Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 360-368

KRINGLE DOMAINS AND PLASMIN DENATURATION

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Received May 28, 1991

The rate of plasmin denaturation was in the order of Lys-plasmin > miniplasmin > microplasmin. Fibrinogen degradation products (FDP) dose dependently increased the denaturation rate of Lys-plasmin and miniplasmin with a maximal rate constant at the FDP/plasmin ratio of about 0.5. The denaturation rate constant of microplasmin was not affected. FDP increased the rate of plasmin denaturation was in parallel with its effect on the interaction among kringle domains. Without FDP only trace amounts of plasminogen dimer could be detected by cross-linking with bis-(sulfosuccinimidyl)-suberate followed by SDS gel electrophoresis. In the low concentration of FDP significant amounts of oligomers of Glu-, miniplasminogens, kringle 1-3 and kringle 1-5 were observed. High concentration of FDP, however, decreased plasminogen oligomer.

Native plasminogen (Glu-Plg) , the proenzyme of plasmin which is the major fibrinolytic protease in blood circulation, consists of five kringle domains and a catalytic domain (1). The kringle domains could bind to fibrin and play an important role in plasminogen activation by tissue-type plasminogen activator, and also in the inhibition by α_2-antiplasmin (2, 3). Second-order autolytic denaturation reaction of plasmin as well as other serine proteases have been studied (4-7). Hydrolysis of the peptide bonds in the active site domain was identified to be the major cause for loss of enzyme catalytic activity (4, 8). FDP or fibrin has been used to enhance plasminogen activation by tissue-type plasminogen activator and other activators (9-17). However, after the plasminogen activation reached the maximum, a rapid decline in plasmin activity was also observed (15). A

The abbreviations used are: Glu-Plg, native plasminogen consisting of Glu^{1}-Asn^{790}; Mini-Plg, Micro-Plg, kringle 1-5, and kringle 1-3, corresponding to plasminogen fragments consisting of Val^{442}-Asn^{790}, Lys^{530}-Asn^{790}, Lys^{77}-Arg^{529}, and Lys^{77}-Val^{337}, respectively; Lys-Plm, Lysine-plasmin; Mini-Plm, miniplasmin; Micro-Plm, microplasmin; FDP, fibrinogen degradation products; PA, plasminogen activator; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BS^3, bis-(sulfosuccinimidyl)suberate; NPGB, *p*-nitrophenyl-*p*'-guanidinobenzoate.

0006-291X/91 $1.50

similar observation has been reported in plasminogen activation by urokinase (18). The decline in the rate of plasmin activity varied with the presence of FDP or lysine in solutions and also of kringle domains in plasmin molecules (15, 18). Autolytic denaturation of plasmin is a second-order reaction and therefore is a kind of protein-protein interaction. These agents influenced the plasmin-plasmin interactions. In this study, we observed a new kind of protein-protein interaction, oligomer formation of plasminogen. The possible correlation between the effect of kringles, FDP, and lysine on plasminogen activation, plasmin denaturation, and oligomer formation of kringles is discussed in this report.

Materials and Methods

Plasminogen and plasmin: Human plasminogen was prepared from pooled human plasma by a modification of the Deutsch and Mertz method (19). Forms 1 and 2 of native plasminogen were separated by chromatography on Lys-Sepharose (20). For this study, form 2 human plasminogen was used exclusively. Urokinase-free human plasmin was prepared by activation of human plasminogen with urokinase-Sepharose as described in a previous report (21).

Preparation of Mini-Plg, Mini-Plm, and kringle 1-3: Mini-Plg and kringle 1-3 were obtained by digesting Glu-Plg with a catalytic amount of porcine pancreatic elastase as described previously (15, 22). Mini-Plm was obtained by activation of Mini-Plg with immobilized urokinase.

Preparation of Micro-Plg, Micro-Plm, and kringle 1-5: Micro-Plg and kringle 1-5 were made by incubating of Glu-Plg with plasmin in an alkaline solution as discussed in a previous report (15, 23). Micro-Plm was prepared from Lys-Plm by incubation at pH 11.0 for 12 h and purified by Lys-Sepharose and soybean trypsin inhibitor-Sepharose (21, 24).

Preparation of FDP: Human fibrinogen (1.0 g) was digested with CNBr (1.3 g) in 70% (v/v) formic acid (100 ml) at 25°C for 17 h. The unreacted reagents were removed by dialysis against distilled water for 24 h, and the product was lyophilized and dissolved (16). The molar concentration of FDP was determined from the difference in the absorbance values (between 280 nm and 320 nm) based on the $\varepsilon^{1\%}$ at 280 nm = 15.5, and molecular weight of 340,000 (18, 25).

Kinetic measurement of the denaturation of Lys-, Mini-, and Micro-Plm: Lys-, Mini-, and Micro-Plm at a final concentration of 0.5 μM were incubated at 37°C in 0.05 M Tris-HCl, 0.038 N NaCl, and 0.01% Tween 80 in the presence of various concentrations of FDP, lysine, or kringle 1-5. Small volumes of plasmin solution were taken at intervals for amidolytic activity measurement with 0.5 mM S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide) in 0.05 M Tris-HCl (pH 7.4), 0.1 N NaCl at 37°C. The initial rate of *p*-nitroaniline released was monitored with a Hitachi 330 spectrophotometer at 405 nm. The molar concentration of catalytically active plasmin in the sample was calculated by comparing the initial rate of hydrolysis of S-2251 with a standard plasmin solution and calibrated by NPGB active site titration as described by Chase and Shaw (26). The rate of plasmin denaturation was plotted according to the second-order equation $(1/A - 1/A_0 = k_2t)$ and analyzed using linear regression, in which A_0 represents the initial molar concentration of plasmin and A represents the molar concentration of plasmin at time t.

Cross-linking of plasminogen and kringle domains with BS^3: Glu-, Mini-, Micro-Plg, kringle 1-3, and kringle 1-5 at a concentration of 0.4 mg/ml were cross-linked with 0.1 mM BS^3 without or with various concentrations of FDP (27). After incubation for 30 min at room temperature, reaction was stopped by adding one-sixth volume of 50 mM ethanolamine, 20 mM N-ethylmaleimide, 50 mM sodium phosphate, pH 7.4. Twenty µl of SDS sample buffer containing 0.03 M sodium phosphate (pH 7.2), 3% SDS, and 6 mM dithiothreitol was added to 30 µl of each sample and denatured at 100°C for 3 min. Samples were then subjected to SDS-PAGE in 5% acrylamide using the buffer system of Weber and Osborn (28).

Reagents: BS^3 was purchased from Pierce. The plasmin substrate S-2251, elastase, NPGB, and lysine were from Sigma. Tween 80 was obtained from Serva. Human fibrinogen was from Kabi. Lysine-Sepharose 4B was obtained from Pharmacia. CNBr was from Merck. All other reagents used were analytical grade.

Results

The second-order reaction rate constant for the plasmin denaturation was derived by plotting the reverse of enzyme concentrations ($1/A-1/A_0$) against time (Fig. 1). The kringle domains on the plasmin molecules had a significant effect on the rate of plasmin denaturation. The rate constant of Lys-Plm denaturation was about four times higher than those of Mini-Plm and Micro-Plm (Table 1). Addition of FDP to Lys-Plm caused a maximal increase in the denaturation constant five times higher than in Lys-Plm without FDP (Table 1). However, Lys-Plm denaturation was slower at higher concentrations of FDP (>0.2 mg/ml) than at lower concentrations of FDP (0.05 mg/ml). FDP only slightly increased the denaturation rate of Mini-

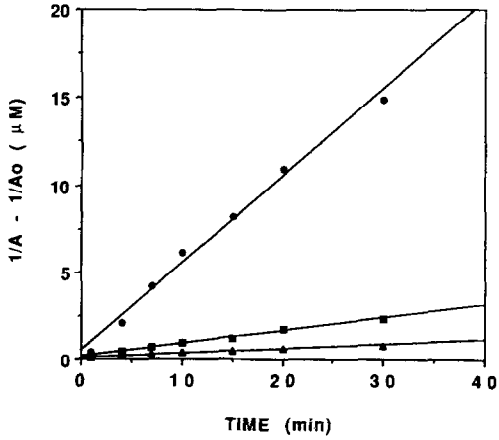


Fig. 1. Denaturation of Lys-, Mini-, and Micro-Plm in the presence of FDP. Lys-Plm (•), Mini-Plm (■), and Micro-Plm (▲) at a final concentration of 0.5 µM were incubated at 37°C in 0.05 M Tris-HCl, 0.038 N NaCl, and 0.01% Tween 80 in the presence of 0.1 mg/ml FDP. The rate of plasmin denaturation was plotted according to the second-order equation ($1/A - 1/A_0 = k_2t$) and analyzed using linear regression as described in Materials and Methods.

Table 1
Effect of FDP on the second-order reaction rate constants of denaturation of Lys-Plm, Mini-Plm and Micro-Plm

	Second-order reaction rate constants (k_2, $\mu M^{-1}min^{-1}$)		
	Lys-Plm	Mini-Plm	Micro-Plm
FDP (mg/ml)			
0	0.13 ± 0.01	0.036 ± 0.001	0.033 ± 0.001
0.05	0.58 ± 0.01	0.071 ± 0.003	0.016 ± 0.001
0.1	0.60 ± 0.02	0.081 ± 0.002	0.021 ± 0.007
0.2	0.43 ± 0.04	0.073 ± 0.003	0.017 ± 0.001
0.4	0.30 ± 0.03	0.071 ± 0.003	0.016 ± 0.001
0.8	0.24 ± 0.03	0.067 ± 0.004	0.012 ± 0.002

Values given are means ± S. E. M. (n = 3).
Lys-, Mini-, and Micro-Plm at a final concentration of 0.5 μM were incubated at 37°C in 0.05 M Tris-HCl, 0.038 N NaCl, and 0.01% Tween 80 in the presence of various concentrations of FDP. The second-order reaction rate constant of plasmin denaturation was calculated as described in Materials and Methods.

Plm. On the other hand, FDP slightly decreased the denaturation of Micro-Plm. Addition of kringle 1-5 efficiently inhibited the effect of FDP on Lys-Plm denaturation. Kringle 1-5 did not cause decrease in denaturation rate of Lys-Plm without FDP (Table 2). The effect of lysine on plasmin denaturation was different from that of kringle 1-5. It can make the Lys-Plm stable in the presence or absence of FDP (Table 3). However, lysine did not cause any significant effect on the denaturation of Mini-Plm (Table 3). In

Table 2
Effect of kringle 1-5 on the second-order reaction rate constants of denaturation of Lys-Plm with and without FDP

	Second-order reaction rate constants (k_2, $\mu M^{-1}min^{-1}$)	
	with FDP (0.1 mg/ml)	without FDP
Kringle 1-5		
0 μM	0.59 ± 0.002	0.083 ± 0.001
0.1 μM	0.38 ± 0.002	0.048 ± 0.002
0.5 μM	0.29 ± 0.003	0.053 ± 0.001
1 μM	0.23 ± 0.002	0.057 ± 0.002
2 μM	0.12 ± 0.002	0.061 ± 0.003
5 μM	0.12 ± 0.002	0.074 ± 0.002
10 μM	0.09 ± 0.004	0.095 ± 0.005

Values given are means ± S. E. M. (n = 3).
Lys-Plm (0.5 μM) was incubated at 37°C in 0.05 M Tris-HCl, 0.038 N NaCl, and 0.01% Tween 80 in the presence of various concentrations of kringle 1-5. The denaturation rate constants were determined.

Table 3
Effect of lysine on the second-order reaction rate constants of denaturation of Lys-Plm and Mini-Plm

	Second-order reaction rate constants (k_2, $\mu M^{-1}min^{-1}$)		
	Lys-Plm		Mini-Plm
Lysine	with FDP (0.1 mg/ml)	without FDP	without FDP
0 mM	0.58 ± 0.003	0.097 ± 0.002	0.036 ± 0.001
1 mM	0.35 ± 0.004	0.032 ± 0.003	0.030 ± 0.001
5 mM	0.21 ± 0.005	0.012 ± 0.002	0.031 ± 0.003
10 mM	0.087 ± 0.004	0.006 ± 0.005	0.036 ± 0.002
50 mM	0.018 ± 0.003	0.010 ± 0.004	0.022 ± 0.005

Values given are means ± S. E. M. (n = 3).
Lys- or Mini-Plm (0.5 μM) was incubated at 37°C in 0.05 M Tris-HCl, 0.038 N NaCl, and 0.01% Tween 80 in the presence of various concentrations of lysine with and without FDP. The denaturation rate constants were determined.

the absence of FDP trace amounts of Glu-Plg dimer could be detected using SDS-PAGE after cross-linking. An increased amount of Glu-Plg oligomers could be detected in the presence of 0.1 mg/ml FDP (Fig. 2). Protein dimer could also be observed in Mini-Plg but not in Micro-Plg (Fig. 2). The oligomer structure could also be observed in kringle 1-5 and kringle 1-3 in the presence of FDP (Fig. 3). The increment of FDP concentration, however, caused a decrease in the amount of Glu-Plg oligomer (Fig. 4).

Discussion

The kringle domains in the plasminogen molecule influenced the effect of FDP on the activation of the plasminogen, especially by tissue-type

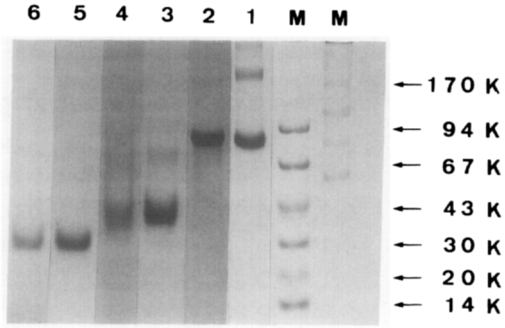


Fig. 2. The dimer formation of Glu-, Mini-, and Micro-Plg with and without FDP. Glu-, Mini-, Micro-Plg, at a concentration of 0.4 mg/ml were cross-linked with 0.1 mM BS^3 without or with 0.1 mg/ml FDP followed by SDS-PAGE as described in Materials and Methods. (27). M:Marker, Lane 1:Glu-Plg with FDP; 2:Glu-Plg without FDP; 3:Mini-Plg with FDP; 4:Mini-Plg without FDP; 5:Micro-Plg with FDP; 6: Micro-Plg without FDP.

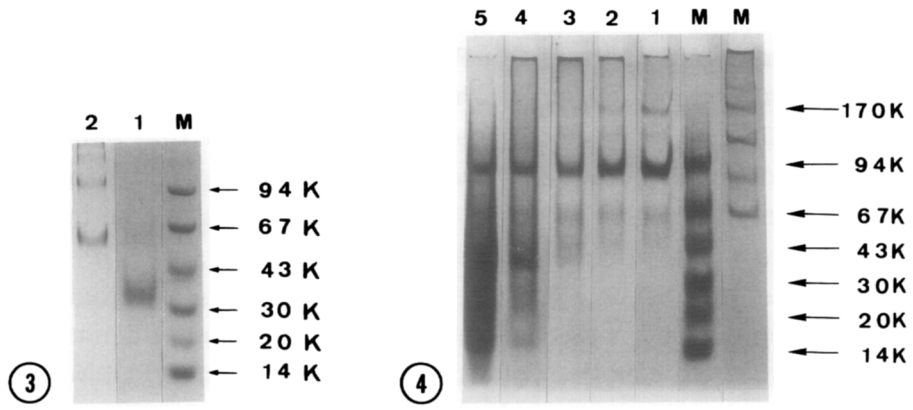
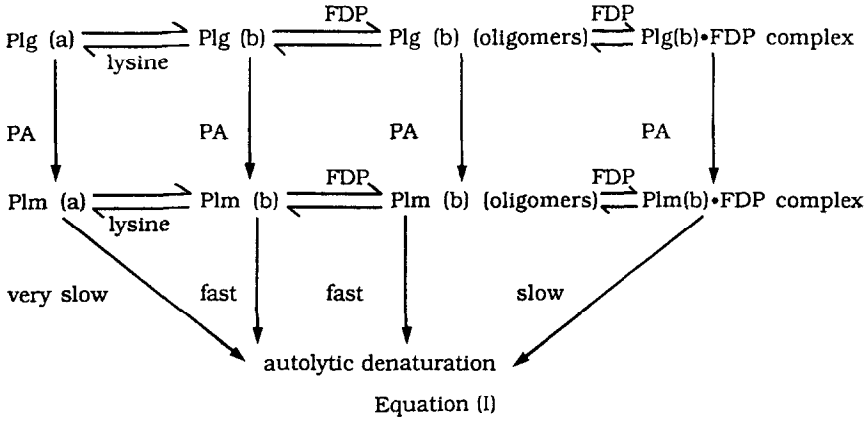


Fig. 3. The dimer formation of kringle 1-5 and kringle 1-3 in the presence of FDP. Kringle 1-5 or kringle 1-3 at a concentration of 0.4 mg/ml were cross-linked with 0.1 mM BS^3 with 0.1 mg/ml FDP followed by SDS-PAGE. M: Marker, Lane 1:Kringle 1-3; 2:Kringle 1-5.

Fig. 4. The dimer formation of Glu-Plg in the presence of various concentrations of FDP. Glu-Plg at a concentration of 0.4 mg/ml was cross-linked with 0.1 mM BS^3 with various concentrations of FDP. M:Marker; Lane 1:Glu-Plg with 0.2 mg/ml FDP; 2:Glu-Plg with 0.4 mg/ml FDP; 3:Glu-Plg with 0.8mg/ml FDP; 4:Glu-Plg with 1.6 mg/ml FDP; 5: Glu-Plg with 3.2 mg/ml FDP.

plasminogen activator (15). The effect of FDP on plasminogen activation is rather complicated since three different components - tissue-type plasminogen activator, FDP and plasminogen - are involved in the system and both tissue-type plasminogen activator and plasminogen are able to bind to FDP (29). The kringle domains also had a significant effect on plasmin denaturation (Table 1). The Lys-Plm with five kringle domains has a higher rate of autolytic denaturation than Mini-Plm or Micro-Plm with only one or non-kringle domain. How do kringle domains in plasmin molecules enhance both denaturation and activation especially in the presence of FDP is not known. Low concentration of FDP significantly increased the denaturation rate of Lys-Plm but not Mini-Plm or Micro-Plm. The effect of FDP on denaturation was offset by adding kringle 1-5 or lysine. The effect of FDP on plasminogen activation was also inhibited by these two reagents (15). At high concentrations of FDP the rate of plasmin denaturation is slower compared to that at low FDP concentrations. The effect of FDP on denaturation of plasmin and activation of plasminogen was mainly through interaction with kringle domains. Binding of FDP to kringle domains might induce a conformational change in kringle domains and facilitate protein-protein interaction. FDP might increase the chance of interaction or affinity among plasminogen molecules which could result in covalently linked oligomer if cross-linking reagent was added. In the samples of Glu-Plg without FDP treated with a cross-linking reagent, mainly monomer was detected using SDS-PAGE. However, in the presence of FDP, dimer and

oligomer of Glu-Plg were detected. In high concentrations of FDP, the amount of oligomer and dimer of Glu-Plg decreased. Plasminogen activation, plasmin denaturation and oligomer formation similarly depend on the interaction of FDP and kringle domains. It seems possible that binding of FDP to kringle domains would lead to conformation changes in kringle domains. The plasmin(ogen) molecule on FDP tends to aggregate and increase the rate of activation and denaturation. However, at high concentrations of FDP the Plg•FDP one-to-one complex was dominant and less plasminogen oligomer was observed. The rate of plasmin denaturation reached a maximal value as the molar ratio of plasminogen to FDP was close to 1:0.5. The maximal amount of oligomer of kringle domains was also observed at the same relative concentration of plasminogen to FDP. The possible effects of FDP, kringle domains, and lysine on plasminogen reactions are summarized in equation (I):



Equation (I)

Plasmin and plasminogen might exist in two forms - (a) and (b) - depending on the conformation of kringle domains. Plm(a) and Plg(a) are the most stable and hence, have less tendency to form oligomers, while Plg(b) and Plm(b), which are less stable, tended to form oligomers more. Lysine shifted the equilibrium from form (b) to (a) and slowed down both denaturation of plasmin and activation of plasminogen. FDP increased the concentration of Plg(b) and Plm(b); more oligomer of plasminogen was detected, and the rate of denaturation of plasmin increased. However, at high FDP concentrations, Plg•FDP complex was dominant and FDP interfered between plasminogen or plasmin molecules. Therefore, less oligomer of plasmin and slow denaturation of plasmin were observed. However, it is also possible that FDP might have provided more than one binding site for kringles. At low FDP concentrations, the complex could be Plg•FDP•Plg. The interaction of plasmin(ogen) molecules on FDP is thus

enhanced. The high concentration of FDP would decrease the concentration of the complex containing more than two plasminogen molecules. Therefore less oligomer could be detected and slow denaturation rate of plasmin was observed. On the other hand, high concentration of FDP did not result in slow plasminogen activation. The rate of plasminogen activation did not parallel the amount of plasminogen oligomer. Plg(b) or Plg(b)•FDP could be the form necessary for plasminogen activation.

Acknowledgments: The expert technical assistance of L. -C. Chang and G. -J. Hsieh are gratefully acknowledged. This work was supported by Grant NSC 79-0412-B006-48, NSC 79-0412-B006-13, and NSC 80-0412-B006-12 from the National Science Council, Republic of China.

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