

Regulation of Fibrinolytic Activity of Neutrophil Leukocyte Elastase, Plasmin, and Miniplasmin by Plasma Protease Inhibitors*

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The effect of solid-phase fibrin on the inactivation of plasmin, miniplasmin, and neutrophil leukocyte elastase (PMN-elastase) by plasma protease inhibitors (α_2 -antiplasmin, α_1 -protease inhibitor, α_2 -macroglobulin) was studied. In Hanks' balanced salt solution, fibrin reduces the second-order rate constant for the inhibition of PMN-elastase by α_1 -protease inhibitor from $8,760 \times 10^4$ to $4 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ and by α_2 -macroglobulin from 121×10^4 to $1.8 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$. The rate constant for miniplasmin inactivation by α_2 -antiplasmin decreases from 99×10^4 to $1 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$, by α_2 -macroglobulin from 78×10^4 to $1.8 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$, and by α_1 -protease inhibitor from $0.11 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ to 0. Plasmin bound to fibrin is completely protected against α_2 -macroglobulin and α_1 -protease inhibitor, whereas the rate constant for the inactivation by its primary plasma inhibitor α_2 -antiplasmin is reduced from 430×10^4 to $1.08 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$. The competition of substrate and inhibitor for the enzyme was also studied, using fibrin preincubated with inhibitor. Under our pseudo-first-order experimental conditions, fibrin completely eliminates those interactions, the second-order rate constant of which is $1.1 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ or less in a system without fibrin surface.

The degradation of fibrin in blood circulation (fibrinolysis) is generally thought to be provided by a serine protease, plasmin, a disulfide bridge-linked two-chain molecule that results from proteolytic activation of the plasma glycoprotein plasminogen (reviewed in Refs. 1 and 2). An alternative pathway of fibrinolysis that comprises polymorphonuclear leukocyte elastase¹ (3) and its interactions with the plasminogen/plasminogen activator system has been suggested and supported by data in the literature (reviewed in Ref. 4). Although plasmin and PMN-elastase display a broad substrate specificity, fibrin-specific proteolysis is accomplished by formation of plasmin on the fibrin surface (5, 6) or elastase release from neutrophil leukocytes adhering to polymerizing fibrin (7). This localization of plasmin is mediated by one or more of the five kringle domains that constitute the amino-terminal heavy chain and recognize lysine residues in fibrin as binding sites. On the other hand, plasminogen is a substrate of PMN-elastase, and the product of

its limited proteolysis is miniplasminogen² that lacks four of the five kringle domains and is more readily activated by plasminogen activators (8). Thus, the fibrin surface can be exposed to three proteases (plasmin, miniplasmin, and PMN-elastase) with the potential capacity to degrade fibrin. *In vivo*, the fibrinolytic activity of these enzymes is regulated by protease inhibitors (α_2 -antiplasmin, α_1 -protease inhibitor, α_2 -macroglobulin) that constitute more than 10% by weight of all plasma proteins and protect the organism from uncontrolled proteolysis by forming inactive enzyme-inhibitor complexes (reviewed in Ref. 9). The interactions between enzymes and their inhibitors have been described by second-order kinetics, and the rate constants for the inactivation of plasmin and miniplasmin by α_2 -antiplasmin and α_2 -macroglobulin, as well as of PMN-elastase by α_1 -protease inhibitor and α_2 -macroglobulin, have been documented (10-18). In addition, PMN-elastase is shown to inactivate α_2 -antiplasmin (8, 19, 20). As far as the relative contribution of plasmin, miniplasmin, and PMN-elastase to fibrinolysis *in vivo* is concerned, it is important to establish the influence of the substrate (fibrin) on the enzyme inhibition by the plasma protease inhibitors. In our study, we examined the inactivation of fibrin-bound enzymes, the competition between the substrate and the inhibitors for the enzymes, and the stability of the enzyme-inhibitor complexes in the presence of fibrin. Our results show that fibrin reduces the rate at which the major plasma protease inhibitors inactivate plasmin, miniplasmin, and PMN-elastase; thus, even in the presence of inhibitors, fibrinolysis by surface-bound enzymes is efficient.

MATERIALS AND METHODS

Human plasma was collected from healthy volunteers. Streptokinase, aprotinin, porcine pancreatic elastase, and the chromogenic elastase substrate (methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-p-nitroanilide) were from Calbiochem. PMN-elastase, human thrombin (1,000 NIH units/mg), α_2 -antiplasmin, p-nitrophenyl p'-guanidinobenzoate, and phenyl-methanesulfonyl fluoride were from Sigma. The synthetic plasmin substrate Spectrozyme PL (H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide) was obtained from American Diagnostica Inc. Human fibrinogen was the product of Chromogenix AB (Mölnådal, Sweden). Lysine-Sepharose 4B, Sepharose 4B, and Sephadex G-25 were purchased from Pharmacia Biotech Inc. Bovine serum albumin and soybean trypsin inhibitor (SBTI)³ were from Serva, lactoperoxidase was from Boehringer (Mannheim), iminodiacetic acid (disodium salt) and 1,4-butanediol diglycidyl ether were from Aldrich-Chemie (Steinheim, Germany), and Na¹²⁵I (carrier-free) was purchased from Izinta Ltd. (Budapest, Hungary). All other reagents were the products of Reanal (Budapest, Hungary).

Plasminogen—This was prepared by affinity chromatography on lysine-sepharose (21) from fresh-frozen citrated human plasma containing 10 units/ml aprotinin and 10 mM benzamidin.

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¹ In this paper, the serine protease elastase (*M*, 30,000), found in the azurophilic granules of the neutrophil polymorphonuclear leukocytes, is referred to as PMN-elastase.

² By miniplasminogen, we mean the elastase-degraded form of plasminogen with NH₂-terminal valine lacking the Glu¹-Val⁴⁴¹ sequence, whereas miniplasmin is the active enzyme generated from this zymogen by plasminogen activators.

³ The abbreviations used are: SBTI, soybean trypsin inhibitor; HBSS, Hanks' buffered salt solution; FDP, fibrin degradation products.

TABLE I

Second-order rate constants for the inactivation of plasmin, miniplasmin, and PMN-elastase by α_2 -antiplasmin, α_1 -protease inhibitor, and α_2 -macroglobulin in solution

The rate constants were determined under the conditions described under "Materials and Methods," in HBSS. The rate constant values are presented in $10^{-4} \text{ M}^{-1} \cdot \text{s}^{-1}$ units \pm S.E. The dashes indicate that the respective interactions were not studied.

Additive	Inhibitor				
	α_2 -Antiplasmin		α_1 -Protease inhibitor		α_2 -Macroglobulin
	None	1.5 g/liter fibrinogen	None	1.5 g/liter fibrinogen	None
Enzyme					
Plasmin	430 \pm 48	160 \pm 119	0.29 \pm 0.04	0.04 \pm 0.01	11 \pm 1.2
Miniplasmin	99 \pm 8	27 \pm 4	0.11 \pm 0.01	0.04 \pm 0.008	78.2 \pm 3.2
PMN-elastase	—	—	8760 \pm 559	8540 \pm 555	121 \pm 19

Miniplasminogen—This was prepared by limited proteolysis of plasminogen as previously described (8).

Plasmin and Miniplasmin—These were generated from the respective zymogens by activation with streptokinase (1,000 units of streptokinase/mg of zymogen).

Determination of Active Enzyme Concentration—This was carried out before each experiment by measuring the hydrolysis rate of synthetic peptide substrate at eight different concentrations. Calculation was performed on the basis of the Michaelis-Menten equation

$$\left(v = \frac{k_{\text{cat}} \cdot E \cdot S}{K_m + S} \right) \quad (\text{Eq. 1})$$

using an extinction coefficient of $8,820 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for *p*-nitroaniline. For plasmin and miniplasmin, a $k_{\text{cat}} = 13.5 \text{ s}^{-1}$ was used for H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide that was determined in a separate experiment with active site-titrated enzymes (22). For PMN-elastase, a k_{cat} of 17 s^{-1} was used for methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide in 100 mM HEPES, 500 mM NaCl, 10% v/v dimethyl-sulfoxide, pH 7.5 (23).

α_2 -Macroglobulin and α_1 -Protease Inhibitor—These were prepared from fresh-frozen citrated human plasma according to published procedures (24). The concentration of active inhibitors was determined as described by Virca and Travis (17) with the following modification: plasmin and soybean trypsin inhibitor were used for α_2 -macroglobulin titration. The concentration of active α_2 -antiplasmin was measured by titration with active site-titrated plasmin.

^{125}I -Labeled Fibrinogen—This was prepared by lactoperoxidase-catalyzed iodination (25). Polystyrene tubes were coated with ^{125}I -fibrin according to the method of Moroz and Gilmore (26). Fibrinolytic assays were performed in the ^{125}I -fibrin-coated tubes by the addition of 200 μl of the appropriate enzyme to be tested. After the indicated incubation times, 190- μl samples were taken, and the released radioactivity of ^{125}I -labeled fibrin degradation products (^{125}I -FDP) was measured in a Wallac 1410 liquid scintillation counter (Pharmacia). Control tubes containing bovine serum albumin in a concentration corresponding to the concentration of the applied enzyme were included in all experiments to estimate the spontaneous release of radioactivity, and these blank values were subtracted from the test values.

Determination of Second-order Rate Constants for Enzyme Inactivation in Solution—Enzymes were preincubated with inhibitors in HBSS (138 mM NaCl, 5.3 mM KCl, 1.3 mM CaCl_2 , 0.8 mM MgSO_4 , 0.34 mM KH_2PO_4 , 0.3 mM Na_2HPO_4 , buffered with 20 mM HEPES-NaOH pH 7.4) at 22°C . After various intervals, samples were diluted into the appropriate synthetic substrate solution for assaying the residual enzyme activities. As saturating substrate concentrations were used, the residual active enzyme concentration was considered to be linearly proportional to the rate of amidolysis of substrates, measured as a change in the optical density at 405 nm ($\Delta A_{405 \text{ nm}}$) with a Beckman DU 7500 spectrophotometer. The second-order rate constants (k'') were calculated as described in the following paragraphs.

1) For the inactivation of PMN-elastase by α_1 -protease inhibitor or of plasmin and miniplasmin by α_2 -antiplasmin, a non-linear curve fitting was applied to the equation,

$$E = \frac{E_0}{1 + E_0 \cdot k'' \cdot t} \quad (\text{Eq. 2})$$

for a second-order reaction with equimolar concentrations of the reactants using Sigmaplot 4.10 software (Jandel Scientific, Corte Madera, CA) (E is enzyme concentration at time t , estimated from the $\Delta A_{405 \text{ nm}}$; E_0 is the initial enzyme concentration).

2) For the inactivation of plasmin and miniplasmin by α_1 -protease inhibitor (a relatively slow reaction), k'' was calculated from a linear regression to the equation $k' = k'' \cdot I$, where I is the concentration of the inhibitor and k' is the pseudo-first-order rate constant. The k' value was determined for three different inhibitor concentrations (at 5-, 10-, and 15-fold molar excess of inhibitor over enzyme) by a non-linear curve fitting to the equation $E = E_0 \cdot e^{-k' \cdot t}$, where E is the enzyme concentration at time t and E_0 is the initial concentration.

3) For the inhibition of plasmin, miniplasmin, and PMN-elastase by α_2 -macroglobulin, a competition experiment (27) was used involving SBTI (for plasmin and miniplasmin) or α_1 -protease inhibitor (for PMN-elastase) in HBSS. Our experimental design is similar to that described by Virca and Travis (17). For the inhibition of plasmin and miniplasmin by SBTI, a k'' of 8,360 and 9,210 $\text{M}^{-1} \cdot \text{s}^{-1}$, respectively, was experimentally determined. For the PMN-elastase- α_1 -protease inhibitor reaction, the k'' value reported in this paper was used. The initial molar ratios of plasmin (miniplasmin) to SBTI to α_2 -macroglobulin were 1:4:9, 1:9:8, or 1:9:3 at initial enzyme concentrations of 80 or 160 nM. The initial molar ratios of PMN-elastase to α_1 -protease inhibitor to α_2 -macroglobulin were 1:2:2 or 1:2:4 at 100 and 200 nM enzyme concentrations. After a 5-min incubation, the appropriate substrate was added, and the amidolytic activity of the enzyme- α_2 -macroglobulin complexes was measured as $\Delta A_{405 \text{ nm}}$. The concentration of enzyme- α_2 -macroglobulin complexes was determined from a calibration curve.

Determination of Second-order Rate Constants for Enzyme Inhibition on the Fibrin Surface—Plasmin (1 nM), miniplasmin (4 nM), or PMN-elastase (2.5 nM) was incubated with ^{125}I -fibrin for 4 min, and thereafter, an inhibitor (α_2 -antiplasmin, α_1 -protease inhibitor, or α_2 -macroglobulin) was added at three different concentrations: 40, 60, and 80 nM. At appropriate intervals, the release of radioactivity from the solid-phase ^{125}I -fibrin was measured. Under the experimental conditions, the enzymes were saturated with substrate that was confirmed by the linear dependence of the proteolytic rate (v), measured as ^{125}I -FDP release (A)/unit of time, on the enzyme concentration in the range below 5 nM for the three enzymes, i.e. at each time point (t),

$$v_t = \frac{dA}{dt} = k_0 \cdot E_t \quad (\text{Eq. 3})$$

where E_t is the enzyme concentration at time t and k_0 is a constant. Under pseudo-first-order conditions of inhibition, $E_t = E_0 \cdot e^{-k' \cdot t}$, where E_0 is the initial enzyme concentration and k' is the pseudo-first-order rate constant for enzyme inhibition. When E_t is replaced in Equation 3, then Equation 4 is as follows.

$$\frac{dA}{dt} = k_0 \cdot E_0 \cdot e^{-k' \cdot t} \quad (\text{Eq. 4})$$

The initial proteolytic rate ($v_0 = k_0 \cdot E_0$) was measured separately in the absence of inhibitor and consequently $dA = v_0 \cdot e^{-k' \cdot t} \cdot dt$. After integration for a period of time from 0 to t , the released radioactivity will be

$$A = \frac{v_0}{k'} \cdot (1 - e^{-k' \cdot t}) \quad (\text{Eq. 5})$$

if the release at time 0 (A_0) is zero. Using a non-linear curve fitting to the last equation and the measured values of released radioactivity A after incubation time t , as well as the previously determined v_0 , the value of the pseudo-first-order inhibition rate constant k' was determined. Measuring k' at three different inhibitor concentrations (I) and using a linear regression to the equation $k' = k'' \cdot I$, the second-order rate constant k'' was calculated.

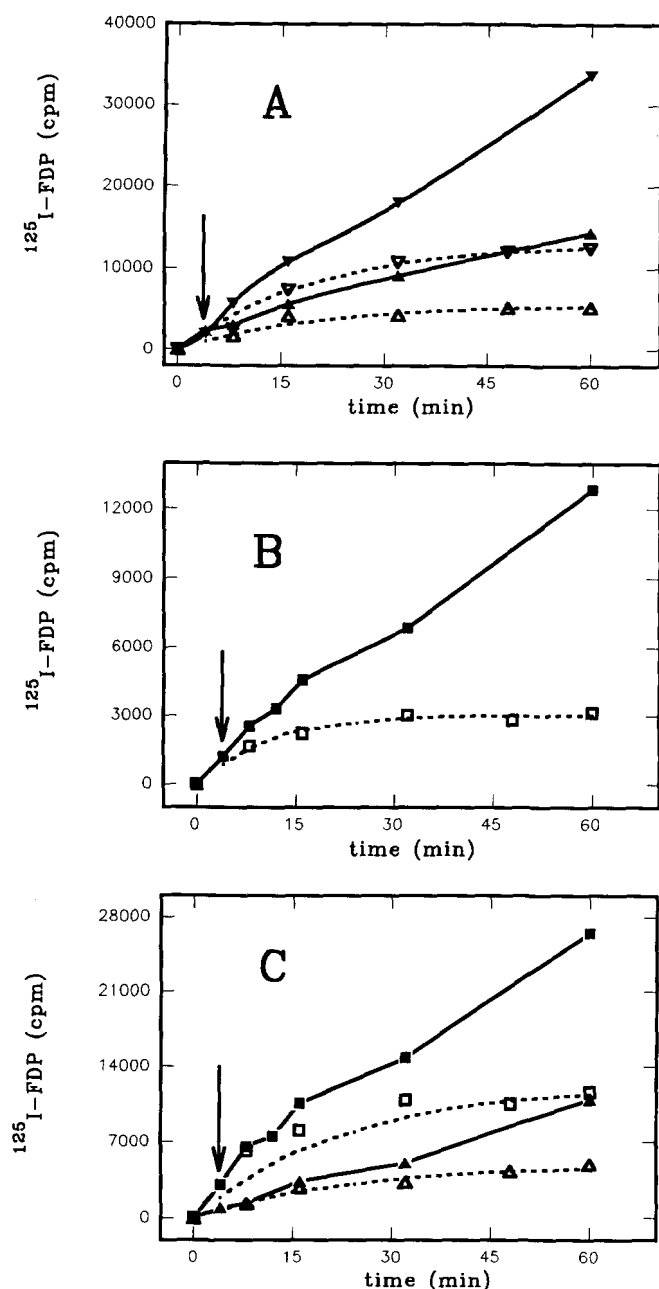


FIG. 1. Inhibition of fibrin-bound plasmin, miniplasmin, and PMN-elastase by α_2 -antiplasmin (A), α_1 -protease inhibitor (B), or α_2 -macroglobulin (C). Plasmin (1 nM, ∇), miniplasmin (4 nM, \blacktriangle), or PMN-elastase (2.5 nM, \blacksquare) was incubated with ^{125}I -fibrin, and ^{125}I -FDP release was measured. At the times indicated by arrows, the respective inhibitor was added at 40 nM final concentration. The release of ^{125}I -FDP in the presence of inhibitor is shown with empty symbols. The dashed lines represent the regression curves, calculated as described under "Materials and Methods." All reactions were performed in HBSS.

Competition between Fibrin and Inhibitors for Enzymes—The inhibitors (40 nM α_2 -antiplasmin, α_1 -protease inhibitor, or α_2 -macroglobulin) were preincubated with solid-phase ^{125}I -fibrin for 2 min. Plasmin (1 nM), miniplasmin (4 nM), or PMN-elastase (2.5 nM) was added, and at intervals (after 4-, 8-, 16-, 32-, and 60-min incubations) ^{125}I -FDP release was measured.

Stability of Enzyme-Inhibitor Complexes—Plasmin (1 nM), miniplasmin (4 nM), or PMN-elastase (2.5 nM) was incubated with inhibitor (α_2 -antiplasmin, α_1 -protease inhibitor, or α_2 -macroglobulin) at different molar ratios. After incubation for 15 min, the enzyme-inhibitor mixtures were transferred to solid-phase ^{125}I -fibrin, and after 16 min, the released radioactivity was measured. Control experiments were performed in the absence of fibrin; enzyme-inhibitor complexes were pre-

pared at the same molar ratios but at 100-fold higher concentrations, and residual amidolytic activity was measured on the respective synthetic substrates. For the α_2 -macroglobulin complexes, the residual-free enzyme was blocked by SBTI (plasmin and miniplasmin) or α_1 -protease inhibitor (PMN-elastase), the amidolytic activity of the α_2 -macroglobulin-enzyme complex was measured, and its concentration was determined from a calibration curve.

RESULTS

The second-order rate constants for the inactivation of plasmin, miniplasmin, and PMN-elastase by different plasma protease inhibitors in the presence or absence of fibrinogen are summarized in Table I. The results show that in solution α_2 -antiplasmin is the major inhibitor for plasmin and miniplasmin and the α_1 -protease inhibitor for PMN-elastase, whereas α_1 -protease inhibitor is only a minor inhibitor for plasmin and miniplasmin. In addition to fibrinogen, the effect of plasminogen (another elastase substrate) on the rate of inhibition of PMN-elastase by α_1 -protease inhibitor was studied; at plasminogen concentrations of physiological relevance (up to 3 μM), the inactivation rate was not affected (data not shown).

The inhibition of fibrin-bound plasmin, miniplasmin, and PMN-elastase by different plasma protease inhibitors is illustrated in Fig. 1. The second-order rate constants, calculated for the various inhibitors and enzymes in the presence of fibrin, are summarized in Table II. Fibrin reduces the second-order rate constant for the inhibition of PMN-elastase by α_1 -protease inhibitor 2,190-fold and by α_2 -macroglobulin 65-fold. The rate constant for miniplasmin inactivation by α_2 -antiplasmin decreases 92-fold and by α_2 -macroglobulin 43-fold, whereas α_1 -protease inhibitor does not inhibit miniplasmin. Plasmin, bound to fibrin, is completely protected against α_2 -macroglobulin and α_1 -protease inhibitor, whereas the rate constant for the inactivation by its major plasma inhibitor α_2 -antiplasmin is reduced 398-fold. These data indicate that the major inhibitors of fibrin-bound enzymes are α_2 -antiplasmin for plasmin, α_2 -macroglobulin for miniplasmin, and α_1 -protease inhibitor for PMN-elastase.

A set of experiments was also carried out in which the inhibitor was preincubated with fibrin. The studies on the competition of fibrin and inhibitors for the enzymes under such conditions were restricted to molar ratios (inhibitor:enzyme) of 10 or more because this situation mimics the biologically relevant one. The results show that α_2 -antiplasmin instantaneously inhibits plasmin and miniplasmin (*i.e.* no ^{125}I -FDP release is detected). The same lack of competing effect on behalf of fibrin is detected in the inhibition of PMN-elastase by α_1 -protease inhibitor and α_2 -macroglobulin, as well as in the inhibition of miniplasmin by α_2 -macroglobulin. On the other hand, α_1 -protease inhibitor does not reduce the rate of plasmin- and miniplasmin-catalyzed degradation of fibrin preincubated with the inhibitors. Similarly, α_2 -macroglobulin does not affect the fibrinolytic activity of plasmin.

The ^{125}I -FDP radioactivity, released by preformed enzyme-inhibitor complexes, corresponds to the calculated proportion of free enzyme in the enzyme-inhibitor mixtures of plasmin with α_2 -antiplasmin, α_1 -protease inhibitor or α_2 -macroglobulin; of PMN-elastase with α_1 -protease inhibitor or α_2 -macroglobulin; as well as of miniplasmin with α_2 -antiplasmin or α_1 -protease inhibitor (*i.e.* fibrin does not influence the stability of the listed complexes). As shown in Fig. 2, fibrin affects only the stability of the miniplasmin- α_2 -macroglobulin complexes; a 4-fold molar excess of inhibitor is necessary for complete inhibition of miniplasmin. This phenomenon may be interpreted as a result of lack of covalent linkage between enzyme and inhibitor, accompanied by high rates of formation and dissociation of the reversible complex.

TABLE II

Second-order rate constants (k'') for the inactivation of fibrin-bound plasmin, miniplasmin, and PMN-elastase by α_2 -antiplasmin, α_1 -protease inhibitor, and α_2 -macroglobulin

The values of k'' are calculated from data of experiments demonstrated in Fig. 1, as described under "Materials and Methods." The rate constant values are presented in $10^{-4} \text{ M}^{-1} \cdot \text{s}^{-1}$ units \pm S.E. The dashes indicate that the respective interactions were not studied. The zero values indicate that under the experimental conditions, no inhibition could be detected. 6AH, 6-aminohexanoate.

Additive Enzyme	Inhibitor			
	α_2 -Antiplasmin		α_1 -Protease inhibitor	α_2 -Macroglobulin
	None	10 μM 6AH	None	None
Plasmin	1.08 \pm 0.07	1.10 \pm 0.13	0	0
Miniplasmin	1.07 \pm 0.1	1.46 \pm 0.13	0	1.83 \pm 0.23
PMN-elastase	—	—	4.12 \pm 0.17	1.85 \pm 0.32

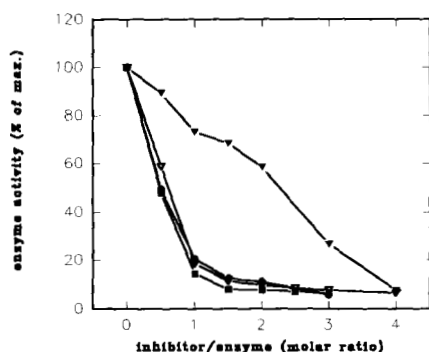


FIG. 2. Stability of the α_2 -macroglobulin-enzyme complexes in the presence of fibrin. α_2 -Macroglobulin and plasmin (●), miniplasmin (▼), or PMN-elastase (■) were preincubated at the indicated molar ratio for 15 min, and then the inhibitor-enzyme complexes were transferred to ^{125}I -fibrin. After a 16-min incubation, samples were taken, and the released radioactivity was measured. Results are expressed in percentages of the radioactivity released by each enzyme in the absence of inhibitor. The empty symbols represent the proportion of miniplasmin amidolytic activity on synthetic substrate that was not protected against SBTI in an enzyme- α_2 -macroglobulin complex, as described under "Materials and Methods."

DISCUSSION

The degradation of fibrin clots that is a prerequisite for proper blood circulation may involve the action of several proteases: plasmin, miniplasmin, and PMN-elastase (4). The generation of their activity is physiologically restricted to fibrin surfaces because plasminogen activators promote active enzyme formation from the zymogen preferentially on the fibrin surface (5, 6). Polymorphonuclear leukocytes also bind to polymerizing fibrin (7); thus, PMN-elastase can be released directly on the substrate surface. On the other hand, the proteolytic activities are regulated by the plasma protease inhibitors, and although inhibition kinetics have been extensively studied in pure systems (10–18), the inactivation of the enzymes on the fibrin surface has not been characterized. By our experimental approach, we determined the second-order rate constants for the inhibition of fibrin-bound plasmin, miniplasmin, and PMN-elastase by α_2 -antiplasmin, α_1 -protease inhibitor, or α_2 -macroglobulin in a milieu of physiological ionic strength and composition (Table II). If we estimate the *in vivo* significance of these kinetic constants using the approach described by Bieth (28),⁴ the fibrin-bound plasmin will be almost completely inactivated in 420 s, miniplasmin in 66 s, and PMN-elastase in 4.2 s at physiological concentrations (9) of α_2 -antiplasmin, α_2 -macroglobulin, and α_1 -protease inhibitor. However, if we consider that α_2 -antiplasmin is inactivated by PMN-elastase (8, 19, 20) and α_1 -protease inhibitor by leukocyte-derived biological oxi-

dants (9), these life span values will certainly be greater in a compartment where polymorphonuclear leukocytes are present (e.g. thrombi).

The comparison of the inhibition kinetic parameters for fibrin-bound enzymes (Table II) with those for free enzymes (Table I) shows that fibrin protects the proteases from inactivation; all of the second-order rate constants are lowered. This protective effect is most profound for the interactions of plasmin with α_2 -macroglobulin (that is completely abolished) and for the inhibition of PMN-elastase by α_1 -protease inhibitor, the rate constant of which is 2,190-fold reduced by fibrin. The latter strongly supports the concept that in the broad range of elastase substrates, fibrin is one of physiological relevance, a fact that is not commonly accepted in the literature. Fibrinogen, the fluid-phase precursor of fibrin, also influences the second-order inhibition rate constants of plasmin, miniplasmin, and PMN-elastase (Table I), but its effect on the protection of enzymes from inactivation is essentially negligible compared with that of fibrin.

Fibrin, preincubated with protease inhibitors, cannot efficiently compete for plasmin, miniplasmin, and PMN-elastase, as evidenced by the lack of degradation products released under pseudo-first-order conditions for the major inhibitors of each enzyme. These results suggest that only enzyme activities generated directly on fibrin bear physiological fibrinolytic significance. On the other hand, under our experimental conditions fibrin completely eliminates those interactions, the second-order rate constant of which is $1.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ or less in a system without fibrin surface (α_1 -protease inhibitor and plasmin or miniplasmin, α_2 -macroglobulin and plasmin).

It is well documented that 6-aminohexanoate lowers the second-order rate constant for the inhibition of plasmin by α_2 -antiplasmin (29, 30). Our results (Table II) show that 6-aminohexanoate concentration, reducing the inhibition constant of plasmin by 50% (29), has essentially no effect on the inhibition of fibrin-bound plasmin by α_2 -antiplasmin. We suggest that this is due to 6-aminohexanoate-induced dissociation of plasmin from fibrin, thus increasing the concentration of free enzyme available for inhibition.

Our results support the concept that in physiological milieu (in the presence of plasma protease inhibitors), fibrin can be efficiently degraded only by enzymes that are generated or released directly on the fibrin surface.

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⁴ J. G. Bieth proposes the so-called delay time of inhibition, $t \approx 5/(k'' \cdot I)$, as a measure for the estimation of the *in vivo* significance of the kinetic parameters.

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