Fibrinolysis with Des-Kringle Derivatives of Plasmin and Its Modulation by Plasma Protease Inhibitors†

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Received January 23, 1998; Revised Manuscript Received April 13, 1998

ABSTRACT: Quantitative characterization of the interaction of des-kringle$_{1-5}$-plasmin (microplasmin) with fibrin(ogen) and plasma protease inhibitors may serve as a tool for further evaluation of the role of kringle domains in the regulation of fibrinolysis. Comparison of fibrin(ogen) degradation products yielded by plasmin, miniplasmin (des-kringle$_{1-4}$-plasmin), microplasmin, and trypsin on SDS gel electrophoresis indicates that the differences in the enzyme structure result in different rates of product formation, whereas the products of the four proteases are very similar in molecular weight. Kinetic parameters show that plasmin is the most efficient enzyme in fibrinogen degradation, and the k_{cat}/K_M ratio decreases in parallel with the loss of the kringle domains. The catalytic sites of the four proteases have similar affinities for fibrin (K_M values between 0.12 and 0.21 μM). Trypsin has the highest catalytic constant for fibrin digestion ($k_{cat} = 0.47$ s^{-1}), and among plasmins with different kringle structures, the loss of kringle$_5$ results in a markedly lower catalytic rate constant ($k_{cat} = 0.0076$ s^{-1} for microplasmin vs 0.048 s^{-1} for miniplasmin and 0.064 s^{-1} for plasmin). In addition, microplasmin is inactivated by plasmin inhibitor ($k'' = 3.9 \times 10^5$ M^{-1} s^{-1}) and antithrombin ($k'' = 1.4 \times 10^3$ M^{-1} s^{-1}) and the rate of inactivation decreases in the presence of fibrin(ogen). Heparin (250 nM) accelerates the inactivation of microplasmin by antithrombin ($k'' = 10.5 \times 10^3$ M^{-1} s^{-1}), whereas that by plasmin inhibitor is not affected ($k'' = 4.2 \times 10^5$ M^{-1} s^{-1}).

The degradation of fibrin in vivo is generally attributed to a serine protease, plasmin, which is formed from plasminogen by plasminogen activators (*1*). Human plasminogen consists of an N-terminal preactivation peptide (Glu^1−Lys^{77}), five kringle domains (Lys^{78}−Arg^{561}), and the protease domain (Val^{562}−Asn^{791}), and the cleavage at Arg^{561} results in the generation of the proteolytically active plasmin with two chains held together by disulfide bridges (*2*−*4*). Lysine-binding sites located on kringle domains recognize lysine residues as binding sites, and thus, they are thought to play a role in plasminogen activation and in the interaction of plasmin(ogen) with substrates, inhibitors, and cell surface receptors (*5*−*12*). Among the kringle domains in plasminogen, kringle$_5$ has the highest affinity for intrachain lysine residues in intact fibrin (*5*, *10*, *13*). Kringles$_{1-3}$ and kringle$_4$, however, prefer C-terminal lysine residues formed in the course of fibrin degradation, and thus, binding of additional plasminogen molecules to fibrin may play a role in the acceleration of fibrinolysis (*7*, *14*). In addition to the binding of extramolecular ligands, the intramolecular interactions between various domains of plasminogen may play a role in maintaining a given conformation of the molecule and it may also influence ligand binding (*15*−*17*). The functions of the kringle domains can be studied with the addition of lysine analogues, e.g., 6-aminohexanoate (*18*, *19*), which block the lysine-binding sites. Plasminogen derivatives lacking various numbers of kringle domains provide an alternative possibility for assessing the role of kringle structures in a given process. PMN-elastase, a protease of neutrophil granulocytes present in thrombi, cleaves plasminogen at Val^{442}, removing the first four kringle domains. The product is miniplasminogen, which can be activated with plasminogen activators to an active protease, miniplasmin[1] (*2*). Miniplasminogen is more readily activated in solution by a tissue-type plasminogen activator than plasminogen, whereas kringle$_{1-4}$ domains are required for a fully developed activation rate enhancement in the presence of fibrin or CNBr cleavage products of fibrinogen (*10*, *20*). In addition to plasmin, miniplasmin, as a member of the elastase-mediated fibrinolytic pathway (reviewed in ref *21*), may be a fibrinolytic protease of physiological importance, as well (*22*−*26*). In the course of digestion of fibrinogen and fibrin, plasmin and miniplasmin yield the same degradation products at similar or different rates depending on experimental conditions (*8*, *24*, *25*, *27*−*29*).

Microplasmin ($M_r = 29\,000$) is an autocatalytic cleavage product of plasmin at basic pH (*30*, *31*). Microplasmin consists of the last 31 amino acid residues of the heavy chain (from Lys^{531} to Arg^{561}) and the protease domain connected with disulfide bridges (Cys^{547}−Cys^{665} and Cys^{557}−Cys^{565}). The preservation of these disulfide bridges is essential for retaining amidolytic activity, as neither molecule's mutant

† This work was suppported in part by Hungarian Grants OTKA T-016497, ETT-T-07-383/93, ETK I11/95, and FKFP-0006/1997 and by U.S.-Hungarian Joint Fund Grant 449/95.

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[1] Abbreviations: HBSS, Hank's balanced salt solution buffered with 10 mM Hepes/NaOH (pH 7.4); microplasmin, des-kringle$_{1-5}$-plasmin; miniplasmin, des-kringle$_{1-4}$-plasmin; 6AH, 6-aminohexanoate; Fg, fibrinogen; PMN-elastase, polymorphonuclear leukocyte elastase.

S0006-2960(98)00180-9 CCC: $15.00
Published on Web 06/05/1998

at these Cys positions nor the protease domain alone has significant activity on synthetic peptide substrates (*32*, *33*). In addition to low-molecular weight substrates, microplasmin digests fibrin surface; in a heterogeneous system, kinetic parameters for association and dissociation of the enzyme−fibrin complex and that for the rate of hydrolysis are remarkably lower than the respective values for plasmin or miniplasmin (*25*).

Second-order rate constants for inhibition of plasmin by plasmin inhibitor, antithrombin, and α_1-protease inhibitor are higher than the respective values for miniplasmin, and both enzymes are protected from inhibitors in the presence of their natural substrates (*9*, *22*, *23*, *34*). Inactivation of (mini)-plasmin is accelerated by heparin both in the presence and in the absence of fibrin (*22*, *35*).

Although microplasmin is not a physiologically relevant protease, the quantitative comparison of the interaction of plasmin derivatives with fibrinogen, fibrin, and plasma protease inhibitors provides a useful tool for evaluating further the role of kringle domains in the regulation of fibrinolysis. Therefore, we have determined kinetic parameters for quantitative comparison of plasmin-, miniplasmin-, microplasmin-, and trypsin-catalyzed degradation of fibrinogen and fibrin and also for their interaction with two plasma protease inhibitors, plasmin inhibitor and antithrombin.

MATERIALS AND METHODS

Human plasma was collected from healthy volunteers. Streptokinase, aprotinin, porcine pancreatic elastase, the chromogenic elastase substrate (methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide), and plasmin inhibitor were from Calbiochem (La Jolla, CA). The chromogenic plasmin substrate Spectrozyme-PL (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide) and bovine thrombin were from American Diagnostica (Hartford, CT) and Merck (Darmstadt, Germany), respectively. Human fibrinogen and S-2222 (*N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide) were purchased from Chromogenix AB (Mölndal, Sweden), and lysine−Sepharose 4B, sulfopropyl-Sephadex C-50, and Sephacryl S-400 HR were the products of Pharmacia Biotech (Uppsala, Sweden). Phenylmethanesulfonyl fluoride and *p*-nitrophenyl *p*′-guanidinobenzoate were from Sigma (St. Louis, MO). Bovine serum albumin and bovine pancreas trypsin were from Serva (Heidelberg, Germany). Unfractionated heparin (199 units/mg specific activity) was purchased from Proquifin. All other reagents were obtained from Reanal (Budapest, Hungary).

Plasminogen and Miniplasminogen Preparation. Plasminogen was purified by affinity chromatography (*36*) on lysine−Sepharose from fresh-frozen citrated human plasma containing 10 units/mL aprotinin and 10 mM benzamidine. Miniplasminogen was prepared by limited proteolysis of plasminogen with porcine pancreatic elastase followed by inhibition of elastase with phenylmethanesulfonyl fluoride and separation of the degradation products on lysine−Sepharose according to our previously published procedure (*20*).

Microplasmin Preparation. Microplasmin, an autodigestive product of plasmin in 0.1 M glycine/NaOH (pH 11.0) buffer was isolated as described (*30*).

Plasmin and Miniplasmin Generation and Determination of the Active Enzyme Concentration. Plasminogen and miniplasminogen were activated with streptokinase (1000 units/mg of zymogen). Determination of the active enzyme concentration was carried out as described in detail elsewhere (*22*, *25*).

Thrombin Purification. Bovine thrombin (50 NIH units/mg) was further purified by ion exchange chromatography on sulfopropyl-Sephadex C-50, as described (*37*), and stored at −70 °C. The specific activity of the final preparation was 1800 NIH units/mg.

Inhibitors. Antithrombin was purified from human plasma, as described previously (*38*). Active inhibitor concentrations were determined with active site-titrated plasmin or thrombin for plasmin inhibitor or antithrombin, respectively (*22*, *23*)

Fibrinogen Purification. Fibrinogen, free of plasminogen and factor XIII, was prepared as previously described (*24*) and dialyzed against Hank's balanced salt solution (138 mM NaCl, 5.3 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM Mg_2SO_4, 0.34 mM KH_2PO_4, and 0.3 mM Na_2HPO_4) buffered with 10 mM Hepes/NaOH (pH 7.4) (HBSS).

Determination of K_M for Fibrinogen and Fibrin. A global K_M of the proteases for fibrinogen and fibrin was determined according to our previously detailed approach (*24*). Briefly, amidolytic activity of the enzymes on synthetic peptide substrates was measured in the absence or in the presence of various concentrations of competing fibrin(ogen). For determination of K_M for fibrinogen, 200 μL of synthetic peptide substrate (Spectrozyme-PL for plasmin derivatives and S-2222 for trypsin) at concentrations of 5−150 μM containing various concentrations of fibrinogen (0−17.6 μM for microplasmin, 0−9 μM for trypsin and miniplasmin, or 0−4.4 μM for plasmin) in 20 mM Hepes and 150 mM NaCl (pH 7.4) was added to 10 μL of protease and the change in absorbance at 405 nm was measured with a Beckman DU-7500 spectrophotometer. K_M for fibrinogen was calculated using nonlinear curve fitting to the equation $v = (v_{max}S)/[S + K_M^{SS}(1 + Fg/K_M^{Fg})]$ for two substrates competing for the protease, where K_M^{SS} and K_M^{Fg} are the Michaelis−Menten constants of the protease for the synthetic substrate and fibrinogen, respectively, S and Fg are the concentrations of the synthetic substrate and fibrinogen, respectively, and v is the rate of *p*-nitroaniline formation calculated by using an extinction coefficient of 8820 M^{-1} cm^{-1} for *p*-nitroaniline.

K_M for fibrin was determined in a similar experimental setting. Fibrin was preformed from 0 to 0.4 μM fibrinogen containing 5−150 μM synthetic peptide substrate with 1 NIH unit/mL thrombin. After 2 min (during this time, thrombin-catalyzed formation of *p*-nitroaniline from the synthetic substrates could not be detected), 10 μL of the fibrinolytic enzyme was added and the change in absorbance at 405 nm measured. Evaluation of experimental data and calculation of K_M for fibrin were carried out as described for fibrinogen.

Detection of the Ethanol-Soluble Degradation Products during the Course of Fibrinogen Digestion and Determination of k_{cat}. For measurement of the formation of ethanol-soluble fibrinogen degradation products during the course of fibrinogen digestion, fibrinogen [3.7 or 11.7 μM in 50 mM Tris-HCl and 150 mM NaCl (pH 7.4)] was incubated at 37 °C with plasmin, miniplasmin, microplasmin, or trypsin at four different concentrations in the range of 2.5−80 nM. After various intervals, samples were withdrawn and the reaction was stopped by the addition of ethanol at 0 °C (final concentrations are 0.25 g/L for the total protein and 20%

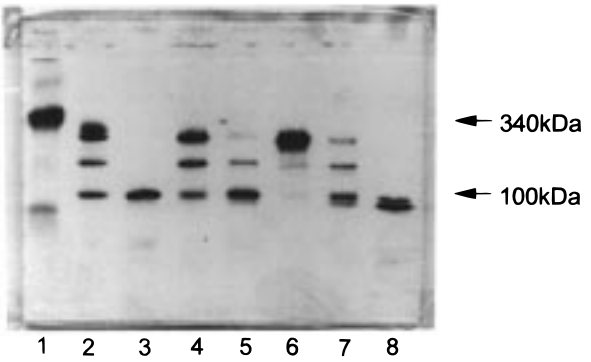

FIGURE 1: Degradation of fibrin by fibrinolytic enzymes. Fibrinogen [5.9 μM in 50 mM Tris-HCl and 150 mM NaCl (pH 7.4)] is converted to fibrin by thrombin (1 NIH unit/mL), and a fibrinolytic protease (at the indicated concentration) is incorporated into fibrin. After incubation at 37 °C for appropriate times, clots are boiled at 90 °C for 5 min in 100 mM Tris-HCl and 100 mM NaCl (pH 8.2) buffer containing 4 M urea and 2% SDS, and SDS gel electrophoresis is performed on a 4 to 15% gradient polyacrylamide gel: lane 1, fibrinogen and plasminogen; lanes 2 and 3, fibrin digested with 10 nM plasmin for 60 and 120 min, respectively; lanes 4 and 5, fibrin digested with 100 nM microplasmin for 60 and 120 min, respectively; and lanes 6−8, fibrin digested with 10 nM trypsin for 10, 20, and 60 min, respectively.

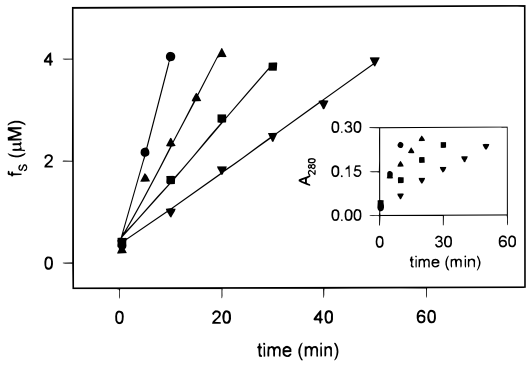

FIGURE 2: Determination of catalytic constants for generation of nonpolymerizable fibrinogen derivatives by plasmin. Symbols represent values from the equation $f_S = S_0 - S - K_M^{Fg} \ln(S/S_0)$ calculated from experimental data presented in the inset, as described in Materials and Methods. Regression values fitted to the equation $f_S = Ek_{cat}t$ are shown by lines. Inset: Formation of ethanol-soluble degradation products after various times of incubation of 3.7 μM fibrinogen and 5 (▼), 10 (■), 15 (▲), or 20 nM (●) plasmin.

for ethanol). At this ethanol concentration, undegraded fibrinogen and X fragments are precipitated, whereas lower-molecular weight degradation products remain soluble, as evidenced by SDS−polyacrylamide gel electrophoresis of unreduced samples (not shown). Fragment X formed by plasmin is clottable, whereas the trypsin-cleaved X-like fragment is probably nonclottable (*29*, *39*). After centrifugation at 20000*g* for 5 min, the absorbance of the supernatant was measured at 280 nm (A_{280}). $A_{280} = 1.6$ reflected 1 g/L protein, as measured with the Lowry method (*40*).

For determination of k_{cat}, two problems arise. (i) Because of the high K_M^{Fg} values, under our conditions, enzymes are not saturated with substrate, and (ii) the consumption of fibrinogen is not negligible during the experiment. To overcome these problems, the integrated form of the Michaelis−Menten equation was used (*41*). After incubation of fibrinogen with the enzyme for time *t*, the ethanol-soluble fraction was considered the nonpolimerizable product of fibrinogen degradation and the concentration of the ethanol-precipitable fraction (considered the residual substrate concentration, *S*) was used to calculate $f_S = S_0 - S - K_M^{Fg} \ln(S/S_0)$, where S_0 is the initial substrate concentration and K_M^{Fg} is independently determined according to the method above. Thereafter, data were fitted to the equation $f_S = Ek_{cat}t$, where the f_S value was determined with incubation of fibrinogen for time *t* with enzyme at concentration *E*. Determination of k_{cat} at various concentrations of fibrinogen (in the physiological range) with various concentrations of the enzyme gives identical values, supporting the reliability of our model. The value of each k_{cat} is derived from 16−20 experiments, four of which are depicted in Figure 2.

Determination of k_{cat} for Fibrin Dissolution. Fibrinogen [5.9 μM in 195 μL of HBSS containing 3 mM $CaCl_2$ (pH 7.4)] was added to thrombin (40 NIH units/mL in 5 μL) and fibrinolytic protease (5 μL) in a microplate well (final concentrations were in the range of 2−32 nM for plasmin and miniplasmin, 20−160 nM for microplasmin, and 0.5−10 nM for trypsin). The turbidity of the fibrin gel network was followed at 340 nm with a Dynatech MR 5000 microplate reader at 37 °C. All further details of the experimental procedure and evaluation of results were performed as described previously (*24*). Briefly, the lysis time (t_e) was arbitrarily defined as the time elapsed until the turbidity of the fibrin gel decreased to the half-maximal value, and the amount of residual undegraded fibrin monomers at t_e was determined by gel filtration on a Sephacryl S-400 HR column. Since in the studied period of fibrin degradation proteases were saturated with substrate, proteolysis proceeded at the maximal rate; thus, $t_e = (Fn_o - Fn_e)/(k_{cat}E)$, where Fn_0 and Fn_e are the concentrations of intact fibrin monomers at time 0 and t_e, respectively, and *E* is the enzyme concentration. By using t_e values for different enzyme concentrations, an Fn_0 of 5.6 μM, and an Fn_e determined independently with gel filtration for the respective enzyme, k_{cat} was calculated from a nonlinear curve fitting to the above equation.

Detection of the Ethanol-Soluble Degradation Products during the Course of Fibrin Digestion. For measurement of the formation of ethanol-soluble fibrin degradation products during the course of fibrinolysis, plasmin (5 nM), miniplasmin (5 nM), microplasmin (100 nM), or trypsin (2.5 nM) was added to fibrinogen [5.9 μM in 50 mM Tris and 150 mM NaCl (pH 7.4)] and clotting was initiated by immediate addition of thrombin (1 NIH unit/mL final concentration). All further details were the same as in our previously published procedure (*24*).

Inhibition of Plasmin Derivatives by Plasmin Inhibitor and Antithrombin. The second-order rate constant (k'') for the microplasmin−plasmin inhibitor reaction in solution in the presence or absence of additives (6-aminohexanoate, fibrinogen, bovine serum albumin, or heparin) was determined according to a previously described procedure (*22*). For studying the effect of fibrin on the rate of inhibition, fibrinogen was added to thrombin (0.7 NIH unit/mL) and microplasmin (133 nM), and after preincubation for 1 min, the reaction was started with plasmin inhibitor (133 nM). After various intervals, the samples were 5-fold diluted in 250 μM Spectrozyme-PL to determine the residual enzyme concentration. Amidolytic activity was a linear function of

Table 1: Kinetic Parameters for Degradation of Fibrinogen[a]

	K_M (μM)	K_M (μM) with 2 mM 6AH	$k_{cat} \times 10^2$ (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
plasmin	2.0 ± 0.2	13.4 ± 1.8	25.6 ± 1.4	128
miniplasmin	8.1 ± 0.9	10.3 ± 1.2	32.8 ± 1.3	40
microplasmin	11.1 ± 2.1	8.5 ± 1.4	29.2 ± 2.1	26
trypsin	3.4 ± 0.2	3.4 ± 0.5	6.0 ± 0.3	18

[a] K_M and k_{cat} values (±standard error) have been determined as described in Materials and Methods.

Table 2: Kinetic Parameters for Degradation of Fibrin[a]

	K_M (μM)	K_M (μM) with 2 mM 6AH	$k_{cat} \times 10^2$ (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
plasmin	0.14[b]	1.26[b]	6.4	460
miniplasmin	0.21[b]	0.82[b]	4.8	230
microplasmin	0.12 ± 0.05	0.28 ± 0.08	0.76	63
trypsin	0.16 ± 0.03	0.16 ± 0.06	47.2	2950

[a] K_M and k_{cat} values (±standard error) have been determined as described in Materials and Methods. [b] From ref *24*.

microplasmin concentration in the presence of the additives or fibrin(ogen). In this system, certain overestimation of the rate constants in the presence of fibrin(ogen) is possible, but its extent is negligible considering the dilution of fibrin(ogen) in the monitoring assay with the synthetic substrate.

For determination of k'' for the microplasmin−antithrombin reaction, inhibitor (375 nM) and enzyme (125 nM) containing additives in HBSS were incubated at room temperature. At various times of incubation, samples were diluted 5-fold into a 150 μM Spectrozyme-PL solution (inhibition of microplasmin by antithrombin was ceased under these conditions) and the residual enzyme concentration was calculated on the basis of the measured ΔA_{405} per minute. The second-order rate constant was computed with linear curve fitting to the equation $1/(I_0 - E_0)\ \ln[E_0(I_0 - E_0 + E)/I_0E] = k''t$ for a second-order reaction of reactants with different initial concentrations, where E is the residual enzyme concentration at time t and E_0 and I_0 are the concentrations of enzyme and inhibitor, respectively (*23*). The effect of fibrin on the inhibition of microplasmin by antithrombin (in the presence or absence of heparin) was studied in a similar setting, as described above.

RESULTS

Microplasmin yields fibrin degradation products with a molecular weight indistinguishable from that of the products of plasmin digestion but requires a 10-fold higher concentration than plasmin to form the products in a comparable time course (Figure 1). Digestion of fibrin with trypsin is faster than that with plasmin at equimolar concentrations, and fragments yielded by this protease are only slightly different from plasmic degradation products. Fibrinogen cleavage patterns for the four proteases are also similar (not shown).

When kinetic parameters for synthetic peptide substrate hydrolysis by any studied protease are detemined in the presence of fibrinogen or fibrin, the apparent K_M for the appropriate peptide substrate increases with increasing concentrations of fibrin(ogen), whereas v_{max} is not changed. This justifies the assumption that the two substrates, the fibrin(ogen) and the synthetic peptide, compete for the protease. K_M values determined according to this approach reflect the affinity of the catalytic site of the enzyme for fibrin(ogen) (Tables 1 and 2). Among the proteases, plasmin has the highest affinity for fibrinogen and there is no significant difference in the K_M values of microplasmin and miniplasmin. Plasmin is the only protease for which the affinity is modified by 6AH; its K_M for fibrinogen in the presence of 2 mM 6AH increases to a value similar to that of the two other plasmin derivatives. When fibrin is the substrate, all proteases have similar affinities. 6AH does not alter the K_M of trypsin at all, and among des-kringle plasmins, microplasmin is the least affected enzyme.

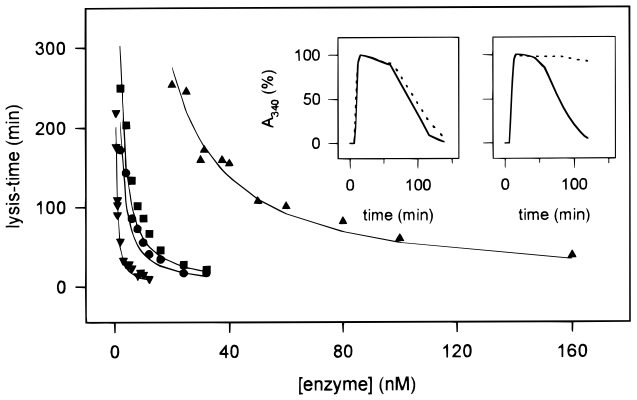

FIGURE 3: Measurement of the turbidimetric lysis time for the determination of catalytic constants for fibrin dissolution. The turbidimetric lysis time, measured as described in Materials and Methods, is a reciprocal function of the concentration of plasmin (●), miniplasmin (■), microplasmin (▲), and trypsin (▼) incorporated into fibrin. Lines represent the curves fitted to the experimental data according to the equation $t_e = (Fn_0 - Fn_e)/(k_{cat}E)$. Inset: Turbidity of the fibrin clot during the course of its formation and degradation by 60 nM microplasmin (left) or 8 nM plasmin (right) in the absence (solid line) or presence (dotted line) of 2 mM 6AH.

Four representative experiments are shown for plasmin (Figure 2) to demonstrate the procedure used for the determination of catalytic constants for fibrinogen digestion. Table 1 contains similar k_{cat} values for plasmin derivatives and a lower value for trypsin.

The turbidimetric lysis time determined for proteases incorporated into the polymerized fibrin gel is a reciprocal function of the enzyme concentration (Figure 3). Comparison of the catalytic rate constants for fibrin degradation by various proteases (Table 2) shows that the values for plasmin and miniplasmin are higher (8- and 6-fold, respectively) than that for microplasmin. Judged on the basis of the calculated k_{cat}/K_M values, trypsin is the most efficient fibrinolytic enzyme, and among plasmin derivatives, there is a difference in this parameter between plasmin and miniplasmin on one hand and microplasmin on the other. The value of k_{cat} determined in HBSS does not differ significantly from previously published values for plasmin and miniplasmin (*24*), which were calculated from experiments in 10 mM imidazole, 150 mM NaCl, and 3 mM $CaCl_2$ (pH 7.4). At saturating fibrin concentrations, fibrinolysis with microplasmin is not affected by 2 mM 6AH, whereas 6AH at this concentration completely inhibits plasmin (Figure 3, inset) and miniplasmin (not shown).

As all studied proteases yield similar fibrin degradation products, monitoring the rate of formation of ethanol-soluble degradation products during the course of fibrin digestion by the various enzymes provides an independent method for assessing their fibrinolytic efficiency (Figure 4). Plasmin

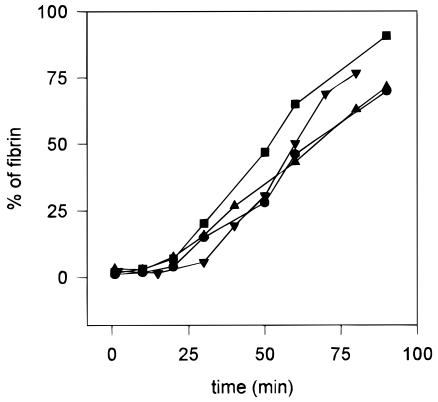

FIGURE 4: Time course of the formation of ethanol-soluble fibrin degradation products. Fibrin is digested with 5 nM plasmin (●), 5 nM miniplasmin (■), 100 nM microplasmin (▲), or 2.5 nM trypsin (▼), and the concentration of ethanol-soluble fibrin degradation products is presented as a percentage of the completely degraded fibrin.

Table 3: Inactivation of Microplasmin by Plasmin Inhibitor and Antithrombin[a]

additive	constant for plasmin inhibitor	additive	constant for antithrombin
none	392 ± 86	none	1.42
3.7 μM fibrinogen	127 ± 28	5.9 μM fibrinogen	0.83
14.7 μM fibrinogen	40 ± 9	14.7 μM fibrinogen	0.11
heparin	419 ± 50	heparin	10.56
6AH	361 ± 65		
3.7 μM fibrinogen and heparin	143 ± 21	5.9 μM fibrinogen and heparin	5.99
3.7 μM fibrinogen and 6AH	208 ± 34	14.7 μM fibrinogen and heparin	3.85
0.15 μM fibrin	197 ± 45	0.44 μM fibrin	0.68
0.44 μM fibrin	46 ± 20	0.44 μM fibrin and heparin	9.17

[a] Second-order rate constants [$k'' \times 10^{-3}$ (M^{-1} s^{-1})] for reaction of microplasmin with inhibitors have been determined as described in Materials and Methods. The concentration of heparin is 250 nM, and that of 6AH is 2 mM.

(5 nM), miniplasmin (5 nM), microplasmin (100 nM), and trypsin (2.5 nM) incorporated into fibrin (5.9 μM) demonstrate comparable kinetics of fibrin degradation.

Microplasmin is inhibited by both plasmin inhibitor and antithrombin; the second-order rate constants of these reactions (Table 3) are lower than the respective values for miniplasmin [9.9×10^5 M^{-1} s^{-1} for plasmin inhibitor and 2.8×10^3 M^{-1} s^{-1} for antithrombin (*23*)]. When inactivation of microplasmin by plasmin inhibitor is measured after preincubation of the enzyme with its substrates, both fibrinogen and fibrin protect the enzyme from inhibition in a concentration-dependent manner. In the presence of 0.44 μM fibrin, the rate of inhibition decreases by a factor of 8.6, and a 10-fold decrease in the k'' value is measured with 14.7 μM fibrinogen (Table 3). Fibrin (0.44 μM) preincubated with plasmin inhibitor, however, cannot protect microplasmin from inactivation; the k'' value is $(337 \pm 40) \times 10^3$ M^{-1} s^{-1}. Heparin (up to 300 nM) does not modify the rate of these reactions. 6AH (2 mM) neither modifies the interaction of microplasmin with plasmin inhibitor nor abolishes the protective effect of fibrinogen. Bovine serum albumin (74 μM) does not protect microplasmin from inhibition (not shown). In the presence of fibrin(ogen), the second-order rate constant for the microplasmin−antithrombin reaction is also diminished (Figure 5A and Table 3). Heparin accelerates inhibition by antithrombin of microplasmin, and the dependence of this effect on heparin concentration is similar to that of miniplasmin; the rate of plasmin−antithrombin reaction is increased already at lower heparin concentrations (Figure 5B). The effect of heparin on microplasmin inhibition is only partially reversed in the presence of 14.7 μM fibrinogen (Figure 5A), whereas that on plasmin or miniplasmin inhibition is abolished completely (not shown).

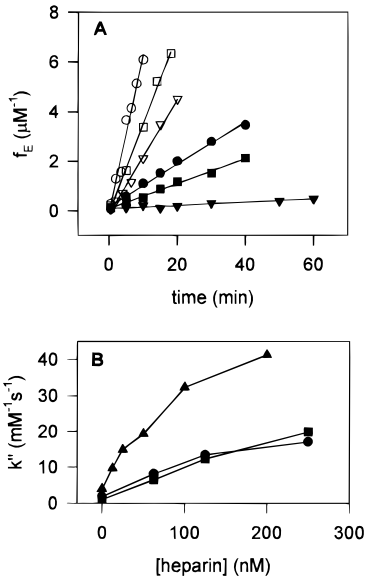

FIGURE 5: Inactivation of microplasmin by antithrombin. (A) Effects of heparin and fibrinogen. Microplasmin (125 nM) and antithrombin (375 nM) are incubated with 250 nM heparin (empty symbols) or without heparin (filled symbols), in the absence (●) or in the presence of 5.9 (■) or 14.7 μM (▼) fibrinogen. The residual enzyme concentration is measured at various times, and the solution to the equation $f_E = 1/(I_0 - E_0)\ \ln[E_0(I_0 - E_0 + E)/I_0E]$ is calculated as detailed in Materials and Methods. As $f_E = k''t$, the slope of the regression line reflects the second-order rate constant for the inhibition. (B) Second-order rate constants (k'') for the inactivation of 125 nM plasmin (▲), miniplasmin (■), or microplasmin (●) with 375 nM antithrombin in the presence of various concentrations of heparin.

DISCUSSION

During the course of fibrin dissolution with clot-enmeshed proteases, fibrin degradation products formed by plasmin, miniplasmin, microplasmin, and trypsin are very similar in molecular weight, as shown on SDS gel electrophoresis (Figure 1). This similarity does not necessarily mean that the three plasmin derivatives yield absolutely identical products. Fibrinogen degradation products formed by trypsin and plasmin run very similarly in starch gels and share common physical properties (*39*); however, the D fragment formed by trypsin has been recently shown to be several amino acid residues longer than the plasmic D fragment (*42*). Fibrin degradation products formed by PMN-elastase and cathepsin G (*24*), two proteases with different primary specificities, however, differ substantially from those presented here for plasmin. These data suggest a major role for the protease domain of plasmin-like enzymes in selecting the susceptible cleavage sites of the substrate.

Among the three plasmin derivatives, the catalytic site of microplasmin has the lowest affinity for fibrinogen (Table 1), and values for plasmin and miniplasmin are consistent with published data [1.9 μM for plasmin and 7.3 μM for

miniplasmin (*34*)]. 6-Aminohexanoate affects only the K_M^{Fg} of plasmin (that of miniplasmin or microplasmin is not influenced), suggesting an essential role for kringle$_{1-4}$ domains in the formation of the plasmin−fibrinogen complex. Each fibrinogen molecule binds four plasminogen molecules, and the affinity of plasminogen for fibrinogen increases as the binding sites are occupied (*43*). One could speculate that this positive cooperativity may be related to the function of kringle$_{1-4}$ resulting in a higher affinity of plasmin for fibrinogen compared to that of miniplasmin. When fibrin is the substrate, however, the catalytic sites of the four proteases possess remarkably similar affinities regardless of the number of kringle domains (Table 2). This result together with the known lower fibrin affinity of PMN-elastase and cathepsin G [0.40 and 0.41 μM, respectively (*24*)] emphasizes the importance of the protease domain in the formation of the enzyme−fibrin complex. A similar conclusion is drawn from our previous study on fibrin surface degradation (*25*). A conformational change of fibrin monomers required for a cooperative binding like that observed for fibrinogen may be limited in the ordered fibrin structure, and thus, K_M values for fibrin depend primarily on the properties of the protease domain. Alternatively, the three-dimensional structure of possible binding sites in polymerized fibrin could be different from that in fibrinogen, explaining the increased enzyme affinities for fibrin.

For determination of catalytic constants for fibrinogen degradation, the concentration of ethanol-soluble degradation products formed by a given protease is measured after various times of incubation and the residual ethanol-insoluble protein concentration is calculated. The ethanol-soluble fraction contains fragment Y- and D-like products, whereas undegraded fibrinogen and fragment X are precipitated, as evidenced by SDS gel electrophoresis (not shown). This approach simplifies the process by ignoring the consecutive formation of various degradation products, which could be considered both substrates and products for the enzyme. On the other hand, the relatively simple fibrinogen cleavage patterns consisting of three main bands suggest that in the studied interval the increase in the amount of the ethanol-soluble fraction can be attributed to the splitting of fragment X to fragments Y and D. The greatly uniform appearance of degradation products formed by the four proteases allows the application of the same experimental procedure for all enzymes. Despite the simplification, values calculated from experimental data obtained at various enzyme and substrate concentrations are consistent with the integrated form of the Michaelis−Menten equation (Figure 2), which supports the assumption that under our experimental conditions the cleavage of only a few sites is of primary importance. The values of k_{cat} (Table 1) determined in our model reflect the rate of the formation of nonclottable degradation products by plasmin, plasmin derivatives, or trypsin. Catalytic constants for fibrin dissolution determined according to our previously published procedure (*24*) characterize the rate of fibrin consumption. Thus, catalytic constants should not be considered to reflect the catalytic properties of the enzyme for the cleavage of a single peptide bond, but as an overall rate constant characterizing a complex process. All four proteases are more efficient in fibrin dissolution than in fibrinogen degradation (Tables 1 and 2). The fact that trypsin is the most efficient enzyme in fibrin degradation, as well as the least efficient enzyme in fibrinogen digestion, emphasizes the predominant role of the structure of the protease domain and the substrate in protease action. Among the three enzymes with identical protease domains, but various kringle structures, however, k_{cat}/K_M decreases in parallel with the loss of kringle domains. The 6-fold fall of the catalytic constant for fibrin dissolution related to the lack of kringle$_5$ in microplasmin is especially remarkable and supports the recently assumed role of this domain in maintaining the conformation of the catalytic domain appropriate for fibrin digestion (*25*). The previously demonstrated intramolecular interaction between kringle$_5$ and the protease domain of plasminogen is consistent with this hypothesis (*15*). Lysis of fibrin clots with a recombinant microplasmin-like molecule is also slower than that by miniplasmin (*44*). An alternative molecular mechanism for the decrease in catalytic efficiency in parallel with the loss of kringle domains can be suggested on the basis of electron microscope studies on the interaction of plasminogen with polymerized fibrin (*45*). Plasminogen binds to fibrin preferentially at end-to-end junctions of monomers, which are 45 nm apart in any filament. As monomers are positioned in a half-staggered manner in protofibrils, potential binding sites are spaced at 22.5 nm. Thus, the catalytic site of plasmin (14 nm) bound to fibrin via kringle domains may reach multiple susceptible bonds at any part of the fibrin molecule. On the other hand, plasmin derivatives of smaller sizes could not reach the cleavage sites in the middle of the coiled coil connecting the D and E domains of fibrin (*45*). This mechanism is consistent with the observation that miniplasmin forms fragments Y, D, and E at a slower rate than plasmin (*29*).

The kringle$_{1-3}$ portion of plasmin has been shown to be of primary importance for the reaction of plasmin with plasmin inhibitor (*9*, *22*, *34*). Second-order rate constants determined for the inhibition of plasmin and miniplasmin by plasmin inhibitor showed a 4-fold decrease related to the loss of the first four kringle domains (*22*), and k'' for microplasmin in the same system (Table 3) is half of the value for miniplasmin, suggesting only a minor role for kringle$_5$. The difference between the rate constants for the inhibition by antithrombin of microplasmin and miniplasmin is also 2-fold, and the dependence of these reactions on heparin concentration is similar; the plasmin−antithrombin reaction is accelerated already at lower concentrations of heparin (Figure 5). Microplasmin preincubated with fibrin(ogen) is protected from inactivation (Table 3) to an extent similar to that of plasmin and miniplasmin (*22*), supporting the primary role of the catalytic domain in this process. On the other hand, the rate of microplasmin inactivation is not changed in the presence of fibrin preincubated with the inhibitor (present result); similar data have been published for plasmin and miniplasmin (*22*).

In summary, our results suggest that the kringle$_5$ domain is of primary importance in maintaining the catalytic efficiency of plasmin and miniplasmin in fibrin dissolution, whereas its role in regulation by plasma inhibitors is minor compared to that of the kringle$_{1-4}$ and the protease domains. In addition, selection of susceptible cleavage sites in the substrate and formation of the enzyme−fibrin complex are mainly dependent on the properties of the protease domain.

ACKNOWLEDGMENT

We are grateful to Ida Horváth and Ágnes Himer for technical assistance.

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BI980180D