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Soluble fibrin preparations inhibit the reaction of plasmin with α_2-macroglobulin

Comparison with α_2-antiplasmin and leupeptin

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The kinetics of plasmin inhibition by α_2-antiplasmin (α_2AP), α_2-macroglobulin (α_2M) and leupeptin were studied in the presence of fibrin monomer (Fn) and CNBr fragments of fibrinogen (Fg-CNBr). Active plasmin was detected in continuous and discontinuous assays using the chromogenic substrate D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251). The two 'fibrin-like' preparations functioned as hyperbolic mixed-type inhibitors of S-2251 hydrolysis. The dissociation constants (K_F) for the binding of plasmin to Fn and Fg-CNBr were 22 nM and 17 nM respectively. Fn and Fg-CNBr inhibited the reaction of plasmin with α_2AP; the extent of inhibition depended on the fibrin concentration. In the presence of 800 nM-Fn or 800 nM-Fg-CNBr, the experimental second-order rate constant ($k''_{app.}$) was decreased from 2.4×10^7 $M^{-1} \cdot s^{-1}$ to 1.2×10^6 and 5.3×10^5 $M^{-1} \cdot s^{-1}$ respectively. The effect of Fn and Fg-CNBr on the rate of plasmin inhibition by α_2M was even greater. The $k''_{app.}$ value was decreased from 4.0×10^5 $M^{-1} \cdot s^{-1}$ to 8.0×10^2 and 1.3×10^3 $M^{-1} \cdot s^{-1}$ in the presence of 800 nM-Fn and -Fg-CNBr respectively. By contrast, the fibrin preparations caused only a small change in the rate of plasmin inhibition by leupeptin. The maximum change in $k''_{app.}$ was 3-fold. All plasmin inhibition curves were linear, suggesting that free and fibrin-bound forms of plasmin remained in equilibrium during the course of reaction with proteinase inhibitors. Fn and Fg-CNBr had no effect on the reaction of miniplasmin with S-2251, α_2AP or α_2M. When ^{125}I-plasmin was incubated with Fg-CNBr and then allowed to react with a premixed solution of α_2AP and α_2M, the Fg-CNBr did not significantly change the percentage of plasmin bound to α_2AP. These experiments demonstrate that the reaction of plasmin with α_2M is inhibited by the non-covalent binding of plasmin to fibrin. We propose that plasmin bound to the surface of a clot is protected from inhibition by α_2M as well as by α_2AP.

INTRODUCTION

In the circulation, fibrin and fibrinogen are digested primarily by one enzyme, plasmin [1]. Other less-well-defined plasmin substrates are located in the extracellular matrix and on cell surfaces [2,3]. In these environments, plasmin may play an important role in cell movement, growth and tumour metastasis [4]. Studies on tissue-type plasminogen activator and urokinase suggest that plasminogen is activated primarily in association with other molecules such as fibrin or fibronectin [5–8] or on the surfaces of cells such as endothelium and hepatocytes [9–11]. At the cell surface, plasmin may provide critical anticoagulant activity and counteract thrombogenesis [12]. Selective activation of plasminogen restricts plasmin to necessary sites and limits systemic fibrinogenolysis.

The plasma proteinase inhibitor α_2-antiplasmin (α_2AP) reacts with solution-phase plasmin extremely rapidly [13,14]; the second-order rate constant for the reaction is $(2–4) \times 10^7$ $M^{-1} \cdot s^{-1}$ [13]. An essential component of this reaction is the non-covalent binding interaction between the Kringle 1 (K1) domain of plasmin and residues 435–452 near the *C*-terminus of α_2AP [14–16]. When the K1 region of plasmin is blocked by association with fibrin [14] or cell-surface plasmin receptors [9], the rate of plasmin inhibition by α_2AP is significantly decreased. This delicate balance provides a second mechanism for localizing fibrinolytic activity and for preventing systemic fibrinogenolysis.

In the plasma, the second most rapid inhibitor of plasmin is α_2-macroglobulin (α_2M). The second-order rate constant for the reaction of solution-phase plasmin with α_2M is approx. 5×10^5 $M^{-1} \cdot s^{-1}$ [17–19]. The plasma concentration of α_2M is three times greater than that of α_2AP. In addition, the reaction of plasmin with α_2M does not depend on the K1 plasmin domain [17]. On the basis of this information, Steiner *et al.* [17] suggested that α_2M may pose a significant inhibitory challenge to plasmin in α_2AP-resistant environments.

The reaction of α_2M with proteinases bound to other macromolecules such as the plasmin–fibrin complex remains poorly understood. Steiner *et al.* [17] demonstrated only a slight decrease in the rate of plasmin inhibition by α_2M when the plasmin was bound to histidine-rich glycoprotein. Binding of pancreatic elastase to elastin also does not affect the rate of inhibition by α_2M [20]. This result contrasts α_2M with the serpin (**serine-proteinase inhibitor**) α_1-proteinase inhibitor (α_1PI); elastin decreases the rate of reaction of elastase with α_1PI more than 1000-fold (20).

In the present investigation the inhibition of plasmin by purified α_2M, α_2AP and leupeptin was studied in the presence of CNBr fragments of fibrinogen (Fg-CNBr) and fibrin monomer (Fn). These soluble 'fibrin-like' preparations mimic the fibrin clot with regard to proteinase-binding interactions [21,22]. Neither is completely representative of fibrin, since the restraints of polymerization are removed and because the Fn and Fg-

Abbreviations used: α_2AP, α_2-antiplasmin; α_2M, α_2-macroglobulin; Fg-CNBr, CNBr fragments of fibrinogen; Fn, fibrin monomer; K1, Kringle 1 domain of plasmin; α_1PI, α_1-proteinase inhibitor; F, Fg-CNBr or Fn; SBTI, soybean trypsin inhibitor; PNPGB, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; BAPNA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; S-2251, D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride; TBS, Tris-buffered saline.

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CNBr are exposed to denaturants during preparation. The purpose of the present study was to determine whether non-covalent plasmin binding interactions might affect the inhibitory activity of α_2M. The soluble fibrin preparations permitted a quantitative analysis of reaction rates. Since Fn and Fg-CNBr significantly decreased the rate of plasmin inhibition by α_2AP, we considered the two preparations appropriate model systems for studying plasmin/proteinase inhibitor reactions. The results obtained with α_2M demonstrate for the first time that plasmin is protected from this inhibitor when the proteinase is bound to fibrin. Thus the reaction of plasmin with α_2M may be modulated by non-covalent plasmin binding interactions, localizing fibrinolysis in a manner analogous to α_2AP.

MATERIALS AND METHODS

Materials

p-Nitrophenyl *p'*-guanidinobenzoate hydrochloride (PNPGB) and N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA) were purchased from Sigma. D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) was from Kabi Vitrum, Stockholm, Sweden. $Na^{125}I$ was from Amersham International, and Iodobeads were from Pierce. Soybean trypsin inhibitor (SBTI) was purchased from Sigma.

Proteins

[Glu^1]Plasminogen was prepared from human plasma by the method of Deutsch & Mertz [23]. The final preparations included carbohydrate variants I and II, with variant II present in slight excess. Miniplasminogen was prepared by digesting plasminogen with porcine pancreatic elastase and purified by chromatography on lysine–Sepharose [24,25].

Miniplasminogen and plasminogen were activated with low-molecular-mass urokinase (Calbiochem) as previously described [26]. The concentration of active proteinase was determined by titration with α_2AP. α_2M and α_2AP were purified from human plasma by the methods of Imber & Pizzo [27] and Wiman [28] respectively.

Human fibrinogen, grade L, was obtained from Kabi Vitrum (Stockholm, Sweden). The protein was dissolved at 5–10 mg/ml in Tris-buffered saline (TBS; 50 mM-Tris/HCl/150 mM-NaCl, pH 7.4) and extracted with lysine–Sepharose to remove plasminogen as previously described [29]. Factor XIII in fibrinogen preparations was inactivated by dialysis against 3.3 M-urea/0.1 M-sodium phosphate, pH 7.4, for 14 h at 22° C, followed by exhaustive dialysis against TBS [30]. Fibrin monomer (Fn) was prepared from urea-treated fibrinogen as previously described, except for the use of 3.3 M-urea instead of NaBr to dissolve the clot [29]. Fg-CNBr was prepared by the method of Blombäck *et al.* [31]. The freeze-dried fragments were reconstituted to a final concentration of 4 mg/ml in TBS as described by Fears [22].

Radioiodination

Plasminogen was radioiodinated with Iodobeads as described by the manufacturer (Pierce). Desalting was performed on Sephadex G-25 (Pharmacia). The specific activity ranged from 1.25 to 2.0 μCi/μg.

Plasmin amidase activity in the presence of Fn and Fg-CNBr

Plasmin (18 nM) and different concentrations of Fn or Fg-CNBr (0–600 nM) were incubated in the sample cuvette of a Hewlett–Packard 8450 diode-array spectrophotometer at 22° C. The incubation buffer was 20 mM-sodium phosphate/150 mM-NaCl/0.05% Tween 80, pH 7.4 (PBS-T). After 5 min, S-2251 (0.065–0.78 mM) was added and the A_{406} was measured every 2 s for 100 s. The velocity of substrate hydrolysis was determined by transforming absorbance measurements into the first derivative function (dA_{406}/dt). The data were analysed by Lineweaver–Burk plots, which were uniformly linear with correlation coefficients ranging from 0.993 to 1.000. Kinetic parameters were derived by averaging the results of three separate experiments. Equivalent studies were performed with miniplasmin and Fn or Fg-CNBr.

Inhibition of plasmin and miniplasmin by α_2AP

The reaction of plasmin with α_2AP was studied in the presence of S-2251 and different concentrations of Fn or Fg-CNBr (0–800 nM). The equilibria and reactions occurring in this system were modelled as follows:

$$\begin{array}{ccc} \text{PS} & & \text{PSF} \\ {\scriptstyle K_m}\uparrow\downarrow & & {\scriptstyle \beta K_m}\uparrow\downarrow \\ \text{S} & & \text{S} \\ + & & + \\ & {\scriptstyle K_F} & \\ \text{P} + \text{F} & \rightleftharpoons & \text{PF} \\ + & & + \\ \alpha_2\text{AP} & & \alpha_2\text{AP} \\ {\scriptstyle k''}\downarrow & & {\scriptstyle \alpha k''}\downarrow \\ \text{P-}\alpha_2\text{AP} & & \text{P-}\alpha_2\text{AP-F} \end{array}$$

Plasmin (18 nM) (referred to as P) and Fn or Fg-CNBr (referred to as F) were incubated in PBS-T for 5 min at 22° C. S-2251 (0.54 mM) (referred to as S) was then added, and the A_{406} was determined every 2 s. At 100 s, the solution was made 18 nM in α_2AP and monitoring was continued for an additional 300 s. The ratio of active plasmin at zero time to that at time t (P_0/P_t) was determined from the velocity of substrate hydrolysis (dA_{406}/dt), assuming that free plasmin and plasmin–F complex are in reversible equilibrium throughout the reaction. In a reversible equilibrium system with [F] > [plasmin], the ratio of free plasmin to plasmin–F complex remains constant, so that differences in the rate of S-2251 cleavage by the two forms of the enzyme do not affect the data. Apparent second-order rate constants ($k''_{app.}$) were determined according to the relationship.

$$\frac{P_0}{P_t} = \frac{k''_{app.} \cdot t \cdot P_0}{\left(1+\frac{[S]}{K_m}\right)} + 1 \qquad (1)$$

This equation is a simplified version of the complete expression which is written in terms of k'' (the second-order rate constant for the reaction of α_2AP with plasmin in the absence of F) instead of $k''_{app.}$:

$$\frac{P_0}{P_t} = P_0 \cdot k'' \left[\frac{1}{\left(1+\frac{[S]}{K_m}\right)\left(1+\frac{[F]}{K_F}\right)} + \frac{\alpha}{\left(1+\frac{[S]}{\beta K_m}\right)\left(1+\frac{K_F}{[F]}\right)} \right] \cdot t + 1 \qquad (2)$$

K_F is the dissociation constant describing the reversible equilibrium between free plasmin and plasmin–F complex. The second-order rate constant for the reaction of α_2AP with plasmin–F complex is k'' multiplied by the constant α. The term $(1+[S]/K_m)$ in eqn. (1) corrects for the presence of substrate during the reaction. To determine $k''_{app.}$ values, the K_m (Michaelis constant) for the hydrolysis of S-2251 by plasmin in the absence of F (0.18 mM) was used regularly. At high concentrations of F this K_m value is an approximation (as shown in eqn. 2); however, our S-2251 hydrolysis experiments indicate that the substrate correction factor has a maximum error of only 35–40% (insignificant compared with demonstrated changes in $k''_{app.}$). For each concentration of F, the plot of P_0/P_t against $P_0 \cdot t$ was linear through more than two half-lives. Significant substrate depletion did not occur within the 400 s time course, since

dA_{406}/dt decreased by less than 5% when plasmin was incubated in the absence of inhibitor. Each concentration of Fn or Fg-CNBr was studied in triplicate.

The reaction of miniplasmin (18 nM) with α_2AP was studied as described above, except that the inhibitor was present in large molar excess (180 nM). Each plot of log (dA_{406}/dt) against time was linear through greater than two half-lives. Apparent pseudo-first-order rate constants ($k'_{app.}$) were calculated according to the following equation:

$$\log\left(\frac{P_t}{P_0}\right) = \frac{k'_{app.} \cdot t}{-2.303\left(1+\frac{[S]}{K_m}\right)} \quad (3)$$

The $k'_{app.}$ is related to the rate constant for the reaction of α_2AP with miniplasmin in the absence of F by the expression:

$$k'_{app.} = k'\left[\frac{1}{\left(1+\frac{F}{K_F}\right)} + \frac{\alpha}{\left(1+\frac{K_F}{F}\right)}\right] \quad (4)$$

The K_m for the hydrolysis of S-2251 by miniplasmin was 0.13 mM [25]. This value is accurate in the presence and absence of F, as demonstrated in the S-2251 hydrolysis experiments presented below. For comparison of data, the $k'_{app.}$ values were converted into apparent second-order rate constants ($k''_{app.}$), assuming a linear relationship between $k'_{app.}$ and α_2AP concentration. All studies were performed at least in triplicate.

Inhibition of plasmin and miniplasmin by α_2M

The inhibition of plasmin and miniplasmin by α_2M was studied by using a modification of the Ganrot assay [32]. Proteinase (3.33 nM) and α_2M (33.3 nM) were incubated with different concentrations of Fn or Fg-CNBr (0–800 nM) in PBS-T at 22° C. At various times, samples of the incubation mixture were removed and the free proteinase in these samples was inactivated with 20 μM-SBTI. In separate control experiments, it was determined that 20 μM-SBTI was sufficient to inactivate all of the plasmin or miniplasmin within less than 4 s, even in the presence of Fn or Fg-CNBr. The amidase activity of α_2M-plasmin and α_2M-miniplasmin complex was not inhibited by SBTI, and the relationship between α_2M–proteinase concentration and S-2251 hydrolysis rate in the presence of SBTI was linear, as previously described [32,33].

The extent of reaction of α_2M with proteinase at various times was determined by measuring the increase in SBTI-resistant amidase activity. Apparent pseudo-first-order rate constants ($k'_{app.}$) were determined from plots of log (P_t/P_0) against time. $k''_{app.}$ values were determined by assuming a linear relationship between $k'_{app.}$ and α_2M concentration. The amidase activity of α_2M-plasmin and α_2M-miniplasmin was not affected by Fg-CNBr or Fn. All experiments were performed in triplicate.

Plasmin inhibition by leupeptin

The reaction of plasmin (18 nM) with leupeptin (10 μM) was studied using the procedure described for miniplasmin and α_2AP. Plasmin was incubated with leupeptin in the presence of S-2251 after pre-incubation with Fn or Fg-CNBr (0–800 nM) for 5 min. The concentration of active proteinase was determined at 2 s intervals from the velocity of substrate hydrolysis (dA_{406}/dt). $k'_{app.}$ values were determined from plots of log (P_t/P_0) against time. Each study was performed in triplicate.

Plasmin inhibition by mixtures of α_2M and α_2AP

^{125}I-Plasminogen was activated with urokinase and incubated with Fg-CNBr (0–800 nM) in PBS-T at 22° C for 5 min. A premixed solution of α_2AP and α_2M was then added. The final concentrations of plasmin, α_2AP, and α_2M were 18 nM, 18 nM, and 720 nM respectively. These concentrations were selected so that the plasmin would distribute nearly equally between the two inhibitors in the absence of Fn and Fg-CNBr. The proteinase inhibitors were allowed to react for 45 min. Reactions were terminated with 0.1 mM-PNPGB. Samples were denatured under non-reducing conditions and subjected to SDS/PAGE on 5% slabs using the Hepes/imidazole, pH 7.3, buffer system described by McLellan [34], as modified by Gonias & Figler [25]. Inhibitor–plasmin complexes were clearly resolved from unchanged ^{125}I-plasmin. Gels were autoradiographed, and each lane was sliced into 3 mm sections for counting in an LKB model 1275 Minigamma γ-radiation counter (efficiency greater than 75%). This procedure quantifies the free ^{125}I-plasmin and the ^{125}I-plasmin covalently bound to α_2M and α_2AP. For the reaction of plasmin with human α_2M, more than 95% of the complex which forms is covalent when analysed using this SDS/PAGE system [19].

Fibrin digestion by plasmin

In the proteinase-inhibition experiments, plasmin was incubated with Fn or Fg-CNBr for 5 min before adding α_2AP or α_2M. In order to determine the extent of Fn or Fg-CNBr digestion, these preparations were treated with plasmin for different periods of time and then analysed by SDS/PAGE under reducing and non-reducing conditions.

RESULTS

Effect of Fn and Fg-CNBr on plasmin amidase activity

The interaction of plasmin with fibrin may be mediated by the K1 domain, the aminohexyl-binding site and/or the enzyme active site [5,35,36]. Any of these interactions may alter plasmin amidase activity. Since S-2251 was used to study the reaction of plasmin with α_2AP, α_2M and leupeptin in the presence of Fn and Fg-CNBr, the effect of the fibrin preparations on plasmin amidase activity was investigated.

For the hydrolysis of chromogenic substrate, the experimentally determined kinetic parameters, $k_{cat.}$ and K_m, reflect both acylation and deacylation rates [37]. Fig. 1 shows Lineweaver–Burks plots for the hydrolysis of S-2251 by plasmin in the presence of different concentrations of Fn (Fig. 1*a*) and Fg-CNBr (Fig. 1*b*). The K_m and $k_{cat.}$ values, determined in the absence of Fn and Fg-CNBr, were 179 ± 4 μM and 11.7 ± 0.1 s^{-1} respectively. Different concentrations of Fn or Fg-CNBr increased the apparent K_m and decreased the apparent $k_{cat.}$ (Table 1). The slopes from the Lineweaver–Burk graphs and the apparent K_m values were plotted against Fn or Fg-CNBr concentration. In both cases, hyperbolic functions were demonstrated. Replots of 1/slope versus 1/[F] were linear (Figs. 1*c* and 1*d*). These results indicate that Fn and Fg-CNBr functioned as hyperbolic mixed-type inhibitors of S-2251 hydrolysis. The dissociation constants (K_F) for the interaction of plasmin with Fn and Fg-CNBr (determined as $-1/x$ intercept in Figs. 1*c* and 1*d*) were 22 nM and 17 nM respectively.

Cleavage of S-2251 by miniplasmin was studied in the presence of Fn and Fg-CNBr (0–800 nM). The rate of substrate hydrolysis was not significantly changed by either fibrin preparation. Other investigators have demonstrated miniplasmin binding to fibrin(ogen) preparations [36]. The results presented here indicate that either the Fn and Fg-CNBr do not bind significant amounts of the miniplasmin or that miniplasmin binding to Fn and Fg-CNBr does not affect S-2251 hydrolysis. Importantly, the unchanged S-2251 hydrolysis rate in the presence of Fn and Fg-CNBr confirms that the plasmin data shown in Fig. 1 are not

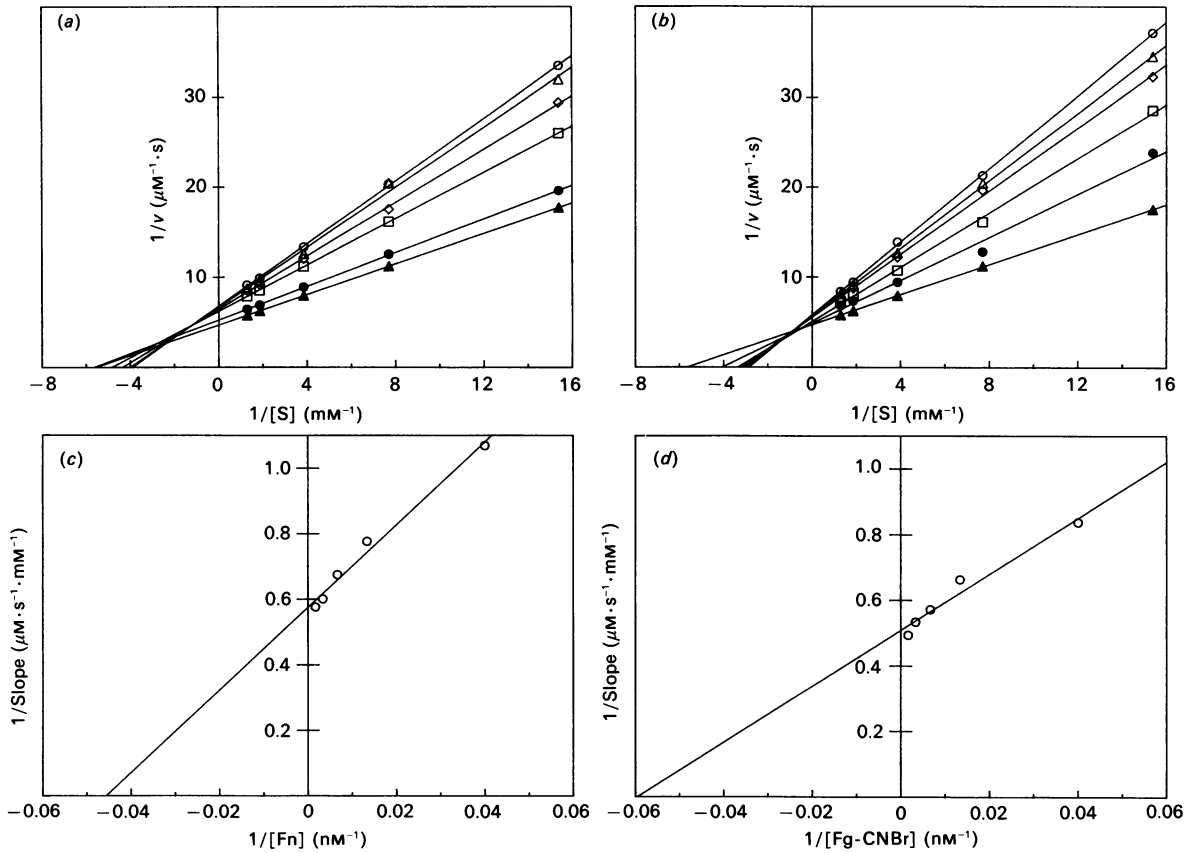

Fig. 1. Steady-state plasmin amidase activity in the presence of Fn and Fg-CNBr

Plasmin (18 nM) was allowed to react with S-2251 (0.065–0.78 mM) at 22° C as described in the text. Fn (*a*) and Fg-CNBr (*b*) were present at concentrations of 0 (▲), 25 (●), 75 (□), 150 (◇), 300 (△), and 600 (○) nM. The slopes from (*a*) were plotted against [Fn] in a double-reciprocal plot in panel (*c*). The slopes from panel (*b*) were plotted against [Fg-CNBr] in a double-reciprocal plot in panel (*d*).

Table 1. Steady-state kinetic parameters for the hydrolysis of S-2251 by plasmin in the presence of Fg-CNBr and Fn

All reactions were conducted at 22° C. The reported values represent the average of triplicate determinations ± S.E.M.

	Fg-CNBr		Fn	
[Fibrin] (nM)	$K_{m,app.}$ (μM)	$k_{cat.,app.}$ (s^{-1})	$K_{m,app.}$ (μM)	$k_{cat.,app.}$ (s^{-1})
0	179±4	11.7±0.1	179±4	11.7±0.1
25	245±4	11.4±0.1	179±5	10.6±0.6
75	297±59	10.9±1.7	207±33	8.9±0.6
150	315±32	10.0±0.9	230±37	8.6±0.3
300	330±114	9.8±1.7	249±45	8.3±0.4
600	349±154	9.6±2.0	256±102	8.2±0.7

attributable to interactions of S-2251 or *p*-nitroanilide with the fibrin preparations.

Modification of Fn and Fg-CNBr by plasmin

Fn and Fg-CNBr were incubated with 18 nM-plasmin under the conditions used in the proteinase-inhibitor experiments. SDS/PAGE demonstrated partial digestion of both preparations within 5 min. More than half of the Fn was converted into Fragment X with small amounts of Fragments Y and D (results not shown). Digestion of the fibrin preparations was not rapid enough to cause significant changes during the short time of reaction with proteinase inhibitors. These studies indicate that the plasmin-binding activity of Fn and Fg-CNBr may have included new sites generated by fibrin cleavage. Therefore the fibrin preparations studied here may most appropriately model the partially lysed fibrin clot.

Plasmin inhibition by α_2AP

Table 2 lists the $k''_{app.}$ values determined for the inhibition of plasmin by α_2AP. The rate of this reaction was decreased as much as 44-fold by Fg-CNBr and 20-fold by Fn. The Fn and Fg-CNBr model systems have not been used before to study the activity of α_2AP; however, the results reported here are consistent with the work of previous investigators [14]. Since Fn and Fg-CNBr both decreased the rate of reaction of plasmin with α_2AP, these preparations were considered appropriate model systems for studying the reaction of plasmin with other proteinase inhibitors.

Plasmin inhibition by leupeptin

Leupeptin is a relatively small proteinase inhibitor (M_r 475.6) that reacts with and inhibits plasmin [38]. There is no evidence for interaction of leupeptin with the plasmin K1 domain. In the absence of Fn and Fg-CNBr, the second-order rate constant for the reaction of plasmin with leupeptin was 4.7×10^3 $M^{-1} \cdot s^{-1}$ (Table 2). In the presence of Fg-CNBr, at concentrations up to 800 nM, the $k''_{app.}$ was decreased 3-fold or less. Equivalent results were obtained with representative concentrations of Fn (400 and 800 nM).

Table 2. Inhibition of plasmin by proteinase inhibitors in the presence of Fn and Fg-CNBr

All reactions were conducted at 22° C. Each $k''_{app.}$ value represents the average (±S.E.M.) of triplicate determinations.

[Fibrin] (nM)	Inhibitor... α_2AP Parameter... $10^{-5} \times k''_{app.}$ ($M^{-1} \cdot s^{-1}$)		α_2M $10^{-3} \times k''_{app.}$ ($M^{-1} \cdot s^{-1}$)		Leupeptin $10^{-3} \times k''_{app.}$ ($M^{-1} \cdot s^{-1}$)
	Fn	Fg-CNBr	Fn	Fg-CNBr	Fg-CNBr
0	235.5±24.0	235.5±24.0	396.7±40.9	396.7±40.9	4.7±0.6
50	114.1±3.8	72.5±7.0	20.6±1.2	45.8±4.8	2.8±0.4
100	77.4±3.6	35.7±1.9	10.9±1.1	23.7±1.5	1.8±0.4
400	24.0±6.0	8.6±0.9	2.8±0.2	2.9±0.2	1.5±0.2
800	12.2±2.1	5.3±0.8	0.8±0.1	1.3±0.2	1.4±0.1

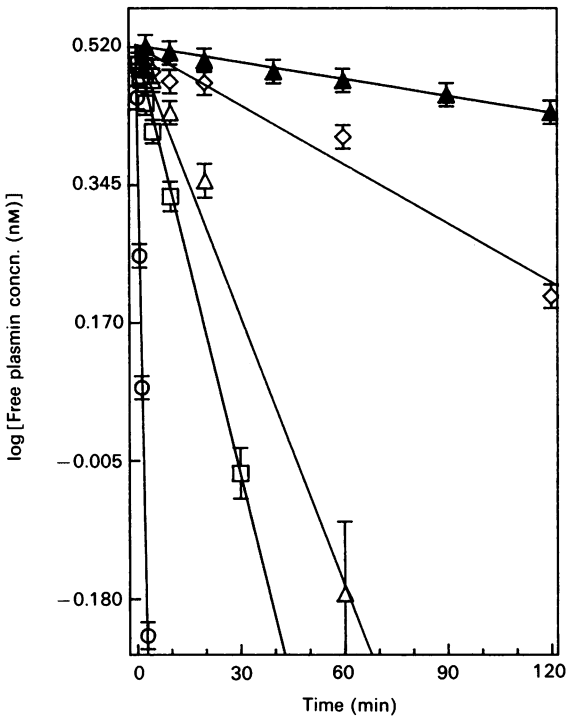

Fig. 2. Reaction of plasmin with α_2M in the presence of Fn

Plasmin (3.33 nM) was allowed to react with α_2M (33.3 nM) for up to 2 h. All reactions were conducted at 22° C. Fn was present at concentrations of 0 (○), 50 (□), 100 (△), 400 (◇), and 800 (▲) nM.

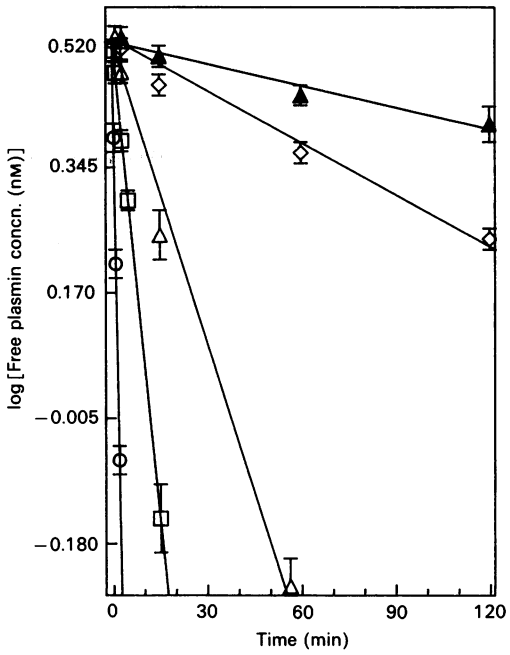

Fig. 3. Reaction of plasmin with α_2M in the presence of Fg-CNBr

Plasmin (3.33 nM) was allowed to react with α_2M (33.3 nM) for up to 2 h. All reactions were conducted at 22 °C. Fg-CNBr was present at concentrations of 0 (○), 50 (□), 100 (△), 400 (◇), and 800 (▲) nM.

Plasmin inhibition by α_2M

The reaction of plasmin with α_2M was studied after preincubating the plasmin with Fn (Fig. 2) or Fg-CNBr (Fig. 3). In the absence of the fibrin preparations, the second-order rate constant for the reaction of α_2M with plasmin was $(4.0 \pm 0.3) \times 10^5$ $M^{-1} \cdot s^{-1}$ (Table 2). This value is in good agreement with previously reported constants [17–19]. Fn and Fg-CNBr significantly decreased the rate of plasmin inhibition by α_2M; the apparent second-order rate constant was reduced by as much as 500-fold by Fn and 300-fold by Fg-CNBr. These studies suggest that binding of plasmin to fibrin significantly reduces the inhibitory activity of α_2M, even though the reaction of plasmin with α_2M is not dependent on the K1 domain [17].

Inhibition of plasmin by mixtures of α_2AP and α_2M

^{125}I-Plasmin was incubated with Fg-CNBr and then reacted with a mixture of α_2AP (18 nM) and α_2M (720 nM) for 45 min. In the absence of Fg-CNBr, approx. 60% of the plasmin bound to α_2M and 40% bound to α_2AP (results not shown). At maximum concentrations of Fg-CNBr (800 nM) the percentage of plasmin bound to α_2AP and to α_2M was not significantly changed. These data confirm that the plasmin-inhibitory activity of α_2M is decreased by Fg-CNBr. The magnitude of this effect is comparable with that demonstrated with α_2AP.

Miniplasmin regulation by proteinase inhibitors

Miniplasmin was allowed to react with α_2AP in the presence of Fg-CNBr (0–800 nM). As shown in Table 3, the rate of miniplasmin inhibition was not changed. Studies performed with Fn (0–800 nM) yielded similar results. The miniplasmin either did not interact with the fibrin preparations or did so in a manner that did not affect the activity of α_2AP.

The reaction of miniplasmin with α_2M was also studied in the presence of Fg-CNBr and Fn. Once again, neither fibrin preparation affected the rate of proteinase inhibition. The data collected with Fg-CNBr are presented in Table 3. These

Table 3. Reaction of miniplasmin with α_2AP and α_2M in the presence of Fg-CNBr

All reactions were conducted at 22° C. Each $k''_{app.}$ value represents the average (±S.E.M.) of triplicate determinations.

[Fg-CNBr] (nM)	Inhibitor... Parameter...	α_2AP $10^{-5} \times k''_{app.}$ ($M^{-1} \cdot s^{-1}$)	α_2M $10^{-6} \times k''_{app.}$ ($M^{-1} \cdot s^{-1}$)
0		2.2±0.4	2.2±0.1
50		3.1±0.6	2.2±0.1
100		2.7±0.6	2.2±0.1
400		2.4±0.6	1.7±0.1
800		2.3±0.3	2.1±0.5

miniplasmin regulation experiments demonstrate than Fn and Fg-CNBr do not affect the activity of α_2AP or α_2M in a non-specific (proteinase-independent) manner.

DISCUSSION

A number of macromolecules regulate fibrinolysis either by stimulating the activity of plasminogen activators or by inhibiting the activity of α_2AP. Fibrin(ogen), fibronectin, histidine-rich glycoprotein, thrombospondin, cell-surface receptors and glycolipids all demonstrate fibrinolysis-regulatory activity [2,6,7,9,11,39–43]. In the present study we have demonstrated that specific 'fibrin-like' preparations (Fn and Fg-CNBr) significantly modify the anti-plasmin activity of α_2M. The $k''_{app.}$ values were decreased 300–500-fold in the presence of 800 nM-Fn or -Fg-CNBr. Similar changes in the inhibitory activity of α_2M have not been demonstrated previously, either with plasmin or with other proteinases such as elastase [20].

Whereas α_2AP is clearly the most rapid plasmin inhibitor in the circulation on the basis of plasma concentration and kinetic rate constants, α_2M is a potent plasmin inhibitor as well. In fact, assuming an α_2M plasma concentration of 3.4 μM [1], the half-life of plasmin in the blood should be less than 0.5 s as a result of reaction with α_2M alone. Clearly this potent anti-plasmin activity of α_2M would be counterproductive in the absence of environment-specific activity modulation, as has been described for α_2AP [5,14]. On the basis of the results of this investigation, we can offer an explanation for previously published qualitative 'clot lysis' experiments, which suggested that α_2M does not significantly retard fibrinolysis *in vitro* [44]. Plasmin that is bound to the fribin clot is probably protected from inhibition by both α_2AP and α_2M.

The reaction of plasmin with α_2AP involves the K1 domain of plasmin; most macromolecules which affect this reaction do so by interfering with the K1–α_2AP non-covalent interaction [9,13,41,45]. A similar mechanism is probably not operational in the case of α_2M [17]. We speculate that the decrease in the rate of reaction of plasmin with α_2M in the presence of Fn or Fg-CNBr results from steric constraints. The additional mass of the bound fibrin molecule limits access of the α_2M to the plasmin active site. The results of the plasmin-inhibition experiments performed with leupeptin support this hypothesis. The reaction of this relatively small proteinase inhibitor with plasmin was only slightly altered by the fibrin preparations.

Any substrate, such as Fn or Fg-CNBr, may competitively inhibit the reaction of a proteinase with an inhibitor by binding to the proteinase active site. This type of interaction probably did not affect the plasmin inhibition rate constants for α_2AP or α_2M, since changes in plasmin-active-site availability were accounted for in the initial rate of S-2251 hydrolysis (dA_{406}/dt at t_0). Miniplasmin cleaved both Fn and Fg-CNBr, and this interaction was not detected in experiments with α_2AP and α_2M. An additional control for this model system was provided by the leupeptin experiments. Based strictly on competition for the plasmin active site, Fn and Fg-CNBr could not have inhibited the reaction of plasmin with α_2AP or α_2M more than 3–4-fold, as was observed with leupeptin.

In the studies presented here, plasmin–inhibitor complexes may have formed by one of two mechanisms: direct reaction of inhibitor with the plasmin–fibrin complex or plasmin dissociation followed by rapid solution-phase proteinase inhibition. Since the plasmin inhibition curves were linear in the presence of Fn and Fg-CNBr, we believe that the reversible equilibrium assumption made in the Materials and methods section (eqns. 1–3) was appropriate. Free and fibrin-bound plasmin must have exchanged at a sufficiently high rate to maintain a constant ratio of these two variably inhibited components. On the basis of the K_F values determined with S-2251, most of the plasmin was bound to Fn or Fg-CNBr when the fibrin preparations were present at concentrations greater than 100 nM. Since the slopes of the plasmin inhibition curves varied as a function of Fn or Fg-CNBr concentration, plasmin dissociation was responsible for at least part of the observed complex-formation with proteinase inhibitors. However, we cannot specify how much plasmin–inhibitor complex formed as a result of direct reaction of α_2AP or α_2M with plasmin–Fn or plasmin–Fg-CNBr.

The molecular interactions mediating the binding of plasmin to fibrin may be heterogeneous. Different domains in the plasmin structure may participate [35]. In addition, different areas of the fibrin may be involved, especially after the fibrin has been partially digested [46,47]. In the experiments presented here, it is possible that the plasmin bound to different sites on the Fn or Fg-CNBr and that each interaction affected the reaction with proteinase inhibitors comparably. Regarding the plasmin domains involved in the fibrin interactions, the data suggest that the K1 domain was principally involved. Miniplasmin, which lacks K1, did not interact with Fn or Fg-CNBr in a manner that was detectable with S-2251, α_2AP or α_2M. It is possible that miniplasmin binds to fibrin without altering the inhibitory activity of α_2AP. The same explanation is less likely for the α_2M experiments.

Effective fibrinolysis requires the generation of plasmin in protected environments. It is clearly established that a number of macromolecules provide plasmin binding sites which limit the activity of α_2AP. On the basis of the present experiments we conclude that plasmin may interact with specific macromolecules which shield proteinase activity from α_2M as well.

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