Kinetics of plasmin inhibition in the presence of a synthetic tripeptide substrate

The reaction with pancreatic trypsin inhibitor and two forms of α_2 -plasmin inhibitor

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The progressive inhibition of plasmin by pancreatic trypsin inhibitor and by α_2 -plasmin inhibitor in the presence of D-valyl-L-leucyl-L-lysine 4-nitroanilide was investigated. The kinetics with plasmin were compared with those with miniplasmin. The kinetic properties of two functionally different forms of α_2 -plasmin inhibitor described by Clemmensen [(1979) in *The Physiological Inhibitors of Coagulation and Fibrinolysis* (Collen, D., Wiman, B. & Verstraete, M., eds.), pp. 131–136, Elsevier, Amsterdam] were characterized. The two forms differ in their plasminogen-binding capability, and this difference can account for a difference in secondary site interaction suggested from the kinetics. The binding of inhibitor to miniplasmin is a simple pseudo-first-order reaction with both pancreatic trypsin inhibitor and the two α_2 -plasmin inhibitor forms. Such simple kinetics are also observed for the reaction between plasmin and the non-plasminogen-binding form of α_2 -plasmin inhibitor. More complicated kinetics are obtained for the reaction between plasmin and the α_2 -plasmin inhibitor form that binds to plasminogen. With both forms of the α_2 -plasmin inhibitor, a complex stable to acetic acid/urea and gel electrophoresis is present and fully developed 15 s after initiation of the reaction with plasmin.

Inactivation of the serine proteinase plasmin (EC 3.4.21.7) by the specific inhibitor α_2 PI (α_2 -anti-plasmin, primary plasmin inhibitor) is essential to the regulation of fibrinolysis. Inactivation kinetics of plasmin have been studied by Christensen & Clemmensen (1977, 1978), Christensen (1979), Wiman *et al.* (1978, 1979), Cederholm-Williams *et al.* (1979) and Wiman (1980a). A reinvestigation of the kinetics by using carefully characterized α_2 PI preparations is of interest, since it has been indicated (Clemmensen, 1979; Wiman, 1980b) that plasma may contain two functionally different forms of the inhibitor. One of these forms binds to plasminogen-Sepharose and the other does not. Much evidence suggests that the so-called lysine-binding sites on plasminogen that are involved in the interaction between the zymogen and α_2 PI are also involved as secondary sites in the interaction between plasmin and α_2 PI. The principal lysine-

binding sites are located on the heavy chain of plasmin, whereas the catalytic site, which is the primary binding site for the inhibitor, is located on the light chain of plasmin (around serine-740). Secondary site interaction has a pronounced influence on the rate of inhibition [see Collen (1980) for a review].

In the present work, the kinetics of the two forms of the α_2 PI are compared with the kinetics obtained with a simple proteinase inhibitor of the Kunitz type, pancreatic trypsin inhibitor. Secondary site interactions are investigated by comparing the kinetics of Lys-plasmin (residues 77–790) and miniplasmin (residues 442–790), and also by studying the effect of the 'lysine analogue', tranexamic acid (*trans*-4-aminomethylcyclohexane-1-carboxylic acid).

A method in which the time course of proteinase inhibition can be followed continuously was introduced by Main & Dauterman (1963). The method, in which proteinase and inhibitor are mixed in the presence of excess substrate, was used to study the inhibition of acetylcholinesterase by organophos-

Abbreviations used: SDS, sodium dodecyl sulphate; α_2 PI, α_2 -plasmin inhibitor; PB α_2 PI, plasminogen-binding α_2 PI; NPB α_2 PI, non-plasminogen-binding α_2 PI.

phorus compounds. This method is applied here to the study of the inhibition kinetics of plasmin by using the synthetic tripeptide substrate D-valyl-L-leucyl-L-lysine 4-nitroanilide.

Materials and methods

Materials

D-Valyl-L-leucyl-L-lysine 4-nitroanilide (S-2251) and tranexamic acid were from Kabi, Stockholm, Sweden. Pancreatic trypsin inhibitor (Trasylol) was from Bayer, Leverkusen, W. Germany. The stock solution contained 20 000 Kallikrein Inhibitor units/ml. This is equivalent to 380 $\mu\text{mol/l}$ (Thorsen & Müllertz, 1974). Human urokinase was from Leo Pharmaceuticals, Copenhagen, Denmark. Glu-plasminogen (residues 1–790) and ^{125}I -labelled plasminogen were prepared from outdated donor blood as previously described (Thorsen *et al.*, 1981). Plasmin was obtained by activating Glu-plasminogen with Sepharose-bound urokinase as described by Wiman & Wallén (1973). The preparation used in the present work contains approx. 25% unactivated plasminogen as estimated from SDS/polyacrylamide-gel electrophoresis and activation experiments with added urokinase. SDS/polyacrylamide-gel electrophoresis of a reduced sample showed the plasmin heavy and light chains to be intact. The plasmin heavy chain migrated as the heavy chain derived from plasmin identified as Lys-plasmin by amino acid sequence analysis. Miniplasminogen (residues 442–790) prepared by elastase-catalysed specific limited proteolysis (Sottrup-Jensen *et al.*, 1977) was generously given by Dr. L. Sottrup-Jensen. Miniplasminogen (stock solution; 7.0 μM) was activated by addition of urokinase to a final concentration of 2000 Ploug units/ml before use. SDS/polyacrylamide-gel electrophoresis of a reduced sample revealed that miniplasminogen was completely converted into miniplasmin. α_2 -Plasmin was prepared by combining the methods of Wiman & Collen (1977) and Christensen & Clemmensen (1978) as described by Thorsen *et al.* (1981). This purification procedure yields a preparation that contains approx. 90% $\alpha_2\text{PI}$ which binds to plasminogen–Sepharose ($\text{PB}\alpha_2\text{PI}$). $\text{NBP}\alpha_2\text{PI}$ is formed spontaneously from $\text{PB}\alpha_2\text{PI}$ at room temperature (I. Clemmensen, unpublished work). The $\text{NPB}\alpha_2\text{PI}$ preparation was obtained from a partially converted $\alpha_2\text{PI}$ preparation from which $\text{NPB}\alpha_2\text{PI}$ as removed by affinity chromatography on a plasminogen–Sepharose 4B column. $\text{NPB}\alpha_2\text{PI}$ was collected in the void volume. On SDS/polyacrylamide-gel electrophoresis $\text{PB}\alpha_2\text{PI}$ has M_r 67 000, $\text{NPB}\alpha_2\text{PI}$ has M_r 65 000 and an inactive $\alpha_2\text{PI}$ form ($\text{I}\alpha_2\text{PI}$) has M_r 60 000 (I. Clemmensen, unpublished work). The $\text{PB}\alpha_2\text{PI}$ preparation used in the present

study contained at least 90% of the M_r -67 000 form. The $\text{NPB}\alpha_2\text{PI}$ preparation contained no detectable $\text{PB}\alpha_2\text{PI}$ (M_r 67 000) but detectable amounts of $\text{I}\alpha_2\text{PI}$ (M_r 60 000).

Determination of protein concentrations

The concentrations of Lys-plasmin and mini-plasmin were determined from the kinetics of hydrolysis of D-valyl-L-leucyl-L-lysine 4-nitroanilide by using a millimolar absorption coefficient, $\epsilon_{410} = 8.48 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for 4-nitroanilide and $k_{\text{cat}} = 13 \text{ s}^{-1}$ (Christensen & Ipsen, 1979). The concentration of plasminogen was determined after its conversion into plasmin (Thorsen & Müllertz, 1974). The concentration of $\alpha_2\text{PI}$ was determined by active-site titration with plasmin as described by Müllertz & Clemmensen (1976).

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1975), and acetic acid/urea/polyacrylamide-gel electrophoresis as described by Thorsen & Müllertz (1979).

Determination of complexes formed between α_2 -plasmin inhibitor and ^{125}I -labelled plasmin

Plasminogen (44 μM) labelled with ^{125}I was activated with urokinase (714 Ploug units/ml) at 37°C in the presence of 11 mM-6-aminoheptanoic acid. The activation mixture was placed on ice after 5 min of incubation, and labelled plasmin obtained by this procedure was then mixed with an excess of $\alpha_2\text{PI}$ (final concentrations 3.8 μM -plasmin + plasminogen and 4.1 μM -inhibitor). Samples were withdrawn at 15, 30, 45, 60, 120, 300 and 600 s after initiation of the reaction between plasmin and inhibitor and added to 1.75 M-acetic acid/9 M-urea to stop the reaction. Each sample was run on an acetic acid/urea/polyacrylamide gel for the separation of the inhibitor–plasmin complex from plasminogen and plasmin that had not reacted. Protein bands were excised and quantified by counting ^{125}I radioactivity in a gamma well counter.

Kinetic measurements

Buffer containing inhibitor and D-valyl-L-leucyl-L-lysine 4-nitroanilide was mixed with an equal volume of buffer containing plasmin by using a manual mixing device with two syringes connected to a flow cell. The dead-time of this simple stopped-flow system is approx. 5 s. The reaction was followed at 410 nm with a Beckman Acta M VI spectrophotometer operating in the derivative mode, dA/dt . This enables a direct measurement of plasmin activity at any time after the reaction is started. Mixing plasma and substrate at the concentration used in this study in the absence of inhibitor produces a constant activity for several minutes. All

experiments were performed in 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.7 at $23 \pm 2^\circ\text{C}$.

Theory

The reaction between a proteinase, E, and an irreversible competitive inhibitor, I, in the presence of substrate, A, may be described by the model (1):

Because $[\text{E}]/[\text{EA}]$ is low at high substrate concentrations, the inhibition proceeds at a rate which is slow compared with that in the absence of substrate. Even with a high value of k_i it is therefore possible with substrate present to add relatively high concentrations of inhibitor and still be able to follow the reaction over a convenient time span. Assuming that steady state is rapidly established with respect to substrate, it is possible to derive eqn. (2) (Main & Dauterman, 1963; Aldridge & Reiner, 1972) for the relation between the plasmin activity, v , the Michaelis constant, K_A , $[\text{A}]$ and $[\text{I}]$:

$$dv/v = -k_i \left(\frac{K_A}{K_A + [\text{A}]} \right) [\text{I}] dt \quad (2)$$

Eqn. (2) describes the decline in enzyme activity during a progressive inhibition and suggests an exponential decrease in v with an apparent first-order constant:

$$k' = k_i \left(\frac{K_A}{K_A + [\text{A}]} \right) [\text{I}] \quad (3)$$

Results

Reaction between Lys-plasmin or miniplasmin and pancreatic trypsin inhibitor

Fig. 1 shows the time course of the decrease in plasmin activity when plasmin is mixed with pancreatic trypsin inhibitor in the presence of 1.2 mM-D-valyl-L-leucyl-L-lysine 4-nitroanilide. The decrease is exponential, as indicated by the semi-logarithmic plot of the results inserted in Fig. 1. This plot is linear for more than 95% of the reaction. No residual plasmin activity could be observed at the end of the experiment, indicating that the inhibition is irreversible within the accuracy of the method. The effect of varying the concentration of inhibitor, I, is shown in Fig. 2. The apparent first-order rate constant, k' , is directly proportional to $[\text{I}]$, as suggested by eqn. (3). The effect of varying the

Fig. 1. Progressive inhibition of plasmin by pancreatic trypsin inhibitor

Buffer (1.0 ml) containing $0.172 \mu\text{M}$ -plasmin was mixed with an equal volume of buffer containing $0.760 \mu\text{M}$ -pancreatic trypsin inhibitor and $2.8 \mu\text{M}$ -D-valyl-L-leucyl-L-lysine 4-nitroanilide in a flow cell. The rate of nitroanilide production measured at 410 nm was recorded by using the spectrophotometer in the dA/dt mode. A semilogarithmic plot of the results is shown in the insert.

Fig. 2. Effect of pancreatic trypsin inhibitor concentration on the inhibition of plasmin and miniplasmin

The apparent first-order rate constant, k' , is plotted as a function of the pancreatic trypsin inhibitor concentration, $[\text{I}]$. Plasmin ($\circ$) or miniplasmin (Δ) is mixed with inhibitor and substrate as described in Fig. 1. Concentrations after mixing: plasmin, $0.086 \mu\text{M}$; miniplasmin, $0.048 \mu\text{M}$; D-valyl-L-leucyl-L-lysine 4-nitroanilide, 0.68 mM .

concentration of the chromogenic tripeptide substrate, A, is shown in Fig. 3. The plot of $1/k'$ versus $[\text{A}]$ yields a straight line. The apparent first-order rate constant, k' , does not depend on the con-

Table 1. *Second-order rate constants for plasmin inhibition by pancreatic trypsin inhibitor and two forms of α_2 -plasmin inhibitor*

The low and high- M_r forms of plasmin are identical with miniplasmin and Lys-plasmin respectively. References: ⁽¹⁾ Wiman (1980a); ⁽²⁾ Wiman *et al.* (1978); ⁽³⁾ Christensen *et al.* (1979); ⁽⁴⁾ Wiman & Collen (1978).

Inhibitor	Plasmin	[Tranexamic acid] (mM)	$10^{-5} \times k_i$ ($M^{-1} \cdot s^{-1}$) (this study)	$10^{-5} \times k_i$ ($M^{-1} \cdot s^{-1}$) (other studies)
Pancreatic trypsin inhibitor	Low- M_r	0	5.6	—
	High- M_r	0	5.6	9.1 ⁽¹⁾
NPB α_2 PI	Low- M_r	1	2.9	—
	Low- M_r	0	2.9	—
	High- M_r	1	2.9	—
	High- M_r	0	3.9	—
PB α_2 PI	Low- M_r	1	3.9	6.0 ⁽³⁾
	Low- M_r	0	4.8	6.5 ⁽²⁾ , 9.0 ⁽³⁾
	High- M_r	1	3.3	—
	High- M_r	0	—	380 ⁽⁴⁾

Fig. 3. *Effect of substrate concentration on the inhibition of plasmin by pancreatic trypsin inhibitor*

The apparent first-order rate constant is plotted in a reciprocal plot of $1/k'$ against the concentration of D-valyl-L-leucyl-L-lysine 4-nitroanilide, [A]. Plasmin ($0.172 \mu M$) is mixed with the inhibitor ($0.76 \mu M$) and various concentrations of substrate as described in Fig. 1.

centration of plasmin (results not shown). The inhibition kinetics of pancreatic trypsin inhibitor in the presence of substrate are thus fully consistent with eqn. (3), and it is possible to calculate the rate constant for the reaction between free plasmin and inhibitor, $k_i = 5.6 \times 10^5 M^{-1} \cdot s^{-1}$ (Table 1). From Fig. 3 it is also possible to determine the Michaelis constant for the steady-state kinetics with D-valyl-L-leucyl-L-lysine 4-nitroanilide, $K_A = 0.18$ mM, which agrees well with the value of 0.15 mM obtained by conventional methods (Christensen & Ipsen, 1979).

Fig. 2 also shows the results obtained when the experiment was performed with miniplasmin instead of plasmin. The results with miniplasmin are superimposed on those obtained with plasmin, indicating that the removal of residues 77–441

(‘Krigle’ 1, 2, 3 and 4) (Sottrup-Jensen *et al.*, 1977) does not change the kinetics.

Reaction between miniplasmin and α_2 -plasmin inhibitor

Most studies on the reaction of α_2 PI have so far been performed with preparations containing mainly the PB α_2 PI form, but in addition various quantities of the NPB α_2 PI form may have been present. The inhibition of miniplasmin by the two forms is shown in Fig. 4. Both inhibitors inactivate miniplasmin in a single first-order reaction in the presence of D-valyl-L-leucyl-L-lysine 4-nitroanilide. The results obtained by varying the concentration of inhibitor or substrate are described by eqn. (3) as indicated by Fig. 4, where $k'(K_A + [A])/K_A$ is plotted against [I]. The rate constant for the inhibition of miniplasmin by NPB α_2 PI is slightly lower than that for the inhibition by PB α_2 PI (Fig. 4 and Table 1). The effect of 1 mM-tranexamic acid on the reaction is also shown in Fig. 4. The reaction with NPB α_2 PI is not affected by this lysine analogue; however, in agreement with the results obtained by Wiman *et al.* (1978), a small effect is observed on the reaction between miniplasmin and PB α_2 PI.

Reaction between Lys-plasmin and α_2 -plasmin inhibitor

Inactivation of plasmin by PB α_2 PI in the presence of substrate is a biphasic reaction with a rapid phase (that cannot be measured by the present method), followed by a more slowly progressing and measurable inactivation (I. Clemmensen, S. Thorsen, S. Müllertz & L. C. Petersen, unpublished work). The kinetics are thus more complicated than predicted from eqn. (3). It is likely that interaction of PB α_2 PI with sites other than those interacting with the substrate (secondary sites) are responsible for this phenomenon. This means that the inhibitor is no

Fig. 4. Inhibition of miniplasmin by two forms of α_2 -plasmin inhibitor

Miniplasmin ($0.096\ \mu\text{M}$) is mixed with substrate ($1.82\ \text{mM}$) and various concentrations of inhibitor as described in Fig. 1. The apparent first-order rate constant k' , multiplied by the 'substrate factor', $(K_A + [A])/K_A$ (see eqn. 3), is plotted as a function of the inhibitor concentration, $[I]$. Δ , $\text{NPB}\alpha_2\text{PI}$; $\blacktriangle$, $\text{NPB}\alpha_2\text{PI}$ plus $2\ \text{mM}$ -tranexamic acid; $\circ$, $\text{PB}\alpha_2\text{PI}$; $\bullet$, $\text{PB}\alpha_2\text{PI}$ plus $2\ \text{mM}$ -tranexamic acid (before mixing).

Fig. 5. Inhibition of plasmin by two forms of α_2 -plasmin inhibitor

Plasmin ($0.172\ \mu\text{M}$) is mixed with substrate and various concentrations of inhibitor as described in Fig. 1. The apparent first-order rate constant, k' , multiplied by the 'substrate factor' $(K_A + [A])/K_A$ (see eqn. 3), is plotted as a function of the inhibitor concentration, $[I]$. $\blacktriangle$, $\text{NPB}\alpha_2\text{PI}$, $1.82\ \text{mM}$ substrate and $2\ \text{mM}$ -tranexamic acid; Δ , $\text{NPB}\alpha_2\text{PI}$ and $1.82\ \text{mM}$ substrate; $\bullet$, $\text{PB}\alpha_2\text{PI}$, $3.62\ \text{mM}$ substrate and $2\ \text{mM}$ -tranexamic acid (before mixing).

longer strictly competitive with respect to substrate and that a model other than that described in eqn. (1) should be used in this case. Other methods appear to be better suited for measuring the kinetics under these conditions, and the rate constant for this initial reaction measured by those methods is given in Table 1 for comparison.

Addition of lysine analogues partially abolishes secondary site interactions (see, e.g., Wiman & Collen, 1978); this is also indicated by the simple monophasic inactivation obtained with plasmin and $\text{PB}\alpha_2\text{PI}$ in the presence of substrate and tranexamic acid (results not shown). The first-order rate constant for the inactivation reaction as a function of the concentration of $\text{PB}\alpha_2\text{PI}$ is shown in Fig. 5. The plot of $k'(K_A + [A])/K_A$ versus $[I]$ does not pass through zero, and the kinetics are therefore more complicated than predicted from eqn. (3). An apparent rate constant of $0.1\ \text{s}^{-1}$ is estimated from the extrapolated intercept with the vertical axis.

The plasmin inactivation by $\text{NPB}\alpha_2\text{PI}$ is monophasic both in the absence and the presence of tranexamic acid (results not shown). Fig. 5 shows that, unlike the kinetics for $\text{PB}\alpha_2\text{PI}$, those for $\text{NPB}\alpha_2\text{PI}$ are in accordance with the simple model described by eqn. (1). The rate constant for the reaction between plasmin and $\text{NPB}\alpha_2\text{PI}$ in the

presence of tranexamic acid is identical, within experimental error, with the rate constant for the reaction between miniplasmin and $\text{NPB}\alpha_2\text{PI}$ (Table 1). Unlike the reaction with miniplasmin, the reaction with plasmin is slightly faster in the absence of tranexamic acid.

Appearance of complexes stable to acetic acid/urea and electrophoresis

The reaction between $\alpha_2\text{PI}$ and plasmin results in a 1:1 complex that is stable to electrophoresis both on a SDS/polyacrylamide gel and on an acetic acid/urea/polyacrylamide gel (Müllertz & Clemmensen, 1976; Thorsen & Müllertz, 1979). The rate of appearance of the stable complex was studied by mixing ^{125}I -labelled plasmin with an excess of inhibitor. After 15, 30, 45, 120, 300 and 600 s, samples of the inhibitor-plasmin mixture were added to $1.75\ \text{M}$ -acetic acid/ $9\ \text{M}$ -urea. Dilution into acetic acid/urea is supposed to cause an efficient quenching of the reaction, since $\alpha_2\text{PI}$ is known to be inactive both at low pH (Moroi & Aoki, 1976; Wiman & Collen, 1977) and at the high concentrations of urea (I. Clemmensen, unpublished work). The amount of stable complex formed was determined after separation of the complex by acetic acid/urea/

polyacrylamide-gel electrophoresis. The complex was quantified by counting the radioactivity of labelled material present in excised protein bands containing the complex, which were well separated from other bands with labelled material containing plasminogen (and possibly plasmin that had not reacted). A complex that is not dissociated under the conditions described above is fully developed 15 s after mixing. This applies to complex-formation with PBa_2PI as well as with NPBa_2PI .

Discussion

The progressive inhibition of plasmin and mini-plasmin was studied in the present work by a method in which proteinase and inhibitor are mixed in the presence of high concentrations of substrate. With a simple irreversible reaction between enzyme and inhibitor, the presence of substrate slows down the inactivation reaction by a factor $\alpha = (K_A + [A])/K_A$. Thus at a suitable high concentration of substrate, it is possible to use an ordinary spectrophotometer to follow the transient reaction between enzyme and inhibitor, even at relatively high concentrations of these components (Fig. 1).

This 'substrate method' has not previously been applied to the study of the inhibition kinetics of proteinases from the haemostatic and fibrinolytic systems. The advantage of the method is that it is possible to follow the reaction at near-physiological concentrations of the proteinase, and that pseudo-first-order kinetics are obtained by addition of inhibitor in excess. This is in contrast with conventional methods, where the components have to be added in nanomolar concentrations (because of the high value of the second-order rate constant for the reaction between enzyme and inhibitor), and where more complicated kinetics are observed (see, e.g., Christensen & Clemmensen, 1978). The 'substrate method' is therefore a valuable supplement to conventional methods.

A simple model (eqn. 1) gives a satisfactory description of the progressive inhibition of mini-plasmin by pancreatic trypsin inhibitor as well as by NPBa_2PI and PBa_2PI . This is also true for the inhibition of plasmin by pancreatic trypsin inhibitor and NPBa_2PI . The results suggest the existence of a strict competition between substrate and inhibitor, and also that the reaction between proteinase and inhibitor is a simple irreversible reaction. This is apparently in conflict with previous results (Christensen & Clemmensen, 1977, 1978; Christensen, 1979; Wiman *et al.*, 1978; Wiman, 1980a), which suggests that the simplest possible model for the inactivation is that described by eqn. (4):

in which PI may be a complex with a 'virgin' inhibitor and PI^* a complex with modified inhibitor (see, e.g., Laskowski & Kato, 1980). However, it has been proposed (Christensen & Clemmensen, 1977, 1978; Wiman *et al.*, 1978, 1979; Wiman, 1980a) that both the dissociation reaction (k_{-1}) and the conversion of PI into PI^* (k_{+2}) are relatively slow reactions. Half-times of 15–1500 min for the first reaction (k_{-1}) and 2–8 min for the second reaction (k_{+2}) have been estimated by the above authors. This indicates that both reactions are insignificant within the time span of the experiments reported in the present work (typically 1–2 min), and also that the method actually measures the second-order rate constant, k_{+1} , for the reaction between free proteinase and inhibitor. The biphasic inhibition pattern observed by Christensen & Clemmensen (1977, 1978) and by Wiman *et al.* (1978) was interpreted in terms of the two-step model shown in eqn. (4). An inhibitor preparation containing a significant fraction that does not bind to plasminogen is also likely to yield biphasic kinetics. This and also the difficulties encountered in the analysis of the kinetics under second-order conditions of the components suggest that the second reaction of the postulated two-step model with a half-time of 2–3 min should be tested by other means. The results with gel electrophoresis and determination of the amount of complex formed at various time intervals after mixing does not exclude the possibility of a two-step mechanism. It is, however, shown by our experiments, which confirm and extend the results obtained by Moroi & Aoki (1976), that a very stable complex that is not dissociated by acetic acid/urea or electrophoresis is already present and fully developed 15 s after mixing.

A comparison of the various second-order rate constants listed in Table 1 may provide an indication of which parts of the native molecules of plasmin and $\alpha_2\text{PI}$ are involved in this reaction. Much evidence indicates that secondary site interactions have a pronounced effect on the plasmin inhibition by $\alpha_2\text{PI}$. Thus it has been shown that the second-order rate constant for the reaction is nearly two orders of magnitudes lower when such interactions are diminished or abolished by addition of lysine analogues (Christensen & Clemmensen, 1978; Christensen, 1979; Wiman & Collen, 1978) or when residues 77–441 are removed from plasmin (Wiman *et al.*, 1978; Christensen *et al.*, 1979). The results listed in Table 1 confirm that $\alpha_2\text{PI}$ is no more efficient as an inhibitor than is pancreatic trypsin inhibitor under these conditions. It is also suggested from the results shown in Fig. 4 that $\alpha_2\text{PI}$ and substrate compete for the same site on miniplasmin in a strict manner without secondary site interactions of the inhibitor. In agreement with the results obtained by Wiman & Collen (1978), lysine

analogues have an effect on the interaction of $\text{PB}\alpha_2\text{PI}$ with the primary site of miniplasmin (Fig. 4). This effect is not observed with $\text{NPB}\alpha_2\text{PI}$.

The fact that the reaction between plasmin and $\text{NPB}\alpha_2\text{PI}$ is described by simple kinetics (eqn. 3) even in the absence of tranexamic acid (Fig. 5) suggests that secondary site interaction does not contribute to this reaction. It is plausible that $\text{PB}\alpha_2\text{PI}$ contains a 'lysine-like' site (complementary to the secondary site on the heavy chain of plasmin) that is modified or removed during the conversion of $\text{PB}\alpha_2\text{PI}$, contrary to what was observed with miniplasmin and $\text{NPB}\alpha_2\text{PI}$. Compared with the pronounced effect of tranexamic acid on the reaction between $\text{PB}\alpha_2\text{PI}$ and plasmin, this is a minor effect that appears only to involve the primary site of plasmin. Since $\text{NPB}\alpha_2\text{PI}$ may be present in significant amounts in plasma (Clemmensen, 1979; Wiman, 1980b), this property of $\alpha_2\text{PI}$ could be of physiological importance.

The plot of the apparent first-order rate constant for the reaction between plasmin and $\text{PB}\alpha_2\text{PI}$ in the presence of tranexamic acid against the concentration of inhibitor (Fig. 5) does not pass through the origin. This could indicate that secondary site interaction is not completely abolished by addition of this lysine analogue, in contrast with what was observed when the secondary site interactions were abolished by replacement of plasmin with miniplasmin (Figs. 4 and 5) or by replacement of $\text{PB}\alpha_2\text{PI}$ with $\text{NPB}\alpha_2\text{PI}$ (Fig. 5).

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