K_i = 2.0$ mM). Substrates tend to displace inhibitory ligands from the active site. Thus in case of simple competition, the apparent K_i observed in the presence of substrate is related to K_i by eqn. (10):

$$K_i' = K_i\left(1 + \frac{s_0}{K_m}\right) \qquad (10)$$

This equation accounts for the inhibition of urokinase activity with synthetic peptide substrates and is likely to be valid also for the inhibition of plasminogen activation. Data on the inhibition of urokinase-catalysed plasminogen activation is superimposable on a binding curve with a dissociation constant equal to K_i. This is fully consistent with other results of the present study, which suggest that the plasminogen concentration used is much lower than the apparent K_m for this substrate. The interaction of *t*-AMCA with tissue activator is approx. 100-fold weaker ($K_i \simeq 250$ mM), and the interaction with plasmin ($K_i = 23$ mM) is intermediate between that of urokinase and tissue activator. This means that the inhibition observed in Fig. 3(*b*) is mainly an effect on the plasmin activity rather than true inhibition of the plasminogen-activation reaction. An additional inhibition of tissue-plasminogen activator at very high concentrations of *t*-AMCA may explain the deviation from the plasmin inhibition curve observed at high concentrations.

Effect of poly-D-lysine on plasminogen activation

Allen (1982) reported an enhancing effect of poly-D-lysine on plasminogen activation catalysed by tissue-plasminogen activator or low-M_r urokinase, but not with high-M_r urokinase. The results shown in Fig. 4 confirm the observation on tissue-plasminogen activator; however, neither low- nor high-M_r urokinase-catalysed plasminogen activation are affected by poly-D-lysine under the conditions employed in the present study. Glu_1- and Lys_{77}-plasminogen activation rates are shown as a function of poly-D-lysine concentrations in Fig. 4. An approx. 10-fold enhancement is obtained by the conversion of Glu_1-plasminogen into Lys_{77}-plasminogen, and a further 10-fold enhancement can be induced by the addition of poly-D-lysine. A bell-shaped curve for the enhancement as a function of the logarithm of the poly-D-lysine concentration is obtained for both Glu_1- and Lys_{77}-plasminogen activation, with a maximal effect at about 10 mg/litre.

Poly-D-lysine and *t*-AMCA are both effectors of tissue-plasminogen-activator-catalysed plas-

minogen activation. Under suitable conditions, each effector induces a considerable enhancement of the reaction rate. The experiments shown in Fig. 5 were performed in order to investigate the combined effects of the reagents. A simple additive effect is seen for Glu_1-plasminogen activation, whereas Lys_{77}-plasminogen activation is apparently inhibited by *t*-AMCA in a manner that is quite different from the inhibition pattern observed in the absence of poly-D-lysine (Fig. 3*b*). In fact the effect appears to be an abolition of the poly-D-lysine enhancement, with a half-maximal effect at about 20 μM-*t*-AMCA. It is notable that the activation of Glu_1-plasminogen is severalfold faster than that of Lys_{77}-plasminogen in the presence of poly-D-lysine when the *t*-AMCA concentration is higher than about 0.6 mM.

The effect of poly-D-lysine on plasminogen activation is quite different from that of *t*-AMCA. First, urokinase-catalysed plasminogen activation is not affected by poly-D-lysine. Secondly, both the activation of Glu_1-plasminogen and Lys_{77}-plasminogen by tissue-plasminogen activator is stimulated by poly-D-lysine. And thirdly an additive effect of *t*-AMCA and poly-D-lysine is observed for the activation of Glu_1-plasminogen (Fig. 5), even under conditions with optimal concentrations of both stimulators.

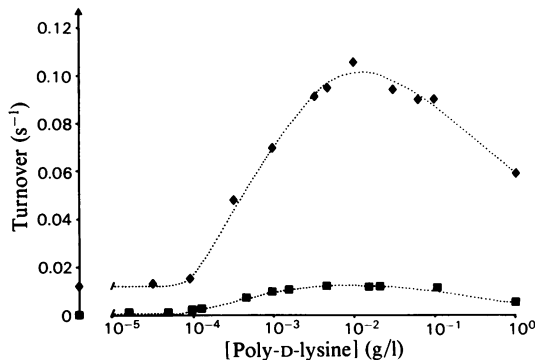


Fig. 4. *Effect of poly-D-lysine on tissue-activator-catalysed plasminogen activation*

The rate of plasminogen activation was measured as described in Fig. 1 (method I). The cuvette contained 0.6 μM-plasminogen, 0.36 mM-Val-Leu-Lys-Nan and various concentrations of poly-D-lysine. In experiments with Glu_1-plasminogen (■) the tissue-plasminogen-activator concentration was 4.0 nM; in experiments with Lys_{77}-plasminogen (◆) it was 1.33 nM.

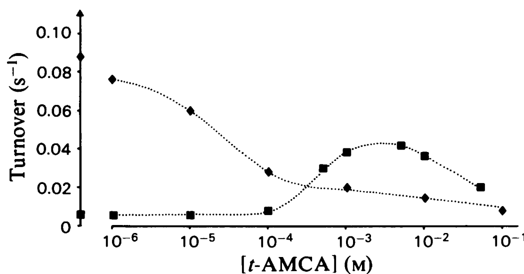


Fig. 5. *Effect of t-AMCA on poly-D-lysine-enhanced plasminogen activation*

The rate of plasminogen activation was measured as described in Fig. 1 (method I). The cuvette contained 1.33 nM-tissue-plasminogen activator, 0.6 μM-plasminogen, 0.36 mM-Val-Leu-Lys-Nan, poly-D-lysine (5 mg/l) and various concentrations of *t*-AMCA. ■, Glu_1-plasminogen; ◆, Lys_{77}-plasminogen.

Effect of plasminogen concentration on tissue-plasminogen activator kinetics

The kinetic results obtained with urokinase in the present study (Fig. 2) and in previous studies (Christensen, 1977; Christensen & Mullertz, 1977; Peltz, 1982; Lucas *et al.*, 1983) all suggest that it is very difficult to saturate this enzyme with its

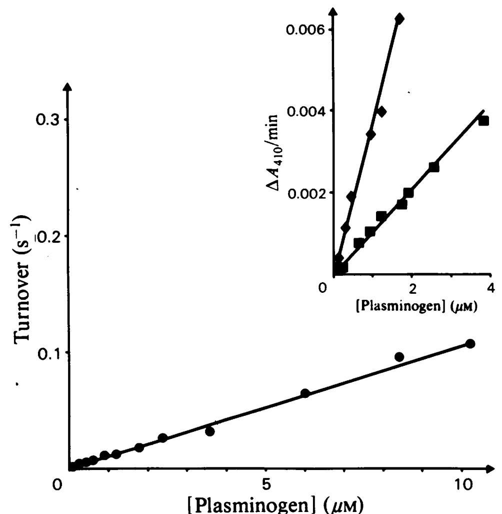


Fig. 6. *Effect of plasminogen concentration on tissue-activator catalysed plasminogen activation*

The rate of plasminogen activation was measured as described in Fig. 1 (method I). The cuvette contained 4 nM-tissue-plasminogen activator, 0.36 mM-Val-Leu-Lys-Nan, poly-D-lysine (16.7 mg/l) and various concentrations of Glu_1-plasminogen. Inset: the rate of plasminogen activation was determined as described in Fig. 2 (method II). The cuvette contained 1.33 nM-tissue-plasminogen activator, 0.36 mM-Val-Leu-Lys-Nan, poly-D-lysine (5 mg/l) and various concentrations of plasminogen. In addition the experiments with Glu_1-plasminogen (■), but not those with Lys_{77}-plasminogen (◆), were performed in the presence of 1 mM-*t*-AMCA.

substrate, plasminogen. The same is true for tissue-plasminogen activator in the absence of effector substances (Hoylaerts *et al.*, 1982; Rijken *et al.*, 1982; Rånby, 1982). Fig. 6 shows tissue-plasminogen-activator-catalysed activation as a function of plasminogen concentration under conditions where the activity is stimulated by the artificial effector substances used in the present study. The rate is still proportional to the substrate concentration, with no indication of substrate saturation, even at relatively high plasminogen concentrations and relatively high turnover rates. The results seem to indicate that the fraction of activator bound to plasminogen is very small, and that the kinetics can be accurately described by assuming a simple second-order reaction. Second-order rate constants (k_a/K_z) are listed in Table 1. In contrast, when stimulated by fibrin or fibrin degradation products, plasminogen activation catalysed by tissue-plasminogen activator has been reported to be saturable with plasminogen at physiological concentration ($K_z \leqslant 2\,\mu$M) (Hoylaerts *et al.*, 1982; Rijken *et al.*, 1982; Rånby, 1982). It remains, however, to be shown whether this effect is due to binding of modulator to the enzyme, tissue-plasminogen activator, or to the substrate, plasminogen. Still another possibility is the association of both enzyme and substrate to the modulator, in which case it is important also to distinguish between various binding modes: independent binding, competition and co-operative binding.

*Analogies between the effector functions of poly-*D-*lysine and those of fibrin*

To what extent it is possible to compare the stimulatory effect of fibrin with that of the model component, poly-D-lysine, is still an open question. Like fibrin-enhanced Lys_{77}-plasminogen activation, poly-D-lysine-enhanced activation is abolished by *t*-AMCA, and the concentration interval for the abolition (10–100$\,\mu$M) is roughly that observed for the abolition of fibrin-stimulated Lys_{77}-plasminogen activation (Hoylaerts *et al.*, 1981). This is also the concentration range over which Lys_{77}-plasminogen is displaced from fibrin (Rákózci *et al.*, 1978; Thorsen, 1975). It is notable that whereas the binding of Lys_{77}-plasminogen to fibrin is highly sensitive to *t*-AMCA, the binding of Glu_1-plasminogen to fibrin (although weaker) is relatively insensitive to anti-fibrinolytic amino acids. Thus the binding of Glu_1-plasminogen to fibrin at *t*-AMCA concentrations higher than about 100$\,\mu$M is stronger than the binding of Lys_{77}-plasminogen (Suenson & Thorsen, 1981; Suenson *et al.*, 1984).

Similar binding properties of plasminogens to poly-D-lysine might explain the fact that the activation of Glu_1-plasminogen in the presence of poly-D-lysine and 1 mM-*t*-AMCA is faster than the activation of Lys_{77}-plasminogen under the same conditions (Fig. 6). Reversal of the enhancement effect at a high excess of modulator is still another property that seems to be common for poly-D-lysine (Fig. 4) and fibrin (Rånby, 1982).

Elucidation of the mechanism of the stimulatory effect of poly-D-lysine awaits more detailed knowledge of the binding of tissue-plasminogen activator and plasminogen to this polymer, just as further evaluation of the analogy between fibrin and poly-D-lysine requires better quantitative determination of plasminogen activation kinetics in the presence of fibrin than is presently available.

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