Purification and Reaction Mechanisms of the Primary Inhibitor of Plasmin from Human Plasma

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The primary inhibitor of plasmin in human plasma was purified by a four-step procedure involving fractional $(\text{NH}_4)_2\text{SO}_4$ precipitation, ion-exchange chromatography on a column of DEAE-Sepharose CL-6B and affinity chromatography on both a plasminogen-CH-Sepharose 4B column and a column of 6-aminohexanoic acid covalently coupled through the carboxylate function to AH-Sepharose 4B. No impurities in the final preparation could be detected when tested by immunoelectrophoresis against a range of specific antisera or against rabbit anti-human serum. On polyacrylamide-gel electrophoresis the inhibitor preparations showed a single band. The dissociation constant for the inhibitor-plasminogen complex was determined to be approx. $3 \mu\text{M}$ at pH 7.8. The reactions of the inhibitor with human plasmin and with bovine trypsin were studied. Comparison of the results obtained confirms the hypothesis previously presented, namely that the reaction of the inhibitor with plasmin involves at least two steps, the initial rapid formation of an enzyme-inhibitor complex followed by a slow irreversible transition to another complex. The results also indicate that the reaction of the inhibitor with trypsin involves just a single, irreversible step, so that this reaction seems to be less complicated than that of the inhibitor with plasmin. The ways in which 6-aminohexanoic acid influences the reactions were studied. The same value for the dissociation constant (approx. $26 \mu\text{M}$) for 6-aminohexanoic acid is obtained for both its effect on the reaction of the inhibitor with trypsin and for competitive inhibition of trypsin. The inhibitory effect of 6-aminohexanoic acid thus seems to be due to its blocking of the active site of trypsin. In contrast with this, the inhibitory effects of L-lysine and 6-aminohexanoic acid on the inhibitor-plasmin reaction occur at concentrations much too low to affect the active site of plasmin. The possible dependence of the reaction of the inhibitor with plasmin on a second site(s) on plasmin is discussed.

The existence in human plasma of a hitherto unknown plasmin inhibitor has been demonstrated (Müllertz, 1974; Collen *et al.*, 1975). This inhibitor, which is present in human plasma at a concentration of approx. $1 \mu\text{M}$ and which has been variously called α_2 -plasmin inhibitor (Moroi & Aoki, 1976), anti-plasmin (Collen, 1976) and the primary inhibitor of plasmin (Müllertz & Clemmensen, 1976), has been purified and has been found to be a glycoprotein with a mol. wt. of about 65000 (Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976; Wiman & Collen, 1977). Our previous studies of the kinetics of the reaction between the inhibitor and human plasmin indicated that the process involves at least two steps, namely the initial rapid formation of an enzyme-inhibitor complex followed by a slow irreversible transition to another complex (Christensen &

Clemmensen, 1977). The initial reaction step is influenced by the presence of L-lysine, whereas the slow transition is not (Christensen & Clemmensen, 1977). This inhibitory action of L-lysine on the inhibitor-plasmin reaction occurs at concentrations much too low to affect the active site of plasmin. To explain it we have suggested that the so-called lysine-binding site(s) of plasmin is involved in the inhibitor-plasmin reaction.

A complex that does not react with plasmin may be formed between the inhibitor and the antifibrinolytic amino acids, such as 6-aminohexanoic acid and analogous compounds that are well-known inhibitors of fibrinolysis. We have now examined these reactions in more detail by investigating the kinetics of the reaction of the inhibitor with both plasmin and trypsin in the presence of 6-aminohexanoic acid, and the interaction of the inhibitor with an affinity chromatography column consisting of 6-aminohexanoic acid

Abbreviation used: Bz-Arg-OEt, α -N-benzoyl-L-arginine ethyl ester.

covalently coupled to AH-Sepharose 4B. The present paper reports a method of purification of the inhibitor using such a column and describes and discusses the kinetic measurements and their results.

Experimental

Materials

Human plasminogen was purified as previously described (Christensen & Müllertz, 1977). Human plasmin was prepared from native plasminogen with an *N*-terminal glutamic acid residue on a column of urokinase-substituted Sepharose 4B (Christensen, 1975), the eluate was concentrated by ultrafiltration, and the concentration of plasmin was determined by titration with *p*-nitrophenyl-*p*'-guanidinobenzoate (Chase & Shaw, 1969). Bovine trypsin was 'Crystalline Trypsin Novo', a gift from Novo A/S, Denmark.

Plasminogen-substituted CH-Sepharose 4B was prepared by coupling 6 μ mol of plasminogen to 45 g of activated CH-Sepharose 4B by the procedure given by the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden). The plasminogen material was a mixture of the two plasminogen forms, one with *N*-terminal glutamic acid and one with *N*-terminal lysine. Before use the gels were washed with a solution of Trasylol (2 μ M) (Bayer, Leverkusen, Germany) in Tris/HCl (0.05 M)/NaCl (0.1 M), pH 7.8, to inactivate traces of plasmin.

6-Aminohexanoic acid-substituted AH-Sepharose 4B was prepared by coupling 378 μ mol of 6-aminohexanoic acid to 45 g of activated AH-Sepharose 4B with the procedure recommended by the manufacturer (Pharmacia).

The primary inhibitor of plasmin in human plasma was purified as described below. Other materials were those described by Müllertz & Clemmensen (1976).

Methods

Purification of the primary inhibitor of plasmin. Batches of the inhibitor were prepared from 500 ml of human citrated plasma. The inhibitor was purified at 4°C in a four-step procedure: (1) fractional $(\text{NH}_4)_2\text{SO}_4$ precipitation as described by Müllertz & Clemmensen (1976); (2) ion-exchange chromatography on DEAE-Sepharose CL-6B as described by Müllertz & Clemmensen (1976); (3) affinity chromatography on a column (35 cm long $\times$ 510 mm² cross-sectional area) of plasminogen-substituted CH-Sepharose 4B equilibrated with a buffer, Tris/HCl (0.05 M)/NaCl (0.1 M), pH 7.4. After washing with the buffer (600 ml), the preparation was eluted with a solution with *trans*-4-aminomethylcyclohexane-1-carboxylic acid (1 mM) in the equilibration buffer. Approx. 40% of the inhibitor material applied showed no affinity to plasminogen. This material did not bind

when re-applied to a column of plasminogen-substituted CH-Sepharose 4B. A substantial amount (30%) of the inhibitor material that did bind could be washed away by the equilibration buffer, indicating that the dissociation constant of the inhibitor-plasminogen complex is rather high. The inhibitor material that was eluted with *trans*-4-aminomethylcyclohexane-1-carboxylic acid was concentrated by ultrafiltration and dialysed against the equilibration buffer. This buffer was also used in step-(4) affinity chromatography on 6-aminohexanoic acid covalently coupled through the carboxylate function to AH-Sepharose 4B. The proteins were eluted with the equilibration buffer, but the inhibitor showed some affinity to the column and was sufficiently retarded to be collected free of contaminating proteins (Fig. 1). The inhibitor material that showed no affinity to plasminogen also showed no affinity to this column.

Determination of concentrations and electrophoretic methods. The concentrations of inhibitor and of total protein were determined after each step as described by Müllertz & Clemmensen (1976). Also crossed immunoelectrophoresis and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were performed as described by these authors.

Kinetic experiments. The kinetic measurements were made in a manner analogous to some described previously (Christensen & Clemmensen, 1977). The desired amount of enzyme and inhibitor were incubated, where appropriate, in the presence of a modifier, after which a small volume of a strong solution of Bz-Arg-OEt was added and the initial velocity of hydrolysis of Bz-Arg-OEt was determined. The increase in volume on addition of Bz-Arg-OEt never exceeded 3.5%. Incubation mixtures had the same concentrations of inhibitor and total concentrations of plasmin or trypsin, each in the range 50–500 nM; 0, 5, 10, 25, 30 and 90 mM-6-aminohexanoic acid, and 1 and 10 μ M-plasminogen were used. Solutions were prepared in a buffer of Tris/HCl (0.05 M)/NaCl (0.1 M), pH 7.8. The temperature was 25°C.

The initial velocity, *v*, of the plasmin- or trypsin-catalysed hydrolysis of Bz-Arg-OEt is proportional to

Fig. 1. Gel-elution pattern of the primary inhibitor of plasmin from 6-aminohexanoic acid-substituted AH-Sepharose 4B

Details are given in the text. Δ , Protein; $\blacktriangle$, concentration of inhibitor.

the concentration of enzyme free to react, e . If v_0 is the initial velocity with no inhibitor present, but at the same concentration of Bz-Arg-OEt and at the same total concentration of enzyme, e_0 , at which v is determined, then $v/v_0 = e/e_0$, and the concentration of enzyme-inhibitor complex formed is given by $[1 - e_0(v/v_0)]$. It is assumed that the dissociation of enzyme-inhibitor complex(es) is so slow that when Bz-Arg-OEt is added, only the amount of enzyme free to react at that time is determined. If the concentration of Bz-Arg-OEt is much greater than the corresponding Michaelis constant, and if a modifier that is a (reversible) competitive inhibitor of the enzyme is present, then the sum of the amounts of free enzyme and of enzyme-modifier complex, but not the enzyme-inhibitor complex(es), present is determined.

Results

The primary inhibitor of plasmin has been purified from human plasma. A four-step procedure was used: fractional $(\text{NH}_4)_2\text{SO}_4$ precipitation; ion-exchange chromatography; affinity chromatography on both plasminogen-substituted CH-Sepharose 4B and 6-aminohexanoic acid covalently coupled through the carboxylate function to AH-Sepharose 4B. Table 1 illustrates the results of the purification procedure. No impurities could be detected in the final preparation by crossed immunoelectrophoresis with rabbit immunoglobulin against human serum proteins (Fig. 2) or with specific antisera against the other known proteinase inhibitors (Müllertz & Clemmensen, 1976). Only one band was seen in polyacrylamide-gel electrophoresis, no dimer or high-molecular-weight impurity was found (Wiman & Collen, 1977). Estimates of the concentration of the purified inhibitor by light-absorption measurements and by electro-immunoassay agreed well. Generally a concentration of 70–80% of this value was determined by titration with plasmin (Christensen & Clemmensen, 1977);

thus in the final preparation 70–80% of the inhibitor was functionally active.

Human plasma seems to contain two different molecular species of the inhibitor, only one of which shows affinity to plasminogen, and only that was purified. The inhibitor material that showed no affinity for plasminogen may contain a certain amount of inactive or inactivated inhibitor, but a substantial amount of inhibitor-plasmin complex was formed when plasmin was added to this inhibitor material in 2-fold excess as revealed by crossed immunoelectrophoresis. Estimated from the difference in area of immunoprecipitate formed in the absence and in the presence of plasmin, the amount of reactive inhibitor material was 75% of the total amount present.

Kinetics of the inhibitor-plasmin reaction

Tables 2–5 show some results obtained for the reaction of the inhibitor with human plasmin. In each experiment the initial concentrations of plasmin and of inhibitor were the same. Of the following three reactions:

it can be seen from Table 2 that only reaction 3 is compatible with the reaction between plasmin and the inhibitor. If $K = 3 \text{ nM}$ and $k_{+2} = 6.5 \cdot 10^{-3} \text{ s}^{-1}$ then theoretical values of $(v/v_0)^c$ shown in Table 2 are obtained, which confirm our previously reported results (Christensen & Clemmensen, 1977). That reaction 1 is not a valid model for this reaction can be seen from the calculated $k_{+1}(\text{I})$ values; that reaction 2 is not, is seen from the fact that plasmin is eventually completely inhibited.

Table 1. Purification of the primary inhibitor of plasmin from human plasma

Total amount of inhibitor was determined by electroimmunoassay and total protein by light-absorption measurement at 280 nm (Müllertz & Clemmensen, 1976). Pure inhibitor (15 nmol) is 1 mg of protein (mol. wt. 65 000).

Purification step	Total volume (ml)	Total protein (mg)	Total amount of inhibitor (nmol)	Purity ratio (mg of inhibitor/mg of protein)	Yield
Plasma	500	40 100	600	0.0010	1
(1) $(\text{NH}_4)_2\text{SO}_4$ (0.8–2.7 M)	430	21 000	420	0.0013	0.7
(2) DEAE-Sepharose CL-6B	106	4014	293	0.005	0.5
(3) Plasminogen-CH- Sepharose 4B	8	10	116	0.8	0.2
(4) 6-Aminohexanoic acid-AH-Sepharose 4B	17	6	88	1.0	0.15

Fig. 2. Crossed immunoelectrophoresis of a final preparation of the primary inhibitor of plasmin with rabbit immunoglobulin against human serum proteins

The sample applied contained 3 μ g of inhibitor. The electrophoresis was run in agarose gel (1 g/100 ml of Tris/barbiturate buffer, pH 8.6); a potential gradient of 1 V/mm was applied for 2 h in the first dimension (anode to the left) and for 17 h in the second dimension (anode at the top). The second-dimension electrophoresis was run into agarose gel containing rabbit immunoglobulin against human serum proteins.

The effect of 6-aminohexanoic acid on the reaction of the inhibitor with plasmin has now been studied. Table 3 shows the v/v_0 values obtained, and Table 4 shows the values of K' , the dissociation constant of the inhibitor-plasmin complex, C_1 , and k_{+2} , the rate constant of the second step, both calculated with the equations that apply to reaction 3 (Christensen & Clemmensen, 1977). It is seen that 6-aminohexanoic acid influences the reaction of the inhibitor with plasmin in a manner similar to that of L-lysine. The rate of the second step is unaffected by the presence of these antifibrinolytic amino acids, but the equilibrium that is reached in the first step is affected, the apparent dissociation constant being $K' = K(1 + m/K_D)$, where m is the concentration of the modifying compound, and K_D is the corresponding inhibition constant. K_D is 5 mM for 6-aminohexanoic acid. The dissociation constant for the 6-aminohexanoic acid-plasmin complex in which 6-aminohexanoic acid interacts with the active site of plasmin (competitive inhibition) is 58 mM (Landmann, 1973; Christensen, 1978). The observed inhibition of the reaction of the inhibitor with plasmin therefore is not due to blocking of the active site of plasmin.

Since affinity chromatography on plasminogen-substituted CH-Sepharose 4B is a useful step in the method of purification of the inhibitor, it is known that the inhibitor binds to plasminogen and that the inhibitor-plasminogen complex dissociates considerably more readily than does the inhibitor-

Table 2. Inhibition of plasmin by the primary inhibitor of plasmin

v/v_0 is the experimental value of the fraction of plasmin free to react after incubation of plasmin and inhibitor at equal concentrations. $(v/v_0)^c$ is the theoretical value of this fraction calculated from reaction 3. k_{+1} (I) is the value of the rate constant of reaction 1 calculated by using the experimental v/v_0 value. Initial velocities (v) of plasmin-catalysed hydrolysis of Bz-Arg-OEt were measured, 50 μ l of Bz-Arg-OEt (20 mM) was added to the incubation mixture (1450 μ l) at time t (i). The buffer was Tris/HCl (0.05 M)/NaCl (0.1 M), pH 7.8, at 25°C.

Incubation time, t (i) (s)	Initial concentrations of plasmin and inhibitor (nM)	v/v_0	$10^{-6}k_{+1}$ (I) ($M^{-1}\cdot s^{-1}$)	$(v/v_0)^c$
20	78	0.19	2.7	0.18
40	78	0.17	1.5	0.17
60	78	0.15	1.2	0.15
120	78	0.14	0.8	0.13
600	78	0	—	0.03
20	156	0.14	2.0	0.13
40	156	0.12	1.2	0.12
60	156	0.12	0.8	0.11
120	156	0.08	0.6	0.09
600	156	0	—	0.02
20	312	0.104	1.4	0.096
40	312	0.091	0.8	0.090
60	312	0.082	0.6	0.084
120	312	0.056	0.5	0.069
600	312	0.01	—	0.015

Table 3. Inhibition of plasmin by the primary inhibitor of plasmin in the presence of 6-aminohexanoic acid

The fraction of plasmin free to react after incubation of plasmin and inhibitor in the presence of 6-aminohexanoic acid is v/v_0 (see Table 2).

Incubation time, t (i) (s)	Initial concentrations of plasmin and inhibitor (nM)	v/v_0		
		Concentration of 6-aminohexanoic acid (mM)...		
		5	10	25
20	78	0.23	0.30	0.37
60	78	0.21	0.25	0.33
120	78	0.18	0.21	0.27
600	78	0	0	0.06
20	156	0.18	0.21	0.29
60	156	0.16	0.18	0.25
120	156	0.13	0.15	0.21
600	156	0	0.03	0.04
20	312	0.13	0.16	0.21
60	312	0.11	0.14	0.19
120	312	0.09	0.11	0.15
600	312	0.02	0.02	0.03

Table 4. Inhibition of plasmin by the primary inhibitor of plasmin in the presence of 6-aminohexanoic acid
Dissociation constants, K' , and rate constants, k_{+2} , of the inhibitor-plasmin reaction in the presence of 6-aminohexanoic acid determined from the measured initial velocities shown in Table 3 by using the equations that apply to reaction 3. From $K' = K(1 + m/K_D)$, where $K = 3 \text{ nM}$ and m is the concentration of 6-aminohexanoic acid, we get $K_D = 5 \text{ mM}$.

Initial concentrations of plasmin and inhibitor (nM)	Concentration of 6-amino-hexanoic acid, m (mM)	Apparent dissociation constant, K' (nM)	$10^3 k_{+2}$ (s^{-1})
78	5	5.4	5.
156	5	6.2	6.5
312	5	6.1	6.5
78	10	10.0	7.
156	10	8.7	6.7
312	10	9.5	7.1
78	25	16.9	6.3
156	25	18.5	6.8
312	25	17.4	6.7

Table 5. Inhibition of plasmin by the primary inhibitor of plasmin in the presence of plasminogen with N-terminal lysine

The values v/v_0 are those obtained from the kinetic measurements, and the values $(v/v_0)^c$ are those calculated from reaction 3 assuming the dissociation constant of the inhibitor-plasminogen complex to be $3 \mu\text{M}$. Initial concentrations of plasmin and inhibitor were both 78 nM (see Table 2).

Incubation time (s)	Concentration of plasminogen (μM)	v/v_0	$(v/v_0)^c$
20	1.0	0.20	0.20
60	1.0	0.15	0.18
120	1.0	0.14	0.15
600	1.0	0	0.03
20	10.0	0.34	0.33
60	10.0	0.30	0.29
120	10.0	0.24	0.24
600	10.0	0	0.05

plasmin complex(es). The existence of a inhibitor-plasminogen complex *in vivo* may be of some physiological importance, and therefore the value of its dissociation constant may be of some interest. We have studied the reaction of the inhibitor with plasmin in the presence of plasminogen, and the results are shown in Table 5. The value of the dissociation constant for the inhibitor-plasminogen complex determined in this way is approx. $3 \mu\text{M}$, that is, 10^3 times the value for the inhibitor-plasmin complex first formed.

Table 6. Inhibition of trypsin by the primary inhibitor of plasmin

For details see Table 2.

Incubation time (s)	Initial concentrations of trypsin and inhibitor (nM)	v/v_0	$10^{-6} k_{+1}$ (l) ($\text{M}^{-1} \cdot \text{s}^{-1}$)
20	61	0.22	2.9
40	61	0.13	2.8
60	61	0.09	2.9
120	61	0.05	2.7
600	61	0	—
20	122	0.14	2.4
40	122	0.07	2.6
60	122	0.05	2.8
120	122	0	—
600	122	0	—
20	244	0.068	2.8
40	244	0.036	2.7
60	244	0.023	2.9
120	244	0	—
600	244	0	—

Kinetics of the inhibitor-trypsin reaction

Tables 6 and 7 show some results obtained for the reaction of the inhibitor with bovine trypsin. The results indicate that this reaction is less complicated than is the interaction between the inhibitor and plasmin, since, as seen from Table 6, reaction 1 is a satisfactory model. The reaction is inhibited in the presence of 6-aminohexanoic acid, and the corresponding inhibition constant is 27 mM (Table 7). The dissociation constant for the 6-aminohexanoic acid-trypsin complex, in which 6-aminohexanoic acid interacts with the active site of trypsin (competitive inhibition) was determined to be 24 mM (Fig. 3).

Discussion

A method of purification of the primary inhibitor of plasmin from human plasma has been presented. Two different molecular species of the inhibitor seem to exist. They are indistinguishable immunochemically, both interact with plasmin, but only one of them interacts with plasminogen, and that is the one that has been purified. The final preparation generally was approx. 17 ml of a solution of the inhibitor ($5\text{--}6 \mu\text{M}$) of which $70\text{--}80\%$ was active. The yield therefore is approx. 15% of the total amount of inhibitor present in plasma. If it is assumed that the inhibitor material that shows affinity for plasminogen constitutes 60% of the total amount of inhibitor in plasma, as it does in the material applied on the plasminogen-substituted column, then the yield of this form is approx. 25% .

Table 7. *Inhibition of trypsin by the primary inhibitor of plasmin in the presence of 6-aminohexanoic acid*
 k'_{+1} (I) is the value of the rate constant of reaction 1 calculated by using the experimental v/v_0 value. k'_{+1} (I) depends on the concentration of 6-aminohexanoic acid, m , thus k'_{+1} (I) = k_{+1} (I)/(1 + m/K_D). If k_{+1} (I) is taken as $2.8 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$ (Table 6), the results lead to K_D of 27 mM.

Incubation time (s)	Initial concentrations of trypsin and inhibitor (mM)	v/v_0			$10^{-6}k'_{+1}$ (I) ($\text{M}^{-1}\cdot\text{s}^{-1}$)		
		Concentration of 6-aminohexanoic acid (mM)...					
		5	30	90	5	30	90
20	61	0.26	0.39	0.54	2.3	1.3	0.70
60	61	0.10	0.17	0.30	2.5	1.3	0.64
120	61	0.06	0.08	0.18	2.1	1.6	0.62
600	61	0	0	0	—	—	—
20	122	0.15	0.24	0.38	2.3	1.3	0.67
60	122	0.05	0.10	0.17	2.6	1.2	0.67
120	122	0.03	0.05	0.10	2.2	1.3	0.61
600	122	0	0	0	—	—	—
20	244	0.080	0.138	0.246	2.4	1.3	0.63
60	244	0.027	0.046	0.092	2.5	1.4	0.67
120	244	0.015	0.023	0.052	2.2	1.5	0.62
600	244	0	0	0	—	—	—

Mechanisms of the reactions of the inhibitor with plasmin and with trypsin

It has previously been found that L-lysine inhibits the first step of the inhibitor-plasmin reaction, and that the corresponding inhibition constant for L-lysine is approx. 24 mM (Christensen & Clemmensen, 1977). We have found the dissociation constant of the L-lysine-plasmin complex, in which L-lysine interacts with the active site (competitive inhibition) to be 192 mM (Christensen, 1978). Therefore the dissociation constant is 25 mM for either an L-lysine-inhibitor complex that does not react with the enzyme, or an L-lysine-plasmin complex that does not react with the inhibitor, even though L-lysine is not situated in the active site of plasmin.

6-Aminohexanoic acid inhibits the reactions of the inhibitor with plasmin and with trypsin. The effect on the inhibitor-trypsin reaction corresponds to competitive inhibition of trypsin, and its most obvious explanation is therefore that a trypsin complex with a compound situated in the active site does not react with the inhibitor and that the only trypsin site of importance is the active site. The rather simple reaction 1 fits the reaction of the inhibitor with trypsin. The interaction of the inhibitor with plasmin is more complicated. As seen from the values of the inhibition constants, the effects of antifibrinolytic amino acids on this reaction are not parallel with the competitive inhibitions of plasmin. It seems to be well established that plasmin possesses at least one site (other than the active site) that interacts with antifibrinolytic amino acids and that the second site(s) bind antifibrinolytic amino acids more strongly than does the active site (Landmann, 1973; Richli & Otawsky, 1975;

Christensen, 1978). The effects of antifibrinolytic amino acids on the reaction of the inhibitor with plasmin may be explained by assuming that the first step of the reaction is either dependent on second site(s) of plasmin or on some site(s) of the inhibitor at which antifibrinolytic amino acids bind. The reactions of the inhibitor with plasmin and with trypsin are both inhibited by 6-aminohexanoic acid. The corresponding inhibition constants are 5 mM for plasmin and 27 mM for trypsin. From this difference we may argue that in both cases it is highly unlikely that the inhibitory effect of 6-aminohexanoic acid arises from a complex it may form with the inhibitor. This is, however, not necessarily a valid argument. The reaction mechanisms are different and a less complicated inhibitor-trypsin reaction possibly does not involve all of the inhibitor sites necessary for the inhibitor-plasmin reaction. But since the inhibitor shows only a weak interaction with a column of AH-Sepharose 4B substituted with 6-aminohexanoic acid and no affinity to lysine-substituted Sepharose 4B (Moroi & Aoki, 1976), the inhibitory effect of 6-aminohexanoic acid on inhibitor-enzyme reactions apparently do arise from complexes it forms with the enzymes. The reaction of the inhibitor with plasmin thus apparently is dependent on the second site(s) of plasmin. Antifibrinolytic amino acids bind more strongly at the second site(s) than at the active site of plasmin. It seems reasonable to suggest that the second site(s) holds the specificity of plasmin, serves to increase the rate of protein-plasmin reactions and serves to position optimally the susceptible areas of specific substrates and inhibitors at the active site. The association of the primary inhibitor of plasmin with plasmin is very fast, the association rate constant being

Fig. 3. Inhibition of trypsin-catalysed hydrolysis of α -N-benzoyl-L-arginine *p*-nitroanilide by 6-aminohexanoic acid

The initial velocities (v , $\text{nm}\cdot\text{s}^{-1}$) obtained at substrate concentrations (s) in the range 0.167–0.667 mM, trypsin concentration, $e_0 = 72 \text{ nM}$, and at concentrations of 6-aminohexanoic acid, $i = 0$ (Δ), 16.67 ($\circ$), and 33.33 ($\square$) mM are illustrated by Lineweaver–Burk plots. The kinetic parameters were obtained from fits to the equation:

$$v/e_0 = k_c/[1 + (K_m(i)/s)]$$

In the insert, $K_m(i)$ is the apparent Michaelis constant obtained at concentration (i) of 6-aminohexanoic acid. Linear competitive inhibition was found. The inhibition constant, $K_i = 24 \text{ mM}$. Experimental conditions: buffer, Tris/HCl (0.05 M)/NaCl (0.1 M), pH 7.8; temperature 25°C. The kinetic parameters for unmodified trypsin-catalysed hydrolysis of α -N-benzoylarginine *p*-nitroanilide obtained were $k_c = 2.6 \pm 0.3 \text{ s}^{-1}$ and $K_m(0) = 0.9 \pm 0.1 \text{ mM}$. These values are in reasonable agreement with those of Erlanger *et al.* (1961) and Yoshida *et al.* (1971).

greater than $10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$ (pH 7.8). The dissociation constant of the complex first formed is approx. 1 nM,

therefore the dissociation rate constant, k_{-1} , is of the same order of magnitude or greater than k_{+2} , the rate constant of the irreversible transformation to the other complex. This means that freshly formed inhibitor–plasmin complex may be a source of plasmin, when the concentration of free plasmin is very low.

Note added in proof

Wiman, B. and Collen, D. have just published a paper reporting on the kinetics of the reaction between antiplasmin and plasmin at pH 7.3 [(1978) *Eur. J. Biochem.* **84**, 573–578]. These authors advocate the proposals of Christensen & Clemmensen (1977), first, that the reaction is a two-step process and secondly that antifibrinolytic amino acids inhibits the first, but not the second step. They have further given a value of the association rate constant k_{+1} .

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